PHYSIOLOGICAL AND MOLECULAR PERSPECTIVES OF STRESS TOLERANCE IN VEGETABLES

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PHYSIOLOGICAL AND MOLECULAR PERSPECTIVES OF STRESS TOLERANCE IN VEGETABLES

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Editorial: Physiological and molecular perspectives of stress tolerance in vegetables

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Editorial on the Research Topic
Physiological and molecular perspectives of stress tolerance in vegetables

Introduction

In 2021, the Intergovernmental Panel on Climate Change (IPCC, 2021) released a recent report on the anthropogenic effects of current climate changes. Climate changes such as persistent drought, increased soil salinity and frequent heat-waves, and reductions in the quantity and quality of water resources pose serious threats to food security for the coming generations, both from a qualitative and quantitative viewpoint (Abdelrahman et al., 2020a,b,c, 2021). For these reasons, the development of climate-resilient crops will play a significant part in revolutionizing farming systems to cope with the projected extreme environmental fluctuations (Schiermeier, 2018; Abdelrahman et al., 2019). To overcome these changes, crops have developed complex mechanisms for stress tolerance, including stress perception, signal transduction, transcriptional activation of stress-responsive target genes, synthesis of enzymatic and non-enzymatic antioxidants, and production of osmoprotectants (Gupta and Huang, 2014; Resende et al., 2020). Emerging technologies from multiple research areas including plant genomics, crop breeding, plant physiology, omics-based techniques, and bioinformatics, present opportunities to improve the efficiency of screening useful agronomic traits that can enhance abiotic stress tolerance in vegetable crops. These interests have prompted us to edit this Research Topic, collecting a total of 12 contributions (six reviews and six original research articles) which cover different Physiological and Molecular Perspectives of Stress Tolerance in Vegetables. In particular, the topics cover both abiotic and biotic stress tolerance/resistance, as well as the potential molecular mechanisms involved. A discussion of these articles is given below.

Key remarks

The physiological and biochemical levels of two different melon (*Cucumis melo*) cultivars were evaluated in response to control, drought, or salt stress conditions (Chevilly et al.). Authors reported distinctive traits for salt tolerance in melon, including phenylalanine, histidine, proline, and the Na^+/K^+ ratio. On the other hand, the characteristic traits for drought tolerance were the hydric potential, isoleucine, glycine, phenylalanine, tryptophan, serine, and asparagine (Chevilly et al.). These obtained results can be useful markers for breeding strategies or to predict which varieties are likely to perform better under drought or salt stress. In another study, Wang et al., functionally characterized the potential role of pumpkin *Regulator of chromosome condensation 1* (*CmRCC1*) gene involved in cold tolerance. Cold stress is the main limiting factor of cucurbit crop cultivation as it affects crop yield and quality; thus, identification of stress responsive genes is a crucial aspect of pumpkin rootstock breeding. Results indicated that *CmRCC1* overexpression in tobacco increased the gravitropic set-point angle in lateral roots, as well as root volume and diameter under cold stress. In addition, *CmRCC1* overexpression maintained photosynthetic activity under cold stress. Thus, this study highlights the positive regulatory role of *CmRCC1* in root architecture, which can be utilized in the future for improving crop yield and quality under cold stress. Song et al. investigated the relationship between antioxidant capacity in leaves and storage properties in different sweet potato (*Pomoea batatas*) cultivars, demonstrating that cultivar 'Xu 32', which showed the best storage property, had higher antioxidant enzyme activity and lower lipoxygenase and malondialdehyde (MDA) contents. The above results revealed that storage property is highly correlated with antioxidant capacity in sweet potato leaves and negatively correlated with α-amylase activity in tuberous roots, which provides a convenient means for the screening of storage-tolerant sweet potato cultivars (Song et al.). In another study, Yi et al. investigated the biological function of radish Aquaporins (*Raphanus sativus*, *RsAQPs*) genes under salt stress conditions. Results indicated that seven *RsAQP* genes, such as *RsPIP1-3*, *1-6*, *2-1*, *2-6*, *2-10*, *2-13,* and *2-14*, exhibited significant upregulation in roots of salt-tolerant radish genotype (Yi et al.). In addition, the overexpression of *RsPIP2-6* enhanced salt tolerance in transgenic radish hairy roots, which was evident by improved growth of transgenic radish under salt stress condition compared with wild-type (WT) plants (Yi et al.). With respect to cluster bean (*Cyamopsis tetragonoloba* L.) drought stress tolerance, RNA-seq analysis of drought-stressed vs. well-watered cluster beans revealed the crucial role of increased wax deposits on the leaf surface in combating drought stress in cluster beans under drought stress condition (Reddy et al.). Thus, further investigation about wax regulatory genes could be important for improving crop drought stress. Khandagale et al. explored the transcriptomic changes in onion (*Allium cepa*) response to *Alternaria porri*, revealing distinctive upregulation of *GABA transporter1, ankyrin repeat domain-containing protein, Xyloglucan endotransglucosylase/hydrolase*, and *Pathogenesis-related protein 5* in resistant onion genotype. Transcriptome profiling of onion response to *Alternaria porri* infection will serve as an important resource for future studies to elucidate the molecular mechanism of onion-*A. porri* interaction and to improve disease resistance in onion. Several review articles in this Research Topic summarized and discussed the recent developments in crop stress tolerance. For example, Kang et al. ummarized and discussed heat stress-responsive genes including those encoding heat shock factors and heat shock proteins, and their functional roles in heat stress tolerance of vegetable crops. Likewise, Hoshikawa et al., investigated the molecular mechanisms involved in heat stress tolerance and the challenges of developing heat-tolerant tomato varieties. Parvathi et al. discussed the progress made in deciphering the multifactorial stress responses of cucurbits and their multifactorial stress-specific traits/mechanisms/pathways and their crosstalk associated traits, both individually and in combination.

Conclusions and future prospects

This special edition brought together interesting studies that reveal the importance of understanding molecular and physiological mechanisms in vegetable crops' response to environmental stresses. Integrated metabolome and transcriptome analysis will be essential components to decipher stress tolerance mechanisms and to identify stress-specific markers that can be utilized in breeding programs to increase yield and productivity under current and future climatic conditions. Although much is known about how plants acclimate to individual stress, little is known about how they respond to a combination of many stress factors simultaneously. Thus, future studies addressing the impact of multifactorial stress combination associated with climate changes is needed to understand how such stress combination is affecting crops. In addition, a proteomic approach has been found to be very important as it helps plant physiologists to understand what is going on in the cell due to an external stimulus. Thus, future studies using proteomics will gain much attention and might provide novel and important information for developing stress-resilient crops.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Distinctive Traits for Drought and Salt Stress Tolerance in Melon (*Cucumis melo* L.)

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Melon (*Cucumis melo* L.) is a crop with important agronomic interest worldwide. Because of the increase of drought and salinity in many cultivation areas as a result of anthropogenic global warming, the obtention of varieties tolerant to these conditions is a major objective for agronomical improvement. The identification of the limiting factors for stress tolerance could help to define the objectives and the traits which could be improved by classical breeding or other techniques. With this objective, we have characterized, at the physiological and biochemical levels, two different cultivars (sensitive or tolerant) of two different melon varieties (Galia and Piel de Sapo) under controlled drought or salt stress. We have performed physiological measurements, a complete amino acid profile and we have determined the sodium, potassium and hormone concentrations. This has allowed us to determine that the distinctive general trait for salt tolerance in melon are the levels of phenylalanine, histidine, proline and the Na^+/K^+ ratio, while the distinctive traits for drought tolerance are the hydric potential, isoleucine, glycine, phenylalanine, tryptophan, serine, and asparagine. These could be useful markers for breeding strategies or to predict which varieties are likely perform better under drought or salt stress. Our study has also allowed us to identify which metabolites and physiological traits are differentially regulated upon salt and drought stress between different varieties.

Keywords: melon, *Cucumis melo*, salt stress, drought stress, amino acids, plant hormones, ion content

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INTRODUCTION

Melon (*Cucumis melo L.*) is a major crop with great agronomic and economic interest, considered a gourmet food in several markets and cultures. One of the main problems for melon farming is that its cultivation demands a lot of water (Cabello et al., 2009). In the current context of anthropogenic global warming and the subsequent climate change, aridity is increasing in traditional cultivation areas, and thus, melon culture is subjected to increasing abiotic stress, which compromises the yield. Specifically, drought stress is increasing, and salt stress is directly related to this water scarcity, given that excessive irrigation increases the salt deposition in the soil and diminishes the phreatic level,

thus enabling the infiltration of sea water. It is estimated that 20% of all arable land and almost half of the land with water availability are affected by salts, significantly reducing yield below the genetic potential of most crops (Botella et al., 2007; Chandna et al., 2014). As a result of salinization, crop yields are declining while arable land is being irreversibly lost (Nawaz et al., 2010). High salinity levels also increase soil pH. In addition, saline stress leads to deterioration of soil structure and prevents the air-water balance, essential for biological processes occurring in the roots (Galvan-Ampudia et al., 2013). Saline soils reduce the biomass production of crops affecting important biochemical and physiological processes in the plant (Serrano et al., 1999).

We have generated considerable knowledge at the biochemical level and physiological level regarding how abiotic stress affects basic physiological processes, the cellular function and even the biochemical targets, but there are still large gaps in our knowledge about the limiting factors for stress responses. More specifically, we are lacking knowledge regarding which traits could be improved by breeding or genetic engineering that would have a major impact on plant growth and development under stress conditions. This explains the low success in breeding novel crops that are adapted to saline soils or are able to maintain yield under drought stress conditions (Ashraf et al., 2009). Proof of this scarcity of results is that there are only two GMO cultivars on the market whose trait is drought tolerance: the Droughtgard maize from BASF and the HB4 soy from Agroceres (Wang et al., 2015; Ribichich et al., 2020). To date, there are no marketed biotechnological crops with enhanced yield under saline conditions.

Several strategies have been developed to identify the limiting factors for stress tolerance. Evaluating the physiological and biochemical response of stress tolerant and stress sensitive plants is a well-established strategy to discover differential traits for abiotic stress tolerance (Taibi et al., 2017, 2018; Chevilly et al., 2021). All these analyses have been performed testing different cultivars from the same variety and a single stress. We have further developed this concept by evaluating, in the same analysis, different stresses and different cultivars of two different varieties to find limiting factors which are not particular to a specific variety or stress. In this report, we have applied this strategy to a pivotal horticultural crop for the economy in the Mediterranean area. There are several reports evaluating Galia melon performance under salt stress in field conditions (Akrami and Arzani, 2018; Akrami et al., 2019), but so far, there are no studies evaluating the plant response at the initial stages of development under controlled conditions. This work has been designed to determine the differences at the physiological and biochemical levels between different melon genotypes under two different abiotic stresses. These varieties had previously been characterized as sensitive or tolerant to abiotic stress. We have subjected these varieties to controlled drought or salinity stress, and have monitored different physiological or biochemical parameters, in order to find changes that are relevant among varieties or treatments. This will allow us to identify the limiting factors in abiotic stress tolerance and will help to define novel breeding strategies.

MATERIALS AND METHODS

Plant Material

The four varieties of pre-commercial melon *(Cucumis melo* L.) seeds used were provided by Enza Zaden and referred to as Cv. 1, Cv. 2, Cv. 3, and Cv. 4. Cv. 1 is a Galia melon (*Cucumis melo* Cv. *reticulatus*) tolerant to abiotic stress, Cv. 2 is a Galia type melon sensitive to abiotic stress; Cv. 3 melon is a Piel de Sapo (a.k.a. Santa Claus Melon; *Cucumis melo* Cv. *inodorus*) tolerant to abiotic stress; and Cv. 4 is a Piel de Sapo sensitive to abiotic stress.

Experimental Design

For different experiments, 20 seeds of each variety were germinated in a Petri dish with moist sterile Whatmann filter paper. After 5 days, seedlings were transferred to a substrate (50% kekkila peat, 25% perlite, 25% vermiculite) in individual plant pots 12 cm diameter × 8 cm height. The experimental design consisted of an aleatory placement where each block was composed by 4 pots per tray and one plant per pot. Each experiment consisted in 5 individuals × 4 varieties × 3 treatments (60 total plants). Plants were watered with Hoagland solution. After 3 weeks, when plants reached the four-leaf phase, irrigation was maintained (control plants), limited (drought stress) or watered with Hoagland solution plus 220 mM NaCl (salt stress). Samples were taken or measurements were performed after 6 days of stress treatment (salt stress) or when the total weight (plant and container) was reduced to 60% of their initial weight (drought stress), at about 9 days. In all cases, the number of samples per experiment (n) refers to biological replicates from different plants (between 3 and 5). All samples for each treatment were collected at the same time. Plants were grown in a phytotron at 25 ± 2°C, humidity of 50–60% and a photoperiod of 16 h light/8 h darkness (200 μmol m^{-2} s^{-1} of light intensity). All experiments were replicated to check the reproducibility of the results.

Physiological Measurements

The water potential (Ψ_w, MPa) was measured with a Schölander pressure pump (model PMS-1000, PMS Instruments, Corvallis, OR, United States). Stomatal conductance (g_s, mmol H_2O $m^{-2}s^{-1}$), the sub-stomatal concentration of CO_2 (C_i), photosynthetic rate (A, μmol CO_2 $m^{-2}s^{-1}$), transpiration (E, mmol H_2O $m^{-2}s^{-1}$), water use efficiency (WUE, μmol CO_2 $mmol^{-1}H_2O$) and leaf temperature through infrared Thermometry (Tleaf, °C), were determined with a CIRAS-3 portable photosynthesis system (PP Systems, Amesbury MA). The measurements were recorded under saturating light conditions (1,500 μmol quanta m^{-2} s^{-1}), with a temperature of 25°C, and ambient CO_2 concentration of 400 mol^{-1} CO_2 and a relative humidity of ∼55%. Chlorophyll fluorescence indices (i.e., Fv/Fm and Quantum yield) were measured with a portable pulse-amplitude modulated chlorophyll fluorometer (PAM-2100, Heinz Walz, Effeltrich, Germany). These measurements of the photosystem II efficiency were performed once the plants were adapted to darkness for 30 min, on the same leaves where stomatal conductance and photosynthesis were determined. All

measures were performed on the third youngest full-developed leaf of each plant, analyzing a total of five plants per variety.

Amino Acid Analysis

One gram of the third youngest leaf was taken, lyophilized and ground with a mortar and pestle in the presence of liquid nitrogen. The resulting powder was homogenized for 30 s with 2 mL of 2% citrate buffer pH 2 (Mulet et al., 2004) and centrifuged for 5 min at 13,000 *g*. The supernatant was filtered through a 25-micrometer pore-size non-sterile filter. 1/10 dilutions of these extracts were injected into an automatic Beckman Gold amino acid analyzer. The analysis was carried out according to the protocol supplied by the manufacturer, using a system of ninhydrin and sodium citrate for detection. Measurements were normalized to dry weight.

Hormone Quantification

Plant hormones were determined following the method of Durgbanshi (Durgbanshi et al., 2005). Briefly, lyophilized samples were ground to powder in the presence of liquid nitrogen. Two hundred milligram per replicate were purified with solid phase extraction columns (SPE; reverse phase and ion exchange), using internal deuterated standards. The analysis was carried out using UPLC-mass spectrometry (Acquity SDS, Waters Corp., Milford, MA). Measurements were normalized to dry weight.

Ion Content Determination

Ions were determined as described (Gisbert et al., 2020). Briefly, samples of the third youngest leaf from 1-month-old plants (about 1 g) were dried at 70°C for 4 days. Dry weight was determined, and ions were extracted by a 30 min incubation in 1 mL of 0.1M HNO_3 at room temperature. Then samples were centrifuged, and the supernatant was diluted with 4 mL of milliQ water and filtered (22 μM). Sodium and potassium were measured in a plasma emission spectrophotometer (Shimadzu), as described (Rios et al., 2012). Measurements were normalized to dry weight.

Statistical Analysis

The ANOVA was performed by using the SPSS software v.25.0 statistical package (IBM SPSS Statistics for Windows, Armonk, NY, USA; IBM Corp.). The means were considered to be significantly different at $p < 0.05$ after Duncan's new multiple range test (MRT) (Duncan, 1955).

RESULTS

Physiological Determinations

Several responses of plants to abiotic stress occur at the physiological level. We investigated whether we could identify differential responses among varieties or cultivars. As expected, the water potential increased upon stress between 1.12 and 1.25 for salt stress and 1.3 and 2.67 for drought stress (expressed as –MPa; **Figure 1A**), thus validating our experimental design. The tolerant cultivars presented higher values. The salinity treatment had a negative effect on stomatal conductance (gs), while the drought stress had a more modest effect on this parameter, observing minor differences when compared to the corresponding control (**Figure 1B**). A similar pattern was found with transpiration (E) and photosynthesis (A), which was stable upon drought stress, but decreased upon salt stress. Interestingly A also decreased upon drought stress in the tolerant Galia cultivar (**Figures 1C,D**). Maximum efficiency of photosystem II (determined as Fv/Fm) and quantum yield presented minor, but in some cases significant changes (**Figures 1E,F**). Water Use efficiency, intrinsic and instantaneous, decreased under drought stress and increased upon salt stress in Galia plants, but was stable in Piel de Sapo (Cv. 3; **Figures 1G,H**).

We also determined the leaf temperature and found a differential response among varieties. In Galia, the leaf temperature decreased in the tolerant variety upon drought stress about 4%, and in the Piel de Sapo the leaf temperature increased in the tolerant variety about 0,3% (**Figure 1I**). We observed minor effects on the sub-stomatical CO_2 concentration (Ci) (**Figure 1J**).

Amino Acid Measurements

Once we had studied the response of the selected varieties and cultivars at the physiological level, we further investigated the level of amino acids. First, we focused on the hydrophobic amino acids (**Figure 2**). In most cases, there was no distinctive pattern. However, for leucine (Leu), the concentration increased under salt stress (between 40 and 115%), and to a minor extent, under drought stress (between 0 and 67%) (**Figure 2B**). Glycine (Gly) can act as an osmolyte and is a precursor of antioxidant molecules, such as the tripeptide glutathione. Its concentration under stress conditions correlated with tolerance to stress, but only for the Piel the Sapo variety (**Figure 2E**). Similarly, phenylalanine (Phe) concentrations under drought stress correlated with sensitivity (**Figure 2F**). Finally, we observed a 7 fold increase in Tryptophan (Trp) concentration in Piel de Sapo sensitive cultivar under drought stress conditions (**Figure 2G**).

We further investigated the polar amino acids. Serine (Ser) concentrations increased, between 2.3- to 4-fold, under salt stress (**Figure 3A**) and similar results were obtained for asparagine (Asn). For other amino acids, such as threonine (Thr), cysteine (Cys), proline (Pro), or glutamine (Gln), we did not find a distinctive pattern (**Figure 3**).

We also studied the charged amino acids and found that an increase in the levels of lysine (Lys) correlate with salt tolerance, but for drought tolerance only in the case of the Galia cultivar (**Figure 4A**). Also, an approximate 4-fold increase of histidine (His) concentration was observed under drought stress conditions for Galia cultivars (**Figure 4C**). Aspartic acid (Asp) levels behaved in disparate manners: in Galia they increased under salt stress in the sensitive cultivar (Cv. 2), while in Piel de Sapo, they increased in the tolerant cultivar (Cv. 3; **Figure 4D**). Glutamic acid (Glu) levels increased under salt and drought stress with respect to the control only in Piel de Sapo (**Figure 4E**). We did not find a distinctive pattern for GSH (**Figure 4F**).

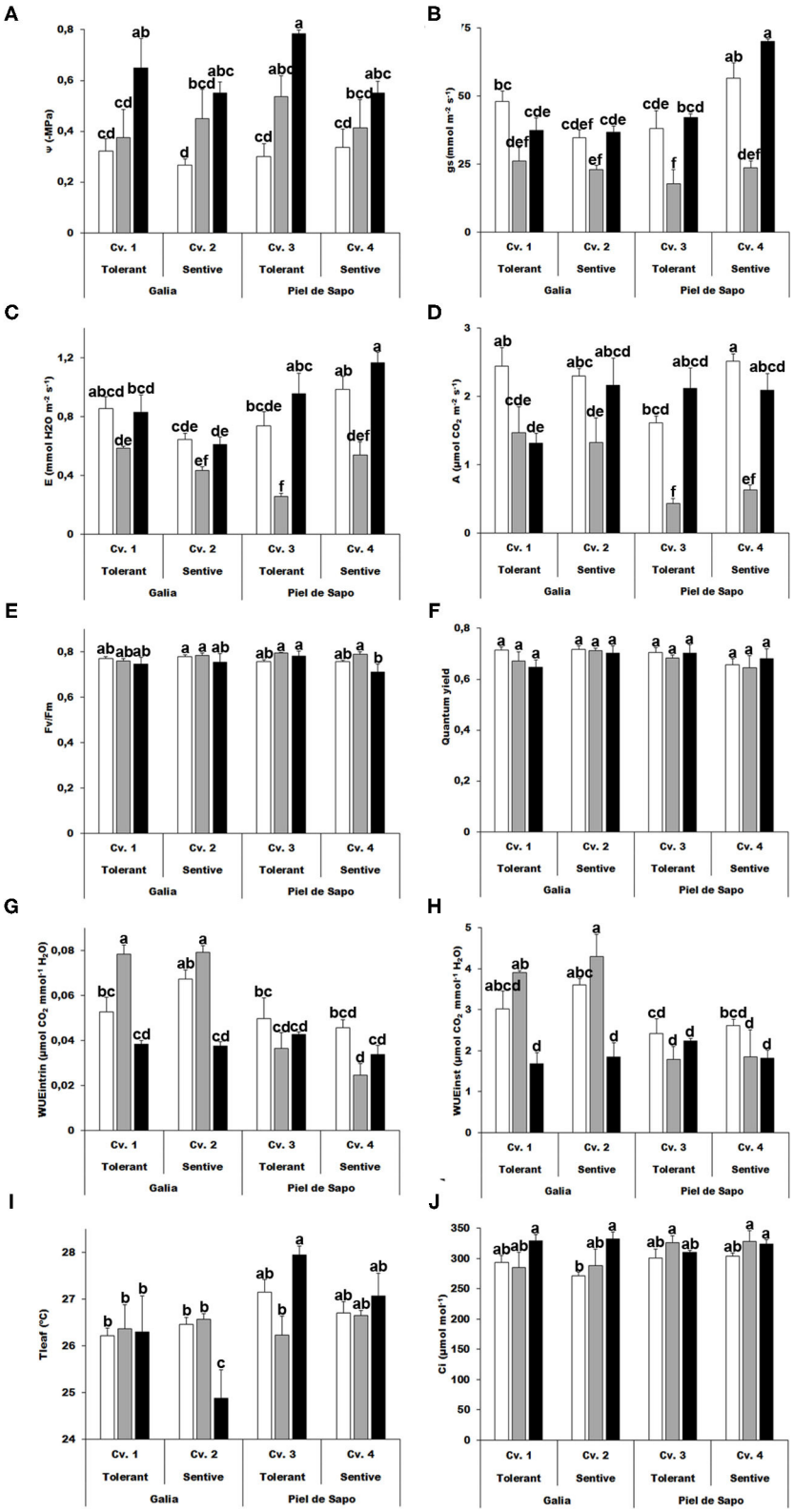

FIGURE 1 | Physiological measurements. Water potential (Ψw) **(A)**; stomatal conductance (gs) **(B)**; transpiration (E) **(C)**; Net photosynthesis (A) **(D)**; Quantum efficiency of photosystem II (Fv/Fm) **(E)**; Quantum yield **(F)**; intrinsic water use efficiency (WUEintr) **(G)**; instantaneous water use efficiency (WUEinst) **(H)**; Leaf temperature (Tleaf) **(I)** and sub-stomatal CO_2 concentration (Ci) **(J)** of Galia tolerant genotype (Cv. 1), Galia sensitive genotype (Cv. 2), Piel de Sapo tolerant genotype (Cv. 3) or Piel de Sapo sensitive genotype (Cv. 4) under control (white bars), salt stress (gray bars) and drought stress (black bars) conditions. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test ($n = 5$). Scale bars are the mean ± standard error (SE). Experiment was replicated with similar results.

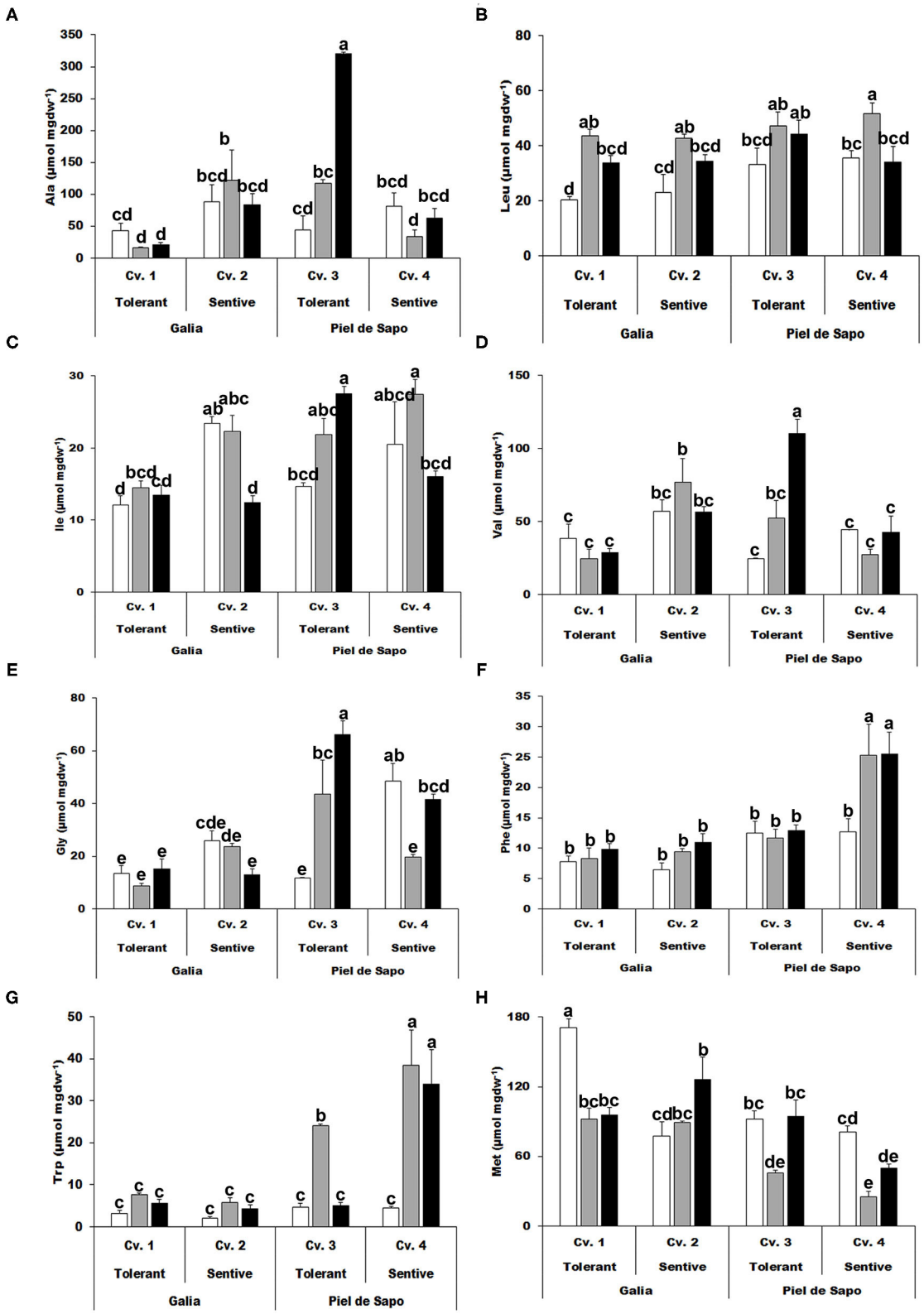

FIGURE 2 | Hydrophobic amino acids. Alanine (Ala) **(A)**; leucine (Leu) **(B)**; isoleucine (Ile) **(C)**; valine (Val) **(D)**; glycine (Gly) **(E)**; phenylalanine (Phe) **(F)**; tryptophan (Trp) **(G)**; and methionine (Met) **(H)** of Galia tolerant genotype (Cv. 1), Galia sensitive genotype (Cv. 2), Piel de Sapo tolerant genotype (Cv. 3) or Piel de Sapo sensitive genotype (Cv. 4) under control (white bars), salt stress (gray bars) and drought stress (black bars) conditions. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test ($n = 3$). Scale bars are mean ± SE. Experiment was replicated with similar results.

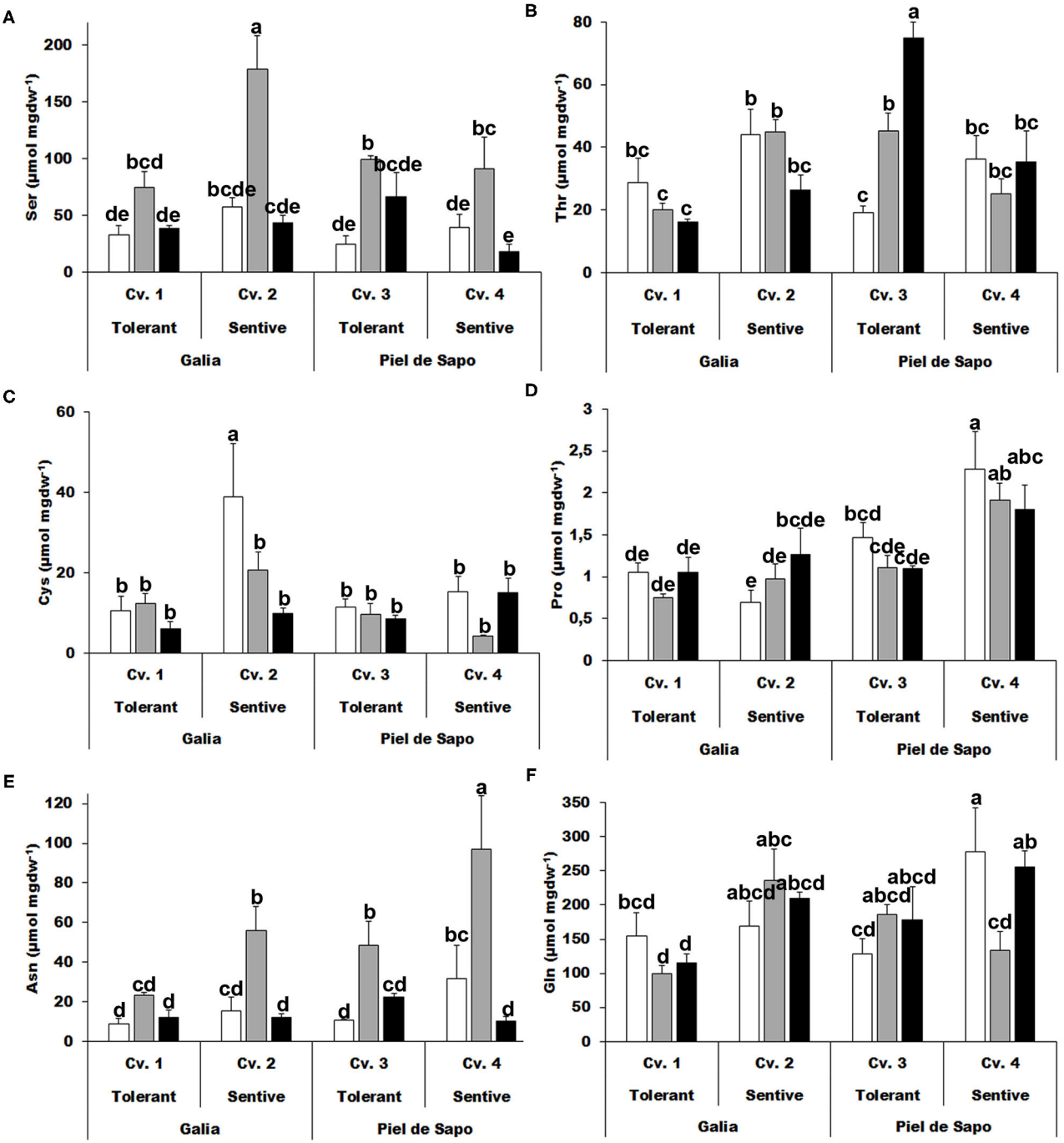

FIGURE 3 | Polar amino acids. Serine (Ser) **(A)**; threonine (Thr) **(B)**; cysteine (Cys) **(C)**; proline (Pro) **(D)**; asparagine (Asn) **(E)**; and glutamine (Gln) **(F)** of Galia tolerant genotype (Cv. 1), Galia sensitive genotype (Cv. 2), Piel de Sapo tolerant genotype (Cv. 3), or Piel de Sapo sensitive genotype (Cv. 4) under control (white bars), salt stress (gray bars), and drought stress (black bars) conditions. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test ($n = 3$). Scale bars are mean ± SE. Experiment was replicated with similar results.

Sodium and Potassium Content

We determined the ion content of the investigated varieties and cultivars under control and stress conditions. As expected, the potassium concentration decreased under salt stress conditions (between 5 and 40%), as sodium competes with potassium. Under drought stress, the potassium concentration also decreased about 10%. Potassium has been described to act as an osmolyte, but according to our results, that is not its main role in the

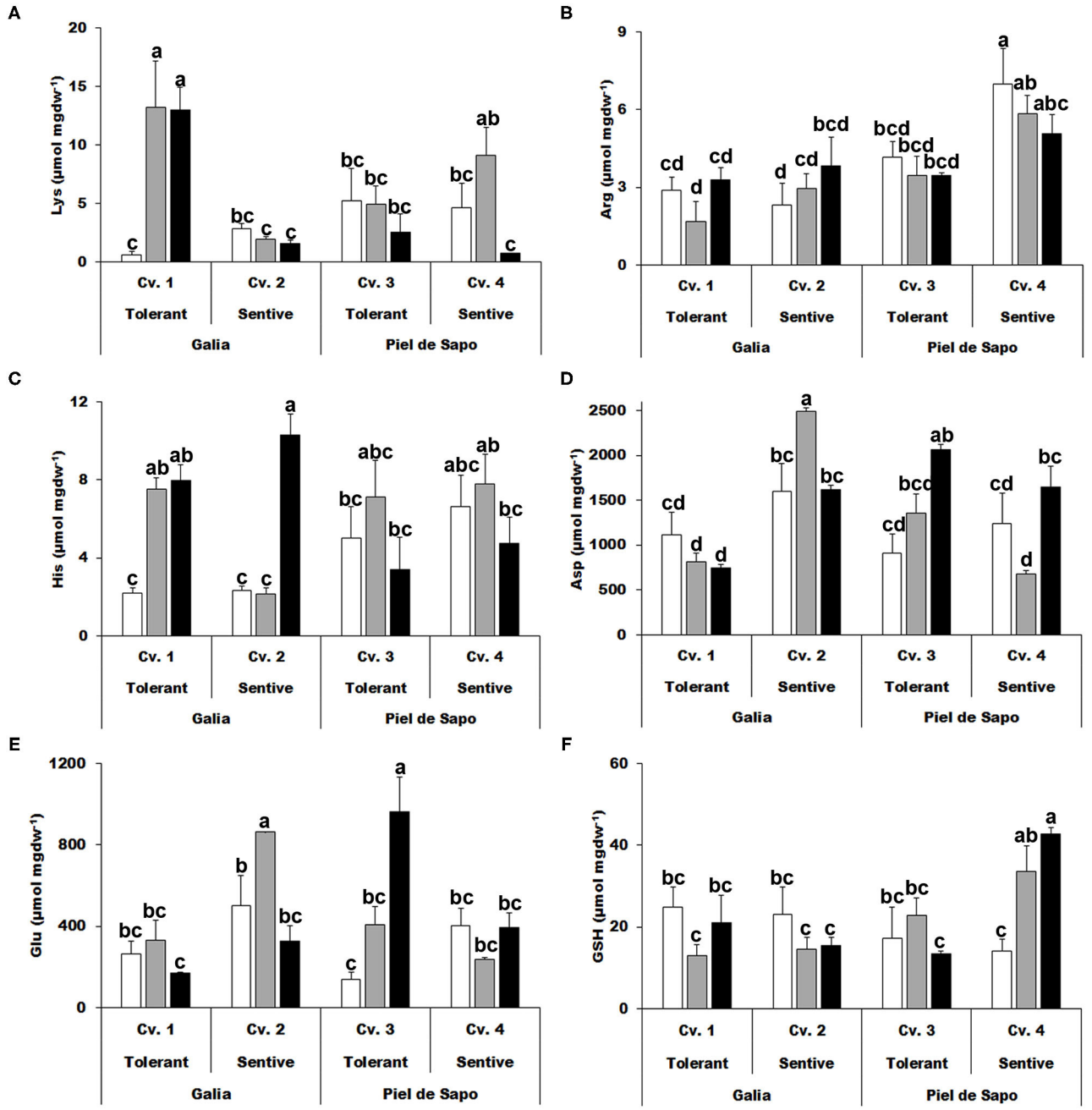

FIGURE 4 | Charged amino acids. Lysine (Lys) **(A)**; arginine (Arg) **(B)**; histidine (His) **(C)**; aspartic acid (Asp) **(D)**; glutamic acid (Glu) **(E)**; and glutathione (GSH) **(F)** concentrations of Galia tolerant genotype (Cv. 1), Galia sensitive genotype (Cv. 2), Piel de Sapo tolerant genotype (Cv. 3), or Piel de Sapo sensitive genotype (Cv. 4) under control (white bars), salt stress (gray bars), and drought stress (black bars) conditions. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test ($n = 3$). Scale bars are mean ± SE. Experiment was replicated with similar results.

investigated plants (**Figure 5A**). Sodium concentrations behaved differently depending on the variety. Sodium levels were higher in the tolerant cultivar in Galia plants, while the levels were lower in tolerant cultivars in Piel de Sapo plants (**Figure 5B**). In all cases, the Na^+/K^+ ratio was higher for tolerant cultivars (**Figure 5C**).

Hormone Determination

One of the most determinant aspects of stress tolerance is the hormonal response. Hormones, such as abscisic acid (ABA) or salicylic acid (SA), are directly involved in the response to abiotic stress, while other hormones, such as indolacetic acid (IAA) or jasmonic acid (JA) are mainly related to growth, but indirectly

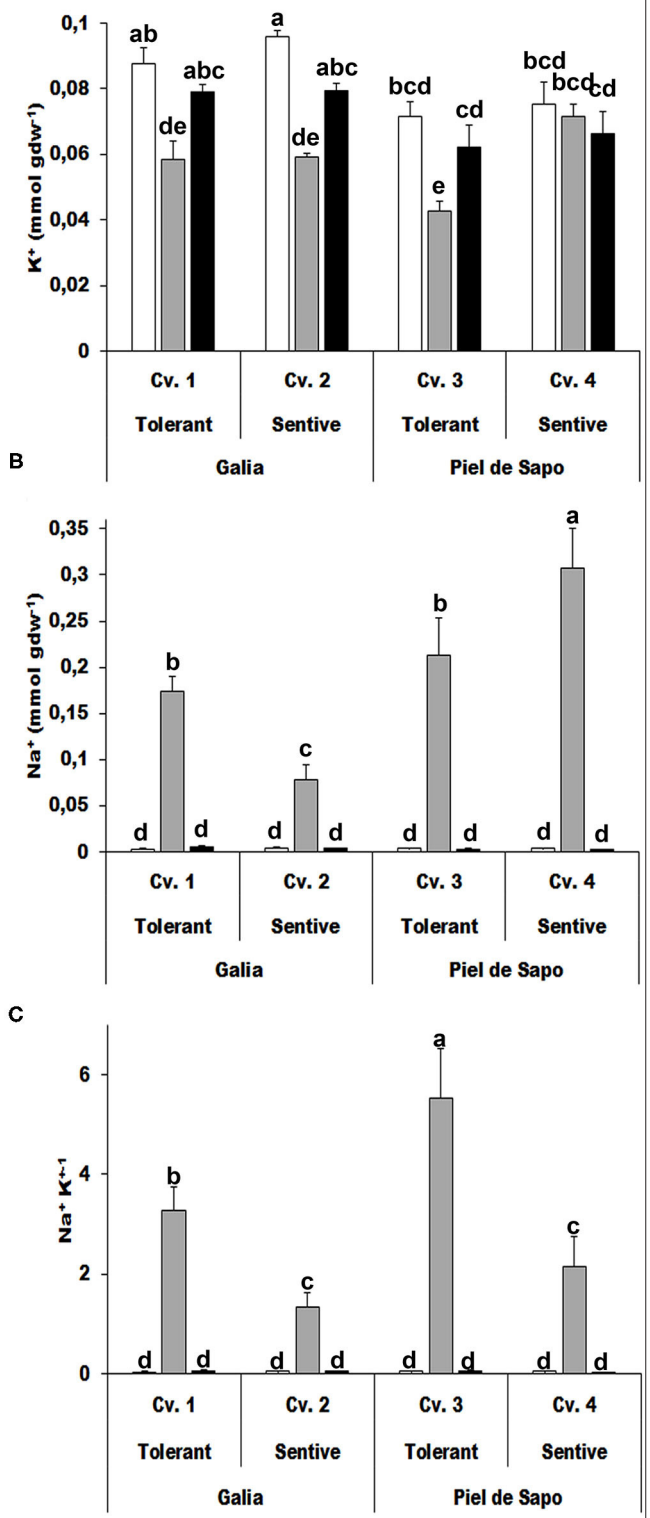

FIGURE 5 | Ion content determination. Potassium content (K^+) **(A)**, sodium content (Na^+) **(B)**, and the Na^+/K^+ ratio **(C)** of the tolerant Galia genotype (Cv. 1), sensitive Galia genotype (Cv. 2), tolerant Piel de Sapo genotype (Cv. 3), or *(Continued)*

FIGURE 5 | sensitive Piel de Sapo genotype (Cv. 4) under control (white bars), salt stress (gray bars), and drought stress (black bars) conditions. Data with different letter differ significantly ($p < 0.05$), as determined by Duncan's MRT test ($n = 5$). Scale bars are mean ± SE. Experiment was replicated with similar results.

may affect the response to abiotic stress. We determined the levels of different hormones under control and stress conditions. IAA concentrations increased 17-fold under salt stress in the tolerant cultivar of the Piel de Sapo variety (**Figure 6A**). Levels of JA decreased upon stress in the tolerant Galia cultivar (70% for salt stress and 43% in drought stress) and increased (1.91-fold for salt stress and 3.53-fold drought stress) in the sensitive cultivar (**Figure 6B**). As expected, ABA levels increased upon stress, but the increase was more pronounced in Piel de Sapo plants under salt stress (between 8 and 11 fold) (**Figure 6C**). SA levels also increased upon stress but, again, only in Piel de Sapo plants (**Figure 6D**).

DISCUSSION

The main objective of this study is to compare physiological and biochemical responses of two cultivars of two different varieties, to both salt and drought stress, in order to find common patterns among different varieties. We included stress tolerant and sensitive cultivars as well to gain further insight into differential responses within varieties. Through the relativization of data (i.e., the ratio of the value under stress with respect to the value under control conditions) we have found that, irrespectively of the variety, tolerance to salt stress correlates with higher ratios (stress/control) for His (3.4 and 1.42 for tolerant vs. 0.92 and 1.18 for sensitive) and Na^+/K^+ (11, 0.37, and 91.3 for tolerant vs. 31.4 and 47.6 for sensitive) and lower Phe (0.93 and 1.06 for tolerant vs. 1.45 and 2.0 for sensitive) and Pro ratios (0.71 and 0.75 for tolerant vs. 1.41 and 0.83 for sensitive). In the case of drought stress, tolerance correlates with increased ratios of Ile (1.87 and 1.1 for tolerant vs. 0.78 and 0.5 for sensitive), Gly (5.68 and 1.12 for tolerant vs. 0.85 and 0.5 for sensitive), Ser (2.72 and 1.17 for tolerant vs. 0.75 and 0.46 for sensitive) and Asn (2.12 and 1.38 for tolerant vs. 0.79 and 0.32 for sensitive), and decrease ratios of Hydric potential (1.63 and 1.33 for tolerant vs. 2.67 and 1.84 for sensitive) and Phe (1.26 and 1.03 for tolerant vs. 2.0 and 1.69 for sensitive) and Trp (1.08 and 1.79 for tolerant vs. 2.0 and 7.61 for sensitive) (**Figure 7A**, **Supplementary Table 1**). All the results are summarized in the form of a heat map in **Figure 7B**. The numerical data of the ratios of the Stress/control for all values are presented in **Supplementary Table 1**.

One interesting aspect of our results is that, among varieties, physiological parameters are not a distinctive trait for abiotic stress tolerance. Several previous studies have determined the effect of stress on melon physiology (Zhang et al., 2021). In a recent study on muskmelon genotypes under drought stress, the net photosynthetic rate, stomatal conductance (Gs), and the transpiration (E) rate decreased (Ansari et al., 2019). Other

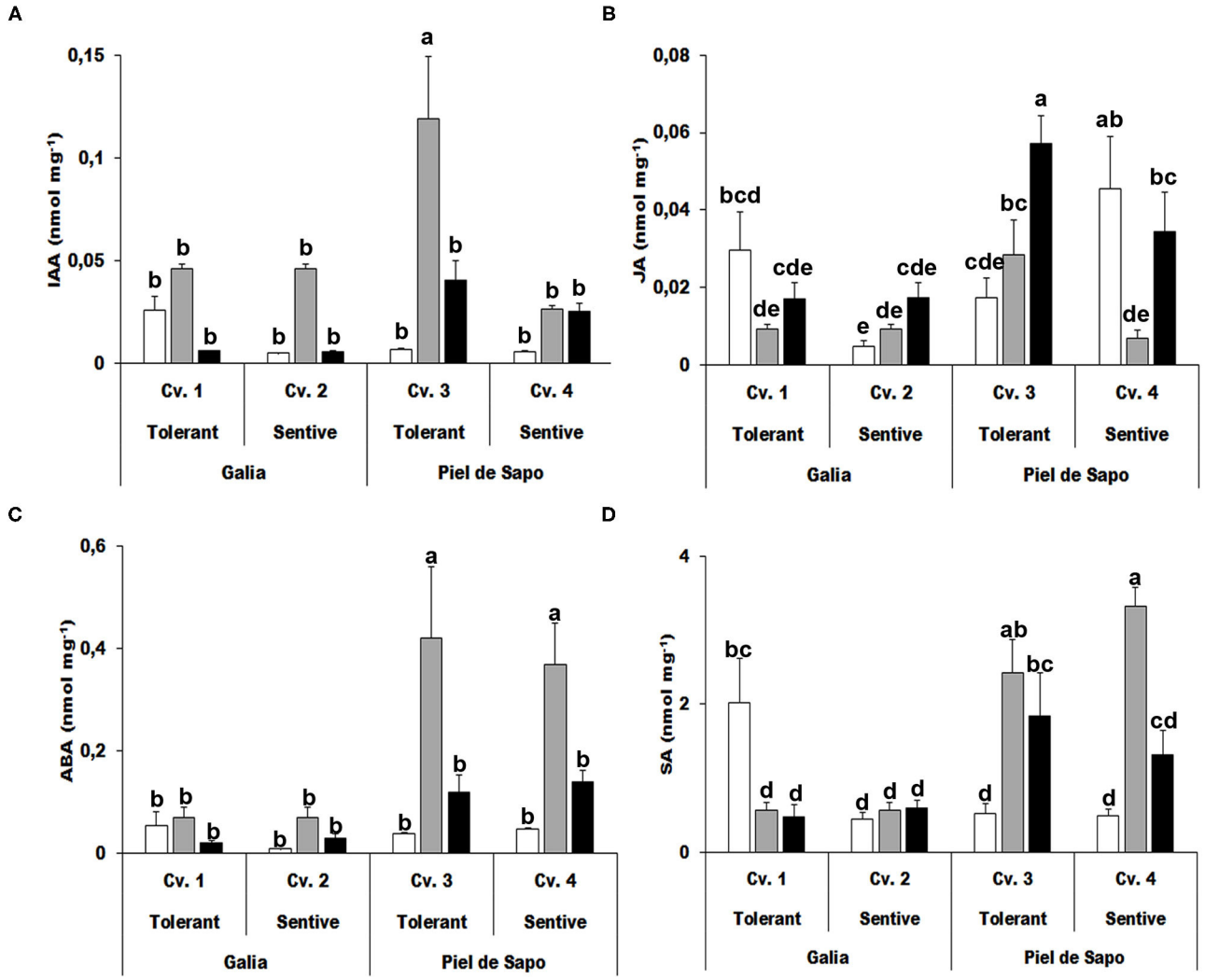

FIGURE 6 | Hormone concentrations. Indolacetic acid (IAA) **(A)**; jasmonic acid (JA) **(B)**; abscisic acid (ABA) **(C)**, and salicylic acid (SA) **(D)** concentrations of Galia tolerant genotype (Cv. 1), Galia sensitive genotype (Cv. 2), Piel de Sapo tolerant genotype (Cv. 3), or Piel de Sapo sensitive genotype (Cv. 4) under control (white bars), salt stress (gray bars), and drought stress (black bars) conditions. Data with different letters differ significantly ($p < 0.05$), as determined by Duncan's MRT test ($n = 5$). Scale bars are mean ± SE. Experiment was replicated with similar results.

reports described similar decreases in stomatal conductance in genotypes different from the ones that we have used in this study (Kusvuran, 2012; Wang et al., 2016). There are reports indicating that during drought stress in melon, there was a significant increase in water use efficiency in drought tolerant genotypes (Akhoundnejad and Dasgan, 2019). In our case, under drought conditions, we did not observe any significant differences with the control values, that is, efficiency is maintained, although in this report plants were grown in field conditions and measures were taken in older plants.

We have also calculated the differential traits between Galia and Piel de Sapo irrespectively of their stress tolerance (**Supplementary Figure 1**). In this case, the Piel de Sapo variety showed higher Stress/control ratio for E, A, WUEintr and WUEinst under salt stress (**Supplementary Figure 1**). In agreement with our results, it has been previously described that under salt stress in muskmelon there is a significant increase in WUEintr and WUEinst with respect to control conditions (Ansari et al., 2018). Our data under saline stress conditions showed no changes for Galia plants, but we confirmed the increase in our conditions for Piel de Sapo cultivars. The fact that most of the differences observed in the physiological traits are variety dependent and not stress dependent may be explained by the differences in the leaf morphology.

There is no description available in the literature regarding the behavior of the free amino acid pools under salt and drought stress in *Cucumis melo* comparing stress and different varieties, so here we have investigated the complete free amino acid profile in our plants under the studied conditions. Salt tolerance correlated with higher levels of His in tolerant plants. His has been related to tolerance against heavy metals as it can chelate them, but its role in salt stress tolerance has not been described. Proline it is known

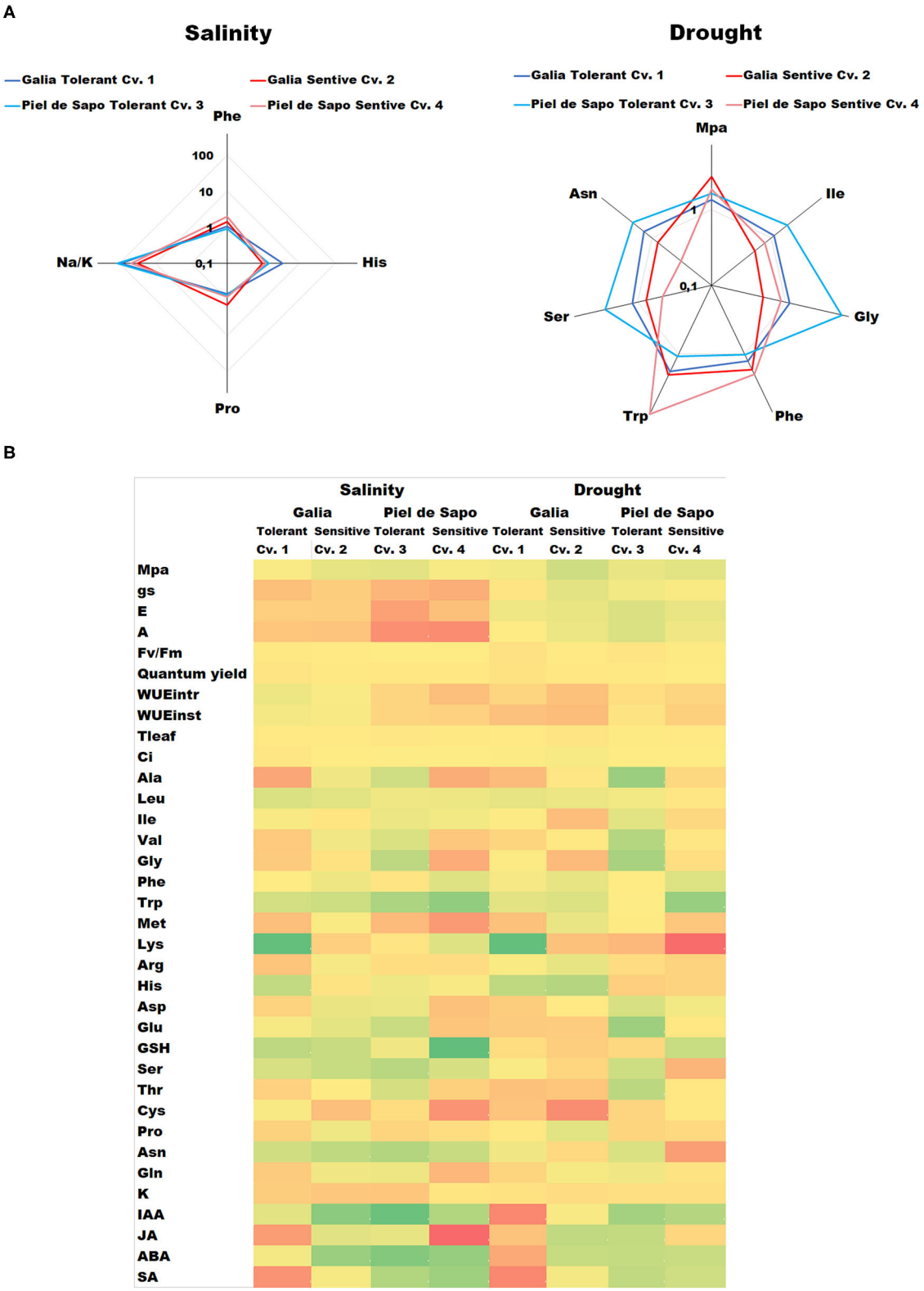

FIGURE 7 | Summary of the main findings of this study. **(A)** Radial diagrams of the ratio between stress/control concentration under salt stress (left) or stress/control concentration under drought stress (right). The tolerant cultivars are shown in shades of blue and the sensitive cultivars in shades of red. The values are represented in a decimal logarithmic scale. **(B)** Heat map of all the results obtained in the present study. Green indicates higher stress/control values, yellow average stress/control values while red indicates lower stress/control values in a decimal logarithmic scale.

to act as and osmolyte, but we have found that tolerant plants have less stress/control ratio than the sensitive ones, pointing out that the increases observed upon salt stress are not determinant for salt stress tolerance. In the case of drought stress, Ile, Gly, Ser and Asn were higher in tolerant varieties. Glycine can act as an osmolyte and also is a component of the tripeptide glutathione. Serine is also a precursor of cysteine and other stress-related molecules. On the other hand, high stress/control ratios of Phe correlated with sensitivity to stress, and it is the only molecule that decreased under conditions of both drought and salt stress. Phe is a precursor of several molecules, among them lignin, a pivotal molecule for cell wall biosynthesis. The accumulation of Phe in sensitive cultivars, irrespectively of the varieties, may be a symptom that basic plant processes like cell wall biosynthesis are more affected by stress than in tolerant cultivars.

Potassium is the major ion in the cytoplasm and thus is largely responsible for the intracellular ionic environment. Sodium is toxic for melon plants and must be extruded from the cell, or accumulated in the vacuole. Regarding ion accumulation, our results suggest that the limiting factor for stress response is the ability to accumulate sodium (Serrano et al., 1999; Rodríguez-Navarro, 2000). Under salt stress, plants can extrude sodium from the root, or take it up, transport it to the aerial part and accumulate it in the vacuoles (Arzani and Ashraf, 2016). The higher Na^+ and Na^+/K^+ ratio of a salt-tolerant variety in our study may be explained by the vacuolar accumulation of sodium in the leaf tissues. Similar results regarding the Na^+/K^+ ratio and water use efficiency were observed in a field trial with *Cucumis melo cv. Huanghemi* (Tedeschi et al., 2017) so the trend is the same, even when we compare field/greenhouse conditions and early development/late develompment. Here we demonstrate that the ability to accumulate sodium and maintain a high Na^+/K^+ ratio is a distinctive trait for tolerant cultivars. We did not find any distinctive pattern with the potassium levels under drought stress. Therefore, the role of potassium as an osmolyte is not a limiting factor for drought tolerance in these melon cultivars.

Melon is a climacteric fruit, so its hormonal levels are subjected to drastic changes (Dunlap et al., 1996). There are several descriptions in the literature of the hormonal levels in cucurbit plants under abiotic stress. For instance, exogenous application of SA increases drought tolerance in muskmelon (Korkmaz et al., 2007), similar to what is observed in other cultivated plants (Souana et al., 2020). In addition, SA and JA levels increase upon spermidine addition and increase tolerance to salt stress (Radhakrishnan and Lee, 2013). Also, JA levels tend to increase in cucumber plants subjected to drought stress (Llanes et al., 2016). It has also been described that under mild or moderate water stress, IAA concentrations tend to increase (Huang et al., 2018). ABA is the main player in the abiotic stress response in plants, and it has also been described to increase upon abiotic stress in melon (Sun et al., 2013). When we studied the phytohormone levels, we did not find any common pattern among the sensitive or tolerant varieties and cultivars studied, although the concentrations of SA and IAA were higher in Piel de Sapo cultivars (between 3- and 7-fold for SA and 4- to 17-fold for IAA).

Taken together, we have performed a complete study of two different melon varieties comparing sensitive and tolerant cultivars and applied statistical tools to the results to find common patterns in salt or drought stress responses that could be useful to predict the behavior of uncharacterized varieties and cultivars and to design novel classical or biotechnological breeding strategies. Varieties or cultivars with increased His content and/or the ability to accumulate sodium (likely in the vacuoles) may display improved tolerance to salt stress, while novel varieties with enhanced levels of Ile, Gly, Ser, and Asn could show better performance under drought stress conditions. High levels of Phe seem correlate with diminished tolerance to abiotic stress. Thus, our results have provided a useful framework for future studies which will examine the ability of these parameters to predict stress tolerance performance in additional melon varieties and cultivars.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, witho undue reservation.

AUTHOR CONTRIBUTIONS

SC, LD-E, and GM-S cultivated the plants, obtained the samples, and performed the experiments. LM and AV did all the physiological determinations and the calculations. JL-N did the amino acid measurements. LY and JM analyzed the results and designed the figures. JB did the statistical analysis and analyzed the results. GM-S, LY, and JM wrote the manuscript. JM designed the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.777060/full#supplementary-material

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Storage Property Is Positively Correlated With Antioxidant Capacity in Different Sweet Potato Cultivars

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Song H-H, Zhou Z-L, Zhao D-L, Tang J, Li Y-H, Han Z, Chen X-Y, Hu K-D, Yao G-F and Zhang H (2021) Storage Property Is Positively Correlated With Antioxidant Capacity in Different Sweet Potato Cultivars. Front. Plant Sci. 12:696142. doi: 10.3389/fpls.2021.696142

Sweet potato decays easily due to its high respiration rate and reactive oxygen species (ROS) accumulation during postharvest storage. In this study, we explored the relationship between antioxidant capacity in leaves and storage properties in different sweet potato cultivars, the tuberous roots of 10 sweet potato cultivars were used as the experimental materials to analyze the storage property during storage at 11–15°C. According to the decay percentage after 290 days of storage, Xu 32 was defined as a storage-tolerant cultivar (rot percentage less than 25%); Xu 55-2, Z 15-1, Shangshu 19, Yushu, and Zhezi 3 as above-moderate storage-tolerant cultivars (rot percentage ranging from 25 to 50%); Sushu 16, Yanshu 5, and Hanzi as medium-storable cultivars (rot percentage 50–75%); and Yan 25 as a storage-sensitive cultivar (rot percentage greater than 75%). Meanwhile, analysis of the α-amylase activity in root tubers of the 10 sweet potato cultivars during storage indicated that α-amylase activity was lowest in the storage-tolerant cultivar Xu 32 and highest in the storage-sensitive cultivar Yan 25. Evaluation of antioxidant enzyme activities and ROS content in the leaves of these 10 cultivars demonstrated that cultivar Xu 32, which showed the best storage property, had higher antioxidant enzyme activity [superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POD)] but lower lipoxygenase (LOX) activity, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents, and superoxide anion radical ($O_2\cdot^-$) production rates compared with those of the storage-sensitive cultivar Yan 25 and the medium-storability cultivars Hanzi, Yanshu 5, and Sushu 16. Additionally, principal component analysis (PCA) suggested that sweet potato cultivars with different storage properties were clustered separately. Correlation and heat map analysis further indicated that CAT, APX, POD, and SOD activities were negatively correlated with α-amylase activity, while LOX activity and MDA and H_2O_2 contents were negatively correlated with the storage property of sweet potato. Combined, our findings revealed that storage property is highly correlated with antioxidant capacity in sweet potato leaves and negatively correlated with α-amylase activity in tuberous roots, which provides a convenient means for the screening of storage-tolerant sweet potato cultivars.

Keywords: sweet potato, storage property, antioxidant capacity, reactive oxygen species (ROS), correlation analysis

INTRODUCTION

Sweet potato (*Ipomoea batatas* L.), which was domesticated in tropical America, is gradually becoming one of the main food crops worldwide (Mwanga et al., 2017). According to the Food and Agriculture Organization (FAO) of the United Nations, global sweet potato production exceeded 140 million tons in 2019, with China accounting for the largest plantation area (FAO, 2019). Sweet potatoes are rich in many nutrients such as vitamins, dietary fiber, and minerals, as well as other ingredients that are beneficial to human health, including flavonoids, carotenoids, and anthocyanins (Wang et al., 2016; Kang et al., 2017). Additionally, starch is the major component of the storage root of sweet potato, accounting for 50–80% of its dry matter (Zhang et al., 2017). Amylase activity has been reported to change in sweet potato roots during storage (Takahata et al., 1995; Zhang et al., 2002). Sweet potato tubers are relatively difficult to store long-term due to their high moisture content and respiration rate, as well as the deterioration of the quality of its flesh during postharvest (Sugri et al., 2017). During postharvest storage, endogenous α-amylase and β-amylase enzyme activities influence the starch structure and reduce the starch content, which greatly affects the commodity value of this tuberous root (Lu et al., 2020). Sweet potato is also susceptible to chilling injury owing to its tropical origins (Li et al., 2018). Combined, these observations are indicative of the importance of postharvest storage for the industrial application of sweet potatoes.

Postharvest senescence includes the loss of texture, membrane injury, and decay (Ali et al., 2020). During postharvest storage, many crops produce reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH), superoxide anion radicals ($O_2\cdot^-$), and singlet oxygen, which contribute to deteriorative changes, such as lipid peroxidation, DNA mutation, enzyme inactivation, and protein denaturation (Tian et al., 2013). Consequently, ROS generation is considered the main reason for the progression of senescence (Mittler, 2002). To resist ROS-mediated damage, plants have evolved a system that maintains a balance between ROS production and elimination involving enzymatic [superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), and peroxidase (POD; EC 1.11.1.7)] and non-enzymatic antioxidants (Miśkiewicz et al., 2000). Despite this, ROS accumulation may exceed the antioxidant capacity, leading to membrane lipid peroxidation and impaired cellular functions (Lurie et al., 1991). Several studies have demonstrated that some plants can delay senescence by eliminating excessive ROS through enhanced antioxidant systems (Zimmermann et al., 2006; Qin et al., 2009). For instance, ultrasonic treatment was shown to effectively decrease the activities of PPO and POD and increase total antioxidant capacity, which help to inhibit the browning of fresh-cut sweet potato, thereby prolonging its postharvest shelf life (Pan et al., 2020). This indicates that antioxidant enzyme capacity is positively correlated with delayed senescence in postharvest fruits and vegetables, which can help prolong their shelf life.

Several studies have investigated the optimization of storage conditions during postharvest sweet potato storage; however, the nature of the endogenous factors that influence the storage characteristics of different sweet potato cultivars remains unclear (Fan et al., 2015; Ji et al., 2017). de Araujo et al. (2021) reported that cold-tolerant sweet potato cultivars have stronger antioxidant enzyme activities compared with those of cold-sensitive cultivars, suggestive of the important role of the antioxidant system in eliminating excessive ROS induced by low temperature. Additionally, under optimal storage temperatures, the activities of antioxidant enzymes increase in nectarines and broccoli, thereby prolonging the postharvest storage period (Zhang Z. et al., 2009; Zhao et al., 2018), while greater antioxidant enzyme activity is also associated with better storage performance in sweet potato cultivars (Tang et al., 2019). However, relatively few studies have systematically evaluated the correlation between the antioxidant system and storage property. Moreover, the screening of storage-tolerant sweet potato cultivars based on the storage property of sweet potato tubers is time-consuming and requires specific storage conditions. In this study, 10 sweet potato cultivars were selected to assess the relationship between the storage property of root tubers and the antioxidant capacity of the leaves. The sweet potato root tubers were stored at 11–15°C for 290 days, following which the rot percentage, weight loss, and α-amylase activity of the different cultivars were assessed, as were differences in antioxidant enzyme activities and ROS-related indexes in the leaves. Furthermore, the relationship between the storage property of the root tubers and the antioxidant capacity of the leaves was investigated by principal component analysis (PCA) and correlation analysis. Combination of this study provides a new method for the rapid screening of sweet potato tuber storability that involves analyzing the biochemical and physiological parameters of sweet potato leaves.

MATERIALS AND METHODS

Plant Materials and Sample Preparation

In this study, 10 sweet potato cultivars—Xu 32, Xu 55-2, Z 15-1, Shangshu 19, Sushu 16, Yanshu 5, Hanzi, Yushu, Zhezi 3, and Yan 25—were selected from the National Sweet Potato Improvement Center (Xuzhou, Jiangsu Province, China). Undamaged root tubers of each cultivar (three replicates of 100 ± 10 tubers) were harvested in the autumn of 2013–2015 and stored for 290 days at 11–15°C. The storage property of the sweet potato cultivars was defined according to the decay percentage of the root tubers. Sweet potato cultivars with a rot percentage of less than 25% were classified as storage-tolerant; those with a rot percentage ranging from 25 to 50% were classified as above-medium storage-tolerant; those with a rot percentage between 50 and 75% were classified as medium-storable; and those with a rot percentage higher than 75% were classified as storage-sensitive (Zhang and Fang, 2006). Each cultivar was assigned a storability score based on the rot percentage. Additionally, the weight loss percentage of the sweet potato tubers was also recorded by determining the tuber weight before and after storage. Tuberous roots without pests, disease, or mechanical damage were selected for the experiment. The stem cuttings of 10 sweet potato cultivars were obtained

from the National Sweet Potato Improvement Center in May 2016 and planted in the greenhouse at the Hefei University of Technology in Hefei, China, at 24°C under a 16/8-h light/dark cycle. After 2 months of growth, the mature leaves (from the second-to-top to the fifth-to-top) of 10 seedlings from each cultivar were sampled, immediately frozen in liquid nitrogen, and ground to a powder. The powder was stored at −80°C for subsequent analysis.

Determination of α-Amylase Activity in Sweet Potato Roots

The α-amylase activity in the tuberous roots of the sweet potato cultivars was determined at 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, and 290 days after storage (DAS) as described by Zhang et al. (2009). Sweet potato root samples (2.0 ± 0.05 g) were homogenized in 4 ml of 0.1 M NaAc (including 6 M $CaCl_2$, pH 5.0) and centrifuged at 20,000 × *g* for 20 min. Then, 0.3 ml of the supernatant was mixed with 0.5 ml of β-limit dextrin and 0.2 ml of 10 mM NaAc and incubated at 30°C. After incubation, 5 ml of 0.01% I_2-KI and 0.4 ml of H_2O were added to 0.1 ml of the reaction solution, and the absorbance was determined at 560 nm. One unit of α-amylase activity was defined as the amount of enzyme needed to degrade 1 mg of β-limit dextrin per minute and was represented as U/g fresh weight (FW).

Determination of Antioxidant Enzymes (i.e., Peroxidase, Catalase, Ascorbate Peroxidase, and Superoxide Dismutase) in Sweet Potato Leaves

The POD, CAT, APX, and SOD activities were determined following the method described by Garcìa-Limones et al. (2002). Sweet potato leaves (2.0 ± 0.05 g) were homogenized in 3 ml of enzyme extract buffer (50 mM K_2PO_4 pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM ascorbic acid (ASA), and 5% polyvinylpyrrolidone (PVP)) at 4°C and centrifuged at 12,000 × *g* for 30 min at 4°C. After centrifugation, the obtained supernatant was considered the crude enzyme solution.

The SOD activity was determined by the photochemical reduction of nitroblue tetrazolium (NBT) in the presence of riboflavin. One unit of SOD activity was defined as the amount of enzyme that inhibited the reduction of NBT by 50%; SOD activity was expressed as U/g FW. The determination of POD activity was based on the increase in absorbance at 470 nm resulting from the oxidation of guaiacol in the presence of H_2O_2. CAT activity was determined as the rate of decrease in absorbance at 240 nm using H_2O_2 as the substrate. APX activity was determined by measuring the changes in absorbance at 290 nm. The reaction system (3 ml total volume) included 50 mM phosphate buffer at pH 7.0, 15 mM ascorbic acid, 15 mM H_2O_2, and the appropriate amount of crude enzyme solution. One unit of POD, CAT, or APX activity was defined as an increase or decrease of 0.01 in the absorbance value per minute and was represented as U/g FW.

Determination of Superoxide Anion Radical Production, Hydrogen Peroxide, and Malondialdehyde Content in Sweet Potato Leaves

The H_2O_2 content and $O_2\cdot^-$ production were determined according to the methods described by Ge et al. (2017). For the determination of $O_2\cdot^-$ production, 2 g of leaf powder was homogenized in 0.1 mM phosphate buffer, pH 7.8, and centrifuged at 12,000 × *g* for 30 min at 4°C; the supernatant was used for $O_2\cdot^-$ determination. Each sample was divided into an experimental group and a control group. Notably, 1 ml each of the supernatant, H_3PO_4 buffer, and 1 mM $HONH_3Cl$ was mixed in a test tube and incubated at 25°C for 1 h. Then, 17 mM *p*-aminobenzenesulfonic acid and 7 mM α-naphthylamine were added and mixed, followed by incubation for an additional 20 min. Absorbance was determined at 530 nm. The $O_2\cdot^-$ production rate was calculated on an FW basis in $\mu mol\cdot g^{-1}\cdot s^{-1}$. For the determination of the H_2O_2 content, 2 g of sweet potato leaf powder was homogenized in 3 ml of precooled acetone and centrifuged at 12,000 × *g* for 30 min. The H_2O_2 content was measured by determining the absorbance at 508 nm. The content of malondialdehyde (MDA), which is considered to be an indicator of the degree of plant oxidative stress, was determined according to the method described by Chen et al. (2018), with slight modifications. Sweet potato samples (2 g) were homogenized in 10 ml of 5% trichloroacetic acid and centrifuged at 12,000 × *g* for 30 min at 4°C. The absorbance of the resulting supernatant was measured at 600, 532, and 450 nm. The MDA content was calculated using the equation: MDA content (nmol/g) = $[6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}] \times V_1 \times V_3/(V_2 \times W)$, where V_1, V_2, and V_3 indicate the total volume of the solution obtained after the reaction (ml), the volume of the extract solution used for the reaction (ml), and the volume of the extract solution (ml), respectively; W indicates the mass of the sample (g).

Determination of Lipoxygenase Activity in Sweet Potato Leaves

Lipoxygenase (LOX) activity was determined by the procedure described by Surrey (1964). Sweet potato leaf powder (2 g) was homogenized in 5 ml of 0.1 M H_3PO_4 buffer, pH 6.8 [4% PVPP (polyvinylpolypyrrolidone) and 1% Triton X-100] and centrifuged at 12,000 × *g* for 30 min at 4°C. The obtained supernatant was considered the crude enzyme solution. The reaction solution contained 0.1 M NaAc buffer, pH 5.5, 0.01 M sodium linoleate, and the appropriate amount of crude enzyme solution. Absorbance was measured at 234 nm. One unit of LOX was defined as a decrease of 0.01 optical density (OD) value in absorbance per minute, and the results were expressed as U/g FW.

Data Analysis

The physiological parameters of the sweet potatoes were analyzed using IBM SPSS 22.0 (IBM Corp., Armonk, NY, United States). The correlation among antioxidant enzyme activities, LOX activity, ROS-related indexes, and storage property of the different sweet potato cultivars, the heat map of the physiological

parameters, and the PCA were assessed using the tools on the OmicShare platform[1] (Gene Denovo, Guangzhou, China).

RESULTS

Determination of the Storage Property of the 10 Sweet Potato Tubers

Decay percentage is one of the basic indexes used to evaluate the storage properties of sweet potatoes. In this study, 10 sweet potato cultivars (i.e., Xu 32, Xu 55-2, Z 15-1, Shangshu 19, Sushu 16, Yanshu 5, Hanzi, Yushu, Zhezi 3, and Yan 25) were selected to evaluate the decay percentage during storage. The storage properties of the different sweet potato cultivars are shown in **Table 1**. Xu 32 was found to be a storage-tolerant cultivar; Xu 55-2, Z 15-1, Shangshu 19, Yushu, and Zhezi 3 above-medium storage-tolerant cultivars; Sushu 16, Yanshu 5, and Hanzi medium-storable cultivars; and Yan 25 a storage-sensitive cultivar. Each sweet potato cultivar was assigned a storability score ranging from 1 (storage-sensitive) to 4 (storage-tolerant). The weight loss percentage of the tubers after 290 days of storage was also determined. The lowest weight loss percentage (18.1%) was observed in Xu 55-2 and highest (67.1%) in Sushu 16; however, no correlation was found between weight loss and storage property (**Table 1**).

[1]https://www.omicshare.com/

Changes in α-Amylase Activity in the Roots of the 10 Sweet Potato Cultivars During Storage

Amylase activity is responsible for starch degradation during the postharvest storage of sweet potatoes. To evaluate the correlation between α-amylase activity and storage property of the different cultivars, α-amylase activity in sweet potato tubers was determined at 30-day intervals during the 290-day storage period. As shown in **Figure 1A**, before storage, the lowest α-amylase activity was observed in the storage-tolerant cultivar Xu 32, while the highest was found in the storage-sensitive cultivar Yan 25. With increasing storage time, the α-amylase activity of the 10 sweet potato root tubers showed an increasing trend, peaking at 290 DAS (**Figure 1B**). During storage, α-amylase activity was lowest in the storage-tolerant cultivar Xu 32 and highest in the storage-sensitive cultivar Yan 25. Between days 30 and 90 of storage, the α-amylase activity of the storage-tolerant and above-medium storage-tolerant cultivars was stable and remained at a low level, whereas that of the storage-sensitive cultivar Yan 25 showed a significant increase during this storage period. These results demonstrated that α-amylase activity was lower in the storage-tolerant cultivar than in the storage-sensitive cultivar at all storage periods evaluated, and further suggested that α-amylase activity is an important indicator of the storage property of the different sweet potato cultivars.

TABLE 1 | Storage property evaluation of 10 sweet potato cultivars, including Xu 32, Xu 55-2, Z 15-1, Shangshu 19, Sushu 16, Yanshu 5, Hanzi, Yushu, Zhezi 3, and Yan 25.

Variety	Xu 32	Xu 55-2	Z 15-1	Shangshu 19	Yushu	Zhezi 3	Sushu 16	Yanshu 5	Hanzi	Yan 25
Decay percentage	<25%	25–50%	25–50%	25–50%	25–50%	25–50%	50–75%	50–75%	50–75%	>75%
Weight loss	33.5 ± 3.2% E	18.1 ± 2.0% F	19.2 ± 3.4% F	42.5 ± 2.5% C	34.6 ± 2.7% E	37.9 ± 6.0% D	67.1 ± 4.1% A	37.6 ± 2.1% D	33.7 ± 6.2% E	51.7 ± 2.4% B
Storage level	Storage-tolerant	Above-moderate	Above-moderate	Above-moderate	Above-moderate	Above-moderate	Medium	Medium	Medium	Storage-sensitive
Storability score	4	3	3	3	3	3	2	2	2	1

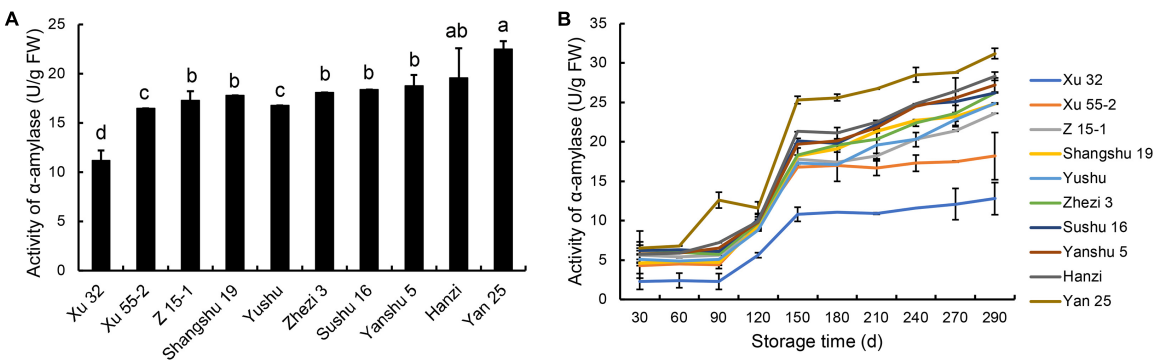

FIGURE 1 | α-amylase activity in the tuberous roots of 10 sweet potato cultivars (i.e., Xu 32, Xu 55-2, Z 15-1, Shangshu 19, Sushu 16, Yanshu 5, Hanzi, Yushu, Zhezi 3, and Yan 25). **(A)** α-amylase activity in the tuberous roots of 10 sweet potato cultivars before storage and **(B)** at 30-day intervals during 290 days of postharvest storage. FW, fresh weight; d, days. Different letters above the columns in this figure and following figures stand for significant difference between two values ($p < 0.05$).

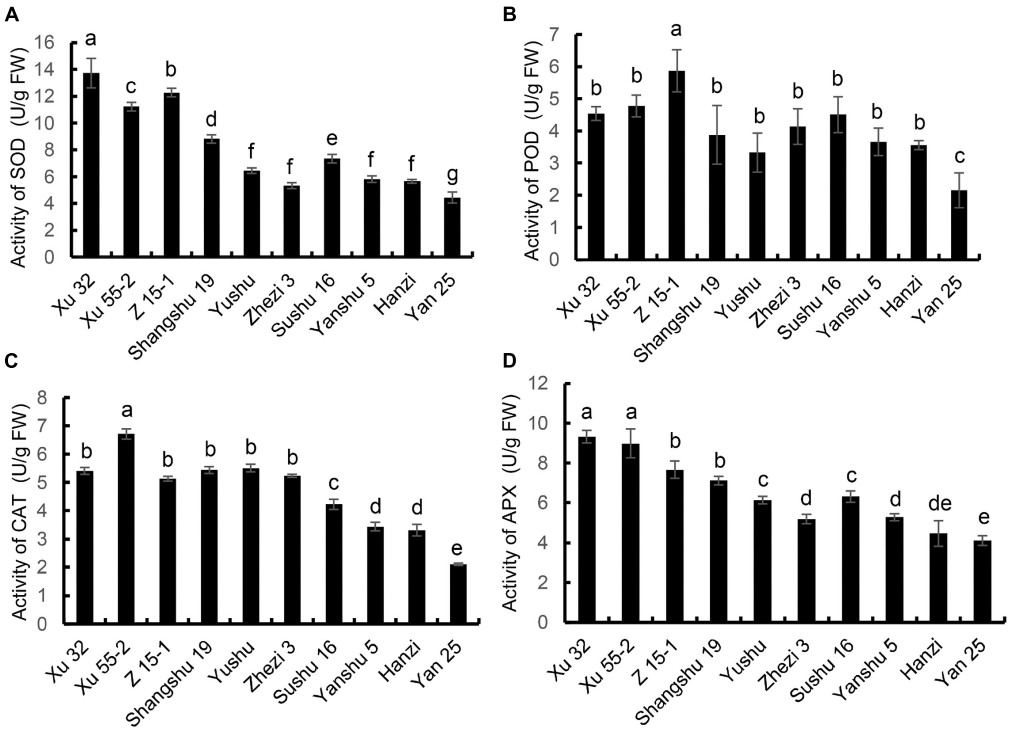

FIGURE 2 | The activities of **(A)** superoxide dismutase (SOD), **(B)** peroxidase (POD), **(C)** catalase (CAT), and **(D)** ascorbate peroxidase (APX) in the leaves of 10 sweet potato cultivars (i.e., Xu 32, Xu 55-2, Z 15-1, Shangshu 19, Sushu 16, Yanshu 5, Hanzi, Yushu, Zhezi 3, and Yan 25). Data are presented as means ± SD (n = 3). FW, fresh weight.

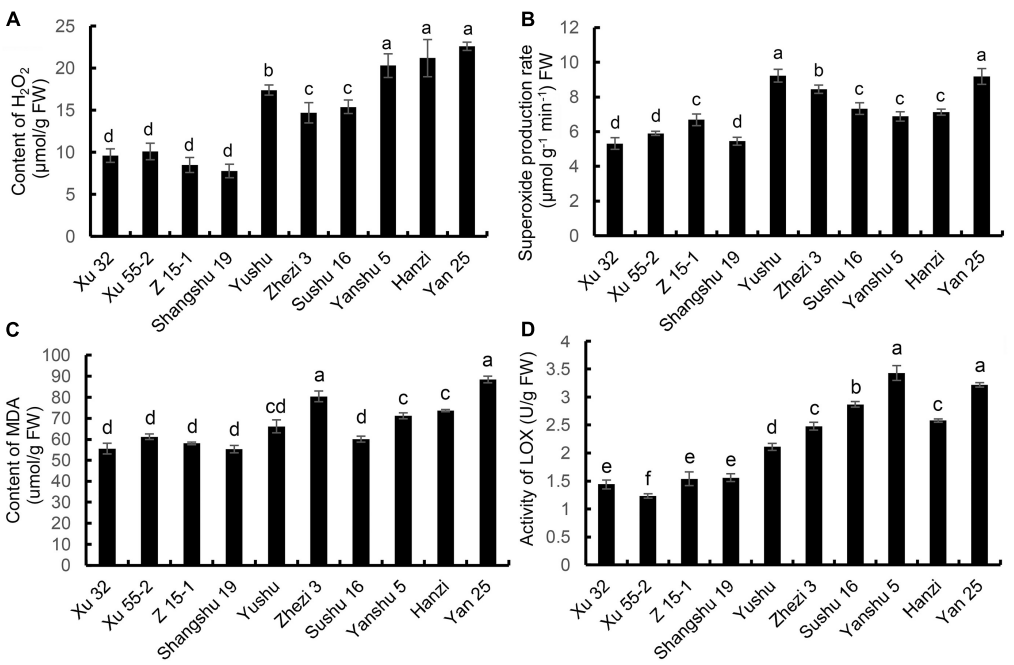

FIGURE 3 | (A) H_2O_2 content, **(B)** $O_2 \cdot^-$ production rate, **(C)** malondialdehyde (MDA) content, and **(D)** lipoxygenase (LOX) activity in the leaves of the 10 sweet potato cultivars. Data are presented as means ± SD (n = 3). FW, fresh weight.

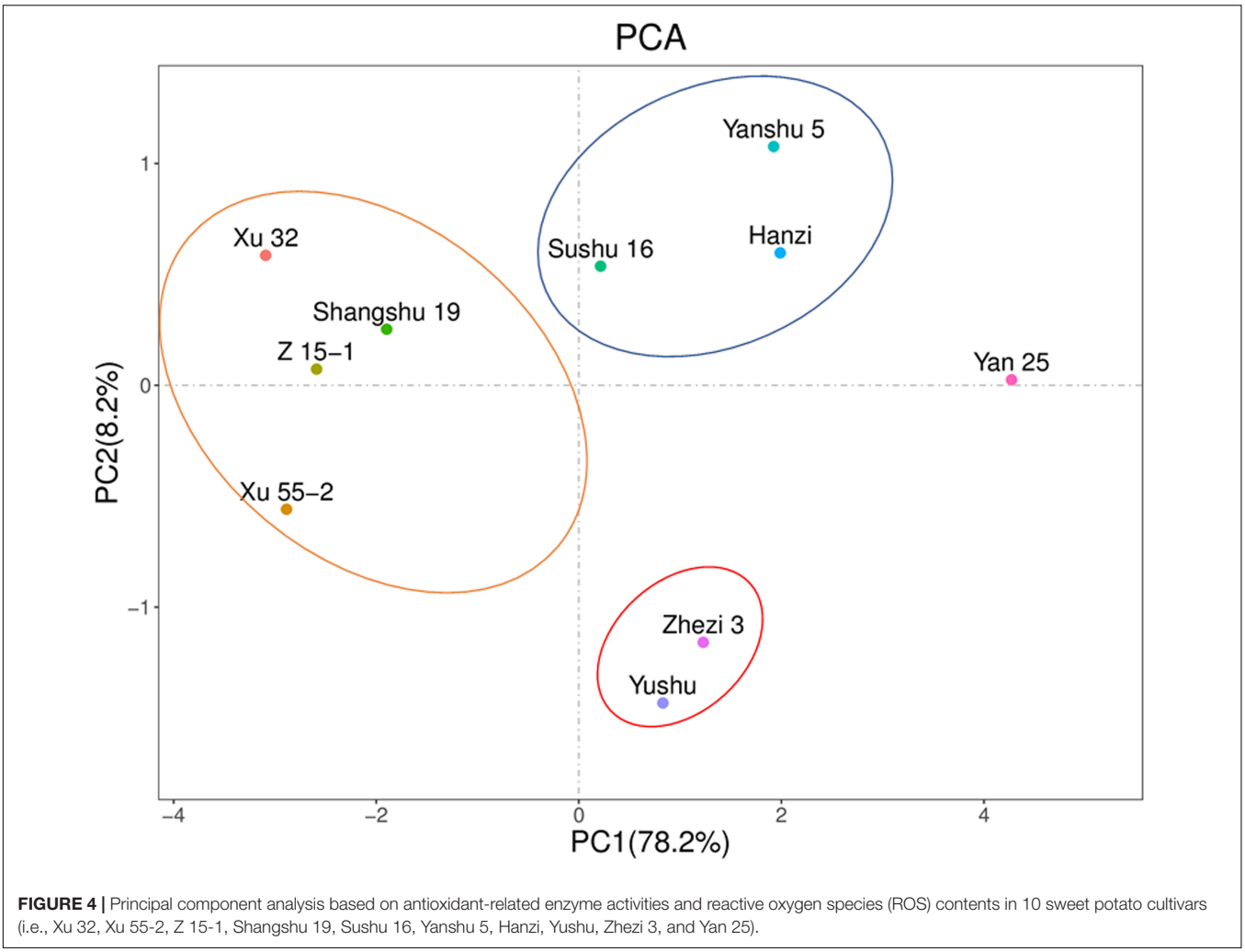

FIGURE 4 | Principal component analysis based on antioxidant-related enzyme activities and reactive oxygen species (ROS) contents in 10 sweet potato cultivars (i.e., Xu 32, Xu 55-2, Z 15-1, Shangshu 19, Sushu 16, Yanshu 5, Hanzi, Yushu, Zhezi 3, and Yan 25).

Analysis of the Activities of Antioxidant Enzymes in the Leaves of the 10 Sweet Potato Cultivars

The activities of antioxidant enzymes are required for ROS scavenging during sweet potato storage. Accordingly, we sought to determine whether a correlation existed between antioxidant enzyme activity in the leaves and tuber storage property. The results showed that SOD activity was highest in the storage-tolerant cultivar Xu 32 and lowest in Yan 25 (**Figure 2A**). SOD activity was 3.1-fold higher in Xu 32 than in the storage-sensitive cultivar Yan 25. As shown in **Figure 2B**, POD enzyme activity was generally consistent with the trend for SOD activity across the 10 sweet potato cultivars, with Yan 25 showing the lowest activity and Z 15-1 the highest (2.73-fold higher compared with that of Yan 25). The activities of CAT and APX in the 10 cultivars are shown in **Figures 2C,D**, respectively. CAT and APX activities were higher in the cultivars that showed better storage property (Xu 32, Z 15-1, Xu 55-2, Shangshu 19, Yushu, and Zhezi 3 vs. Sushu 16, Yanshu 5, Hanzi, and Yan 25). The Xu 55-2 cultivar displayed the highest CAT activity and Yan 25 the lowest. Meanwhile, APX activity was highest in Xu 32 and lowest in Yan 25. The above results indicated that the better the storage property, the higher the activities of antioxidant-related enzymes.

Changes in Superoxide Anion Radical, Hydrogen Peroxide, and Malondialdehyde Contents and Lipoxygenase Enzyme Activity in the Leaves of the Different Sweet Potato Cultivars

The changes in H_2O_2 content in the leaves of the sweet potatoes are shown in **Figure 3A**. The H_2O_2 content was lowest in the Xu 32, Xu 55-2, Z 15-1, and Shangshu 19 cultivars and highest in Yan 25. The H_2O_2 content in Yan 25 was 2.89-fold higher than that of Shangshu19. The H_2O_2 content in Xu 32, Xu 55-2, and Z 15-1, the cultivars with stronger storage performance, was significantly lower than that of Sushu 16, Yanshu 5, Hanzi, Yushu, and Zhezi 3, cultivars with reduced storage property. $O_2\cdot^-$ production and MDA content showed a pattern similar to that for the H_2O_2 content (**Figures 3B,C**). The rate of $O_2\cdot^-$ production was lowest

in the storage-tolerant cultivar Xu 32 and highest in the storage-sensitive cultivar Yan 25 (1.72-fold that of Xu 32). The MDA content showed a gradual increase with decreasing the storage property of sweet potato. Yan 25 exhibited the highest MDA content, which was 1.6-fold that of Shangshu 19, the cultivar that displayed the lowest MDA content. As shown in **Figure 3D**, LOX activity was generally low in the leaves of the Xu 32, Xu 55-2, Z 15-1, and Shangshu19 cultivars and was lowest in Xu 55-2. Overall, LOX activity was higher in the leaves of the Yan 25, Sushu 16, and Yanshu 5 cultivars than in those of Zhezi 3, Sushu 16, and Hanzi. LOX activity in Yanshu 5 and Yan 25 was 2.79- and 2.62-fold, respectively, that of Xu 55-2. These findings indicated that the cultivars with better storability had lower $O_2\cdot^-$, H_2O_2, and MDA contents, as well as lower LOX enzyme activity.

Principal Component Analysis of Antioxidant Enzyme Activities and Reactive Oxygen Species Metabolites in the Leaves of the Different Sweet Potato Cultivars

The PCA showed that PC1 and PC2 accounted for 78.2 and 8.2%, respectively, of the variability in the data (**Figure 4**). Storage-tolerant and storage-sensitive cultivars were clearly clustered in PC1. Yanshu 5, Hanzi, and Sushu 16 were clustered together, as were Xu 32, Xu 55-2, Z 15-1, and Shangshu 19, Yushu, and Zhezi 3. The variety showing the highest positive loading on PC1 was Yan 25, and the variety that showed the lowest loading on PC2 was Yanshu 5. These observations suggested that a positive correlation exists between antioxidant capacity and storage property among the different sweet potato cultivars.

Analysis of the Correlation Between Physiological Indexes and Storage Property in the Different Sweet Potato Cultivars

Then, the correlation among storage properties (i.e., α-amylase activity and rot percentage) of sweet potato roots and leaf parameters [i.e., ROS production (H_2O_2, $O_2\cdot^-$, and MDA) and antioxidant enzyme activities (i.e., POD, APX, SOD, CAT, and LOX)] was analyzed (**Figure 5**). A positive correlation was found among storage property and POD, APX, SOD, and CAT activities, as well as among α-amylase activity, LOX activity, and $O_2\cdot^-$, H_2O_2, and MDA contents. In addition, SOD, POD, CAT, and APX activities and storage properties were negatively correlated with LOX, α-amylase activities and $O_2\cdot^-$, H_2O_2, and MDA contents. SOD activity was significantly and positively correlated with APX activity (r = 0.948) and highly and negatively correlated with H_2O_2 contents (r = −0.841). POD activity was highly and positively correlated with SOD activity (r = 0.764) and highly and negatively correlated with H_2O_2 levels (r = −0.767). CAT activity was highly and positively correlated with APX activity (r = 0.799) and negatively correlated with LOX activity (r = −0.850). LOX activity showed a negative correlation with APX activity (r = −0.853) and a positive correlation with the H_2O_2 content (r = 0.877). A negative correlation was found between H_2O_2 content and SOD activity (r = −0.841). Besides, there was a significant and negative correlation between α-amylase activity and storage property (r = −0.915) and a positive correlation between α-amylase activity and MDA content (r = 0.777). Overall, the correlation analysis indicated that the storage property of sweet potato roots is positively associated with antioxidative enzyme activity and negatively correlated with ROS metabolites in sweet potato leaves. The positive correlation detected among the activities of the antioxidant enzymes suggested that they are activated and cooperated in scavenging ROS.

Heat Map Analysis of Antioxidant-Related Indexes and Cluster Analysis of the Relationship Among the Different Sweet Potato Cultivars

To further verify the relationship between the antioxidant capacity and storage property of the different sweet potato cultivars, we generated a heat map of the antioxidant enzyme- and ROS-related indexes in the sweet potato leaves and the storage property of sweet potato (α-amylase activity). As shown in **Figure 6**, the sweet potato cultivars (i.e., Xu 32, Xu 55-2, Shangshu 19, and Z 15-1) with better storability were clustered together and showed higher activities of antioxidant related enzymes (i.e., POD, SOD, APX, and CAT), but significantly lower LOX and α-amylase activities, $O_2\cdot^-$ production rates, and H_2O_2 and MDA contents relative to the storage-sensitive cultivars (i.e., Sushu 16, Yanshu 5, Hanzi, Yan 25, Yushu, and Zhezi 3). Xu 32, the cultivar with the best storage property, had the lowest α-amylase activity, while the storage-sensitive cultivar, Yan 25, had the highest.

DISCUSSION

Owing to its tropical origins, the tuberous roots of the sweet potato are susceptible to chilling stress (Li et al., 2018). Moreover, sweet potato decays easily during storage due to its high water content (Sugri et al., 2017). Even at appropriate storage temperatures, crops still undergo deteriorative changes resulting from the activity of internal factors, such as ROS, a key contributor to postharvest senescence (Wang et al., 2019; Guo et al., 2021). ROS can be produced in plants during many metabolic reactions, but particularly in chloroplasts and mitochondria during senescence. Throughout this process, the antioxidant defense system, comprising both enzymatic and non-enzymatic antioxidants, is activated to scavenge excessive ROS, thereby preventing cellular damage (Nie et al., 2020). Antioxidant capacity was reported to be related to the storage properties of different sweet potato cultivars (de Araujo et al., 2021), while increased antioxidant enzyme activity was found to be positively correlated with sweet potato storability (Tang et al., 2019). However, whether a correlation exists between antioxidant enzyme activity in sweet potato leaves and the storage properties of the tubers has not been determined. To address this, in this study, we evaluated whether the antioxidant capacity of

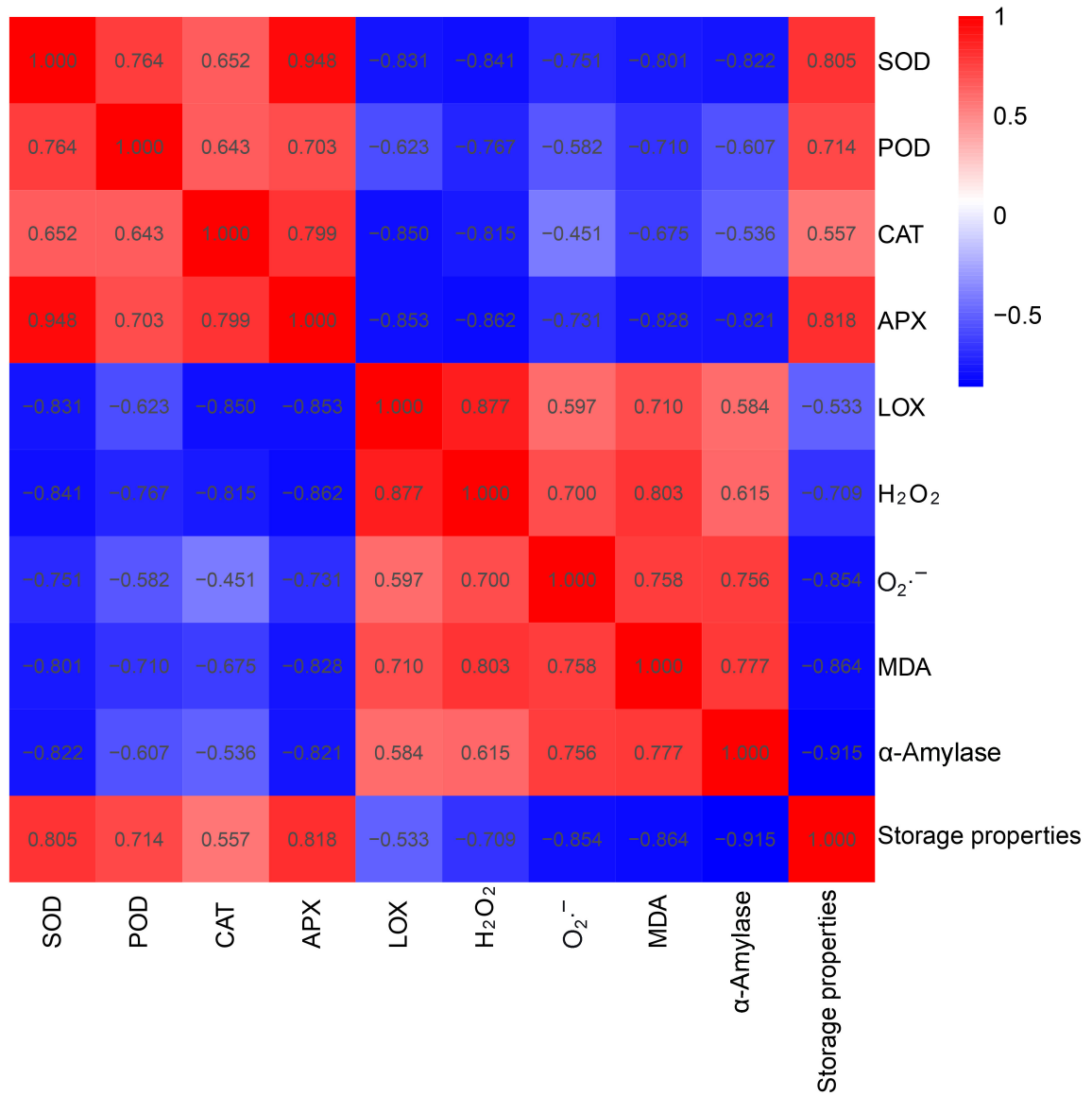

FIGURE 5 | Correlation analysis among SOD, POD, APX, CAT, and LOX activities, superoxide anion ($O_2 \cdot^-$) production, and MDA and H_2O_2 contents in the leaves and storage property and α-amylase in the roots of 10 sweet potato cultivars (i.e., Xu 32, Xu 55-2, Z15-1, Shangshu 19, Sushu 16, Yanshu 5, Hanzi, Yushu, Zhezi 3, and Yan 25).

sweet potato leaves is positively correlated with the storage property of sweet potato using 10 sweet potato cultivars as the experimental material.

The storability of the 10 cultivars was first determined based on decay percentage at 290 DAS. We found that Xu 32 is a storage-tolerant cultivar; Yan 25 is a storage-sensitive cultivar; Xu 55-2, Z 15-1, Shangshu 19, Yushu, and Zhezi 3 are above-medium storage-tolerant cultivars; and Sushu 16, Yanshu 5, and Hanzi are medium-storable cultivars. We also determined the weight loss percentage of the tubers at 290 DAS but found no association between weight loss and decay percentage. Accordingly, only the latter was used to categorize the storage property of sweet potato tubers. Starch constitutes an important carbohydrate reserve in tuberous roots of sweet potatoes, and amylase activity is required for starch degradation during storage (Lu et al., 2020). In this study, we found that α-amylase activity was lowest in the storage-tolerant cultivar Xu 32 and highest in the storage-sensitive cultivar Yan 25. A significant increase in α-amylase activity was observed in tubers during storage, especially in the more storage-sensitive cultivars, suggesting that a correlation exists between α-amylase activity and storage property of tubers (**Figure 1**), which was consistent with the results of Lu et al. (2020). We further found that sweet potato tuber storability is highly correlated with the antioxidant capacity of the sweet potato leaves, which provides a convenient means for the screening of storage-tolerant sweet potato cultivars.

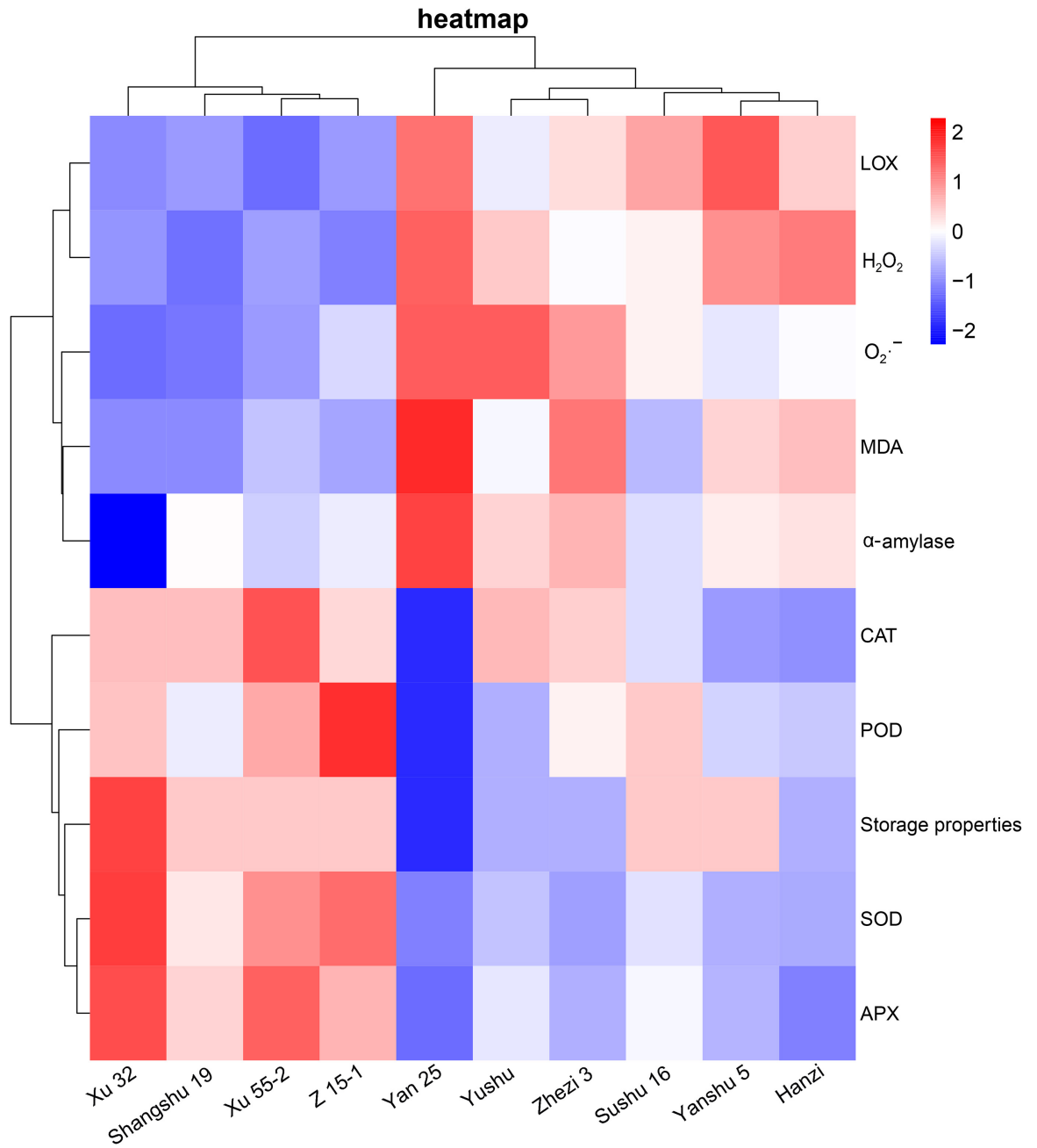

FIGURE 6 | Heat map analysis of antioxidant enzyme (i.e., SOD, POD, APX, CAT), α-amylase, and LOX activities and ROS-related indexes (i.e., $O_2^{\cdot-}$, H_2O_2, and MDA) in 10 sweet potato cultivars (i.e., Xu 32, Xu 55-2, Z15-1, Shangshu 19, Sushu 16, Yanshu 5, Hanzi, Yushu, Zhezi 3, and Yan 25).

Numerous studies have shown that ROS accumulates in fruit and vegetable during storage. For instance, ROS accumulation in longan postharvest leads to a gradual increase in cell membrane permeability and the destruction of cell membrane structure (Lin et al., 2005). Additionally, hydrogen sulfide treatment can increase the antioxidant capacity of strawberry, thereby prolonging its shelf life (Hu et al., 2012). Combined, these observations suggest that antioxidant capacity is intrinsic to a specific cultivar and is a key determinant of postharvest senescence. However, the relationship between the storage property of different sweet potato cultivars and the antioxidant capacity of sweet potato leaves still needs further investigation. In this study, we found that antioxidant enzyme (i.e., CAT, POD, APX, and SOD) activity in the leaves of the storage-tolerant cultivars Xu 32, Xu 55-2, and Z 15-1 remained at higher levels compared with those of the storage-sensitive cultivars Yan 25, Sushu 16, Yanshu 5, and Hanzi, whereas the opposite was seen for LOX activity. Besides, the storage-tolerant cultivar Xu 32 and the

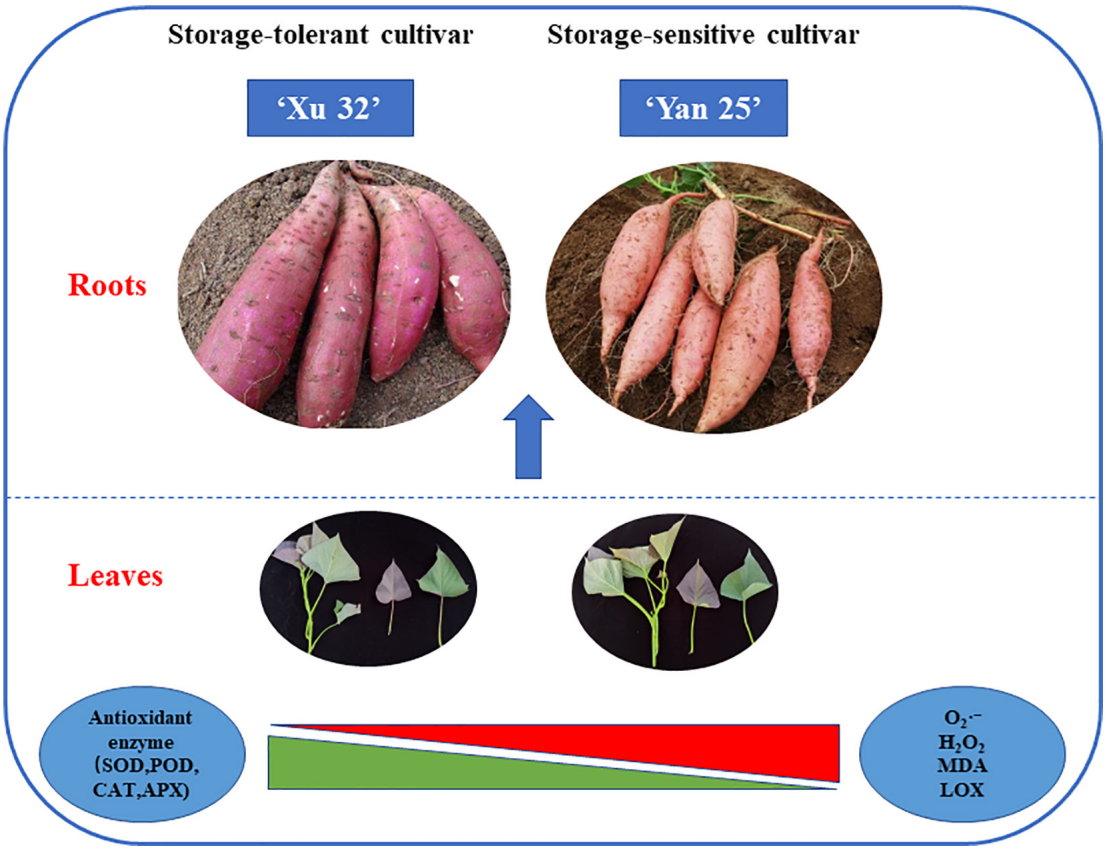

FIGURE 7 | Schematic diagram showing that ROS and MDA levels; LOX, SOD, POD, APX, and CAT activities in sweet potato leaves are highly associated with the storage property of different sweet potato tubers.

above-moderate storage-tolerant cultivars Xu 55-2 and Shangshu 19 contained lower levels of ROS metabolites when compared with those of the storage-sensitive cultivar Yan 25, all of which suggested that antioxidant capacity is positively correlated with sweet potato storability.

Furthermore, correlation and heat map analysis showed that there was a prominent association between the antioxidant capacity of sweet potato leaves and the storage property of sweet potato tubers, while antioxidant enzyme activity was negatively correlated with the levels of ROS metabolites and positively correlated with storage property. Moreover, α-amylase activity was found to be negatively correlated with storage property, suggesting that α-amylase activity is also a valuable index for evaluating the storage potential of sweet potato tubers. PCA indicated that sweet potato cultivars with similar antioxidant enzyme activities, such as Xu 32, Xu 55-2, Z 15-1, and Shangshu 19, were clustered together, as were Yanshu 5, Hanzi, and Sushu 16 (**Figure 3**). Overall, these results were consistent with the storage properties of the different cultivars and sweet potato varieties with similar antioxidant enzyme activities. The growth environment, soil, fertilizer and water management, temperature, light, and other external conditions can all affect the storage performance of sweet potato, while the ecological environment can significantly affect sweet potato quality (Yan et al., 2017). Rosenthal and Jansky (2008) reported that antioxidant activity in potato growing in a high-yield production environment was usually the highest and increased during storage, indicative of the importance of antioxidant enzymes for potato storability. Additionally, ultrasound treatment can inhibit the browning of fresh-cut sweet potatoes by reducing PPO and POD activities while improving total antioxidant capacity (Pan et al., 2020). Moreover, low-temperature conditioning at 10°C can induce antioxidant enzyme activity in tuberous roots and protect tubers from chilling injury when subjected to subsequent cold storage at 4°C (Li et al., 2018). Together, these findings suggest that the antioxidative enzyme system is critical for protecting the sweet potato from postharvest senescence and decay. As shown in **Figure 7**, antioxidant enzymes are required for maintaining ROS metabolic balance, while accumulated ROS may negatively influence the storage property of sweet potato tubers.

Overall, this study provides strong evidence that the antioxidant capacity of leaves in different sweet potato cultivars is positively correlated with their storability. We further found that α-amylase activity in sweet potato tubers is negatively correlated with storage property, suggesting that α-amylase activity may represent a valuable index for evaluating the storage potential of sweet potato tubers. Finally, given that the characterization of storage property in different sweet potato cultivars is a

time-consuming process, this study provides a convenient means for evaluating the storage properties of sweet potatoes by measuring the antioxidant capacity in sweet potato leaves.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

H-HS, K-DH, G-FY, and HZ conceived and designed the experiments. H-HS, Z-LZ, and D-LZ performed the experiments. Z-LZ, D-LZ, HZ, and X-YC analyzed the data. K-DH, G-FY, and H-HS wrote the manuscript. K-DH, G-FY, and ZH interpreted the data and revised the manuscript. All authors contributed to the article and approved the submitted version.

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CmRCC1 Gene From Pumpkin Confers Cold Tolerance in Tobacco by Modulating Root Architecture and Photosynthetic Activity

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Low-temperature stress is the main limiting factor of cucurbit crop cultivation as it affects crop yield and quality. The identification of genes involved in cold tolerance is a crucial aspect of pumpkin rootstock breeding. Here, we examined the function of a pumpkin Regulator of Chromosome Condensation 1 (*CmRCC1*) gene in the root development and cold stress responses of tobacco (*Nicotiana benthamiana*). *CmRCC1* expression was differentially induced in pumpkin root, stem, and leaf under cold stress. Transient transformation showed that CmRCC1 is located in the nucleus. *CmRCC1* overexpression in tobacco increased the gravitropic set-point angle in lateral roots, as well as root diameter and volume. The expression of auxin polar transport factors, *PIN1* and *PIN3*, decreased and increased in *CmRCC1*-overexpressed plants, respectively. Yeast two-hybrid verification and luciferase complementation imaging assay showed that CmRCC1 interacts with CmLAZY1. Furthermore, the decreases in maximum quantum yield of PS II, the effective quantum yield of PS II, and electron transfer rate and the increases in quantum yield of nonregulated energy dissipation and malondialdehyde content were compromised in transgenic plants compared with wild-type plants under cold stress. The results suggest that *CmRCC1* plays an important role in the regulation of root architecture and positively modulates cold tolerance.

Keywords: *CmRCC1*, cold stress, root architecture, photosynthesis, pumpkin

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INTRODUCTION

The Regulator of Chromosome Condensation 1 (RCC1) superfamily of proteins is characterized by 350–500 residue domain, known as the RCC1-like domain (RLD), which was first reported in human RCC1 in 1987 (Ohtsubo et al., 1987). RCC1 consists of seven homologous repeats of 51–68 amino acid residues. It combines with chromatin and a nuclear Ras-like G protein, Ran, to establish a RanGTP concentration gradient, which affects the formation and function of the nuclear envelope, spindle formation, nuclear transport, and the cell cycle during tumorigenesis (Ren et al., 2020). Since the initial identification of RCC1, a number of proteins that contain one or more RLDs have been discovered. In human cells, these RCC1 superfamily

proteins can be subdivided into five subgroups based on structural criteria (Hadjebi et al., 2008).

Recent studies have been reported the functions of RCC1 superfamily proteins in plants. *Arabidopsis thaliana* contains 24 RCC1 family proteins, among which UV RESISTANCE LOCUS 8 (UVR8), a UV-B photoreceptor, has been studied the most (Rizzini et al., 2011; Christie et al., 2012; Wu et al., 2012; Jenkins, 2014). UV-B absorption induces the instant monomerization of UV-B RESISTANCE 8 (UVR8) and interaction with CONSTITUTIVELY PHOTOMORPHOGENIC 1, the central regulator of light signaling, to secure plant acclimation and promote survival in sunlight (Rizzini et al., 2011). RCC1/UVR8/GEF-like 3 (RUG3), another RCC1 family protein, interacts with ataxia telangiectasia-mutated protein in the mitochondria of *Arabidopsis* to synergistically regulate *nad2* mRNA splicing and complex I biogenesis (Kühn et al., 2011). As an upstream regulatory element of reactive oxygen species (ROS) homeostasis, RUG3-mediated mitochondrial retrograde signaling plays an important role in DNA damage repair and mitochondrial function restoration in the root apical meristem (Su et al., 2017). The *Tolerant to Chilling and Freezing 1* (*TCF1)* gene in *Arabidopsis* encodes a protein containing six predicted tandem RCC1 repeats that show a similarity to yeast and human RCC1 (Ohtsubo et al., 1989; Renault et al., 1998). *TCF1* regulates cold acclimation and freezing tolerance by modulating *Blue-Copper-Binding gene* (*BCB*) to adjust lignin accumulation and consequently cell wall remodeling (Ji et al., 2015). *Sensitive to ABA 1* (*SAB1*) encodes a RCC1 family protein and physically interacts with ABI5, which results in reduced ABI5 phosphorylation and protein stability, decreased ABI5 DNA-binding activity, and increased the H3K27m2 methylation of *ABI5* promoter in *Arabidopsis* (Ji et al., 2019). Four out of eight RLD proteins in *Arabidopsis* were identified as LAZY1/LAZY1-LIKE (LZY) interactors, and RLDs regulate PIN-dependent auxin transport in various developmental processes, including gravitropic set-point angle (GSA) control (Furutani et al., 2020). A newly discovered RCC1 family protein, PLASTICITY OF ROSETTE TO NITROGEN 1, confers the plasticity of rosette diameter in response to changes in nitrogen availability in *Arabidopsis* (Duarte et al., 2021). Additionally, 56 *RCC1* genes have been identified in upland cotton (*Gossypium hirsutum*), among which *Gh_A05G3028* and *Gh_D10G2310*, the homologous genes of *AtTCF1* and *AtUVR8*, were dramatically induced under salt treatment, and the silencing of these two genes exhibited a salt-sensitive phenotype (Liu et al., 2019).

As the most important environmental stress, low temperature can limit the growth of plants and affect the distribution and yield of crops (Stitt and Hurry, 2002; Zhang et al., 2004). Low-temperature stress negatively affects plant growth morphology, physiology, and biochemistry by limiting cell survival, cell division, photosynthetic efficiency, and water transport (Beck et al., 2007; Sanghera et al., 2011). In recent years, extreme weather occurs frequently around the world and further increases the risk of low-temperature damage to plants, which remarkably reduces the economic benefits of agricultural production. Solving the adaptation problem of plants under chilling injury has always been a hot topic worldwide (Rigby and Porporato, 2008; Augspurger, 2013; Hatfield and Prueger, 2015). Therefore, studying the response mechanism of plants to chilling injury and discovering the functional genes of plants for cold resistance are of great importance to cope with global climate anomalies.

Pumpkin (*Cucurbita maxima*) is a typical warm-loving vegetable. It is often used as the rootstock in grafting many kinds of cucurbit crops because of its developed root system and strong resistance to soil-borne pathogens and abiotic stresses. Pumpkin rootstocks can reduce water loss by limiting the transpiration of grafted seedlings, promote the absorption and transportation of water and nutrients in grafted seedlings, and regulate the osmotic pressure in cells to alleviate the damage of plants under low-temperature stress (Schwarz et al., 2010). However, the possible molecular regulatory mechanisms underlying pumpkin response to cold stress are not yet illustrated. In this study, the Regulator of Chromosome Condensation 1 (*CmRCC1*) gene was characterized from a cold-tolerant pumpkin rootstock. The expression patterns of *CmRCC1* in response to cold treatment were analyzed through quantitative real-time polymerase chain reaction (qRT-PCR). *CmRCC1* was overexpressed in transgenic tobacco (*Nicotiana benthamiana*) plants to evaluate its function in root development and cold stress tolerance. Root morphology assays revealed that *CmRCC1* overexpression altered the root architecture under normal growth conditions. Moreover, *CmRCC1*-overexpressed (*OxCmRCC1*) plants showed good performance under cold stress. Generally, our results suggest that *CmRCC1* plays important roles in plant cold response and can be a candidate gene to improve the cold tolerance of crops in the future.

MATERIALS AND METHODS

Plant Materials and Cold Treatment of Pumpkin Seedlings

"Qingyan No. 1," a pumpkin rootstock with low temperature tolerance, was used as the experimental material in this study. The pumpkin seeds were soaked with 1‰ $KMnO_4$ for 15 min to conduct surface disinfection. Afterward, the seeds were soaked in warm water at 55°C, cooled naturally, soaked for 12 h, and placed in a growth chamber at 30°C for germination. Then, the seeds were sown in 10 cm × 10 cm pots with peat–vermiculite–perlite medium (2:1:1). The growth conditions were as follows: photoperiod, 12/12 h; day/night temperature, 28/18°C; light intensity, 16,000 Lx; and air humidity, 70–85%. Pumpkin seedlings at three-leaf stage were exposed to 4°C in a growth chamber (Ningbo Saifu DGX-260, China) for cold stress. The root, stem, and third true leaf of each plant were sampled at 0, 3, 6, 12, and 24 h after low-temperature treatment. The samples were frozen at −80°C in liquid nitrogen before qRT-PCR analysis.

Subcellular Localization of CmRCC1

The full-length coding sequence (CDS) of *CmRCC1* was amplified by PCR using 2× High-Fidelity Master Mix (Tsingke, Inc.,

Beijing, China), and the fragments were inserted into the *Bgl* II site of the pCAMBIA1305.4-N-GFP vector by using ClonExpress II One Step Cloning Kits (Vazyme, Piscataway, NJ, United States) to generate *35S*::GFP-CmRCC1 fusion protein under the control of the Cauliflower mosaic virus (CaMV) 35S promoter. The construct and negative control (pCAMBIA1305.4-N-GFP) were transformed into *Agrobacterium tumefaciens* strain GV3101 and infiltrated into tobacco leaves according to previously described method (Sheludko et al., 2007). Leica SP8 confocal microscope was used to detect the GFP fluorescence signal with 4,6-diamidino-2-phenylindole (DAPI) as the nucleus marker.

Total RNA Extraction and Reverse Transcription

Total RNA was isolated using TransZol reagent (TransGen Biotech Inc., Beijing, China) in accordance with the manufacturer's protocol. The extracted total RNA was dissolved in diethylpyrocarbonate-treated water. The cDNA template for gene cloning was synthesized from 2 μg of RNA using HiScript II One Step RT-PCR Kit (Vazyme, Piscataway, NJ, United States). While for qRT-PCR, the cDNA was synthesized from 1 μg total RNA using HiScript II Q RT SuperMix for qPCR (+g DNA wiper; Vazyme, Piscataway, NJ, United States).

Generation of *CmRCC1* Transgenic Tobacco Plants

The CDS of *CmRCC1* was cloned into the pHellgate8 vector to generate the *35S*::*CmRCC1* construct by ClonExpress II One Step Cloning Kits. The construct was transformed into *A. tumefaciens* strain GV3101 and then transferred into tobacco plants using the leaf disc method (Horsch et al., 1985). Transgenic tobacco seeds were screened on MS medium suspended with kanamycin (50 mg/L). T_2 homozygous lines were used for further experiments.

Root Morphology Assays

The roots of three uniform plants from each replicate were harvested and washed with deionized water. The root morphology was scanned using Imagery Scan Screen (Epson Expression 11000XL, Regent Instruments, Canada). Root image analysis was conducted *via* the WinRHIZO 2003a software (Regent Instruments, Canada).

Yeast Two-Hybrid Verification

The open reading frames (ORFs) of *CmRCC1* and *CmLAZY1* from "Qianyan No. 1" roots were amplified using sequence-specific primers (**Supplementary Table S1**) and incorporated into pGBKT7 and pGADT7 vectors (Clontech, United States), respectively, to verify the protein–protein interactions of CmRCC1 with CmLAZY1. According to the manufacturer, the recombinant plasmids, pGADT7-CmLAZY1 and pGBKT7-CmRCC1, pGADT7 and pGBKT7-CmRCC1, pGADT7-T and pGBKT7-lam (negative control), and pGADT7-T and pGBKT7-p53 (positive control), were introduced into the yeast strain, Y2H Gold. The transformants were grown on SD/−Leu/−Trp and SD/−Leu/−Trp/−Ade/-His media to evaluate the interactions.

Luciferase Complementation Imaging Assay

As described previously, the ORF of *CmLAZY1* was cloned into pCAMBIA-nLUC to yield the fusion construct, pCAMBIA-CmLAZY1-nLUC, and the ORF of *CmRCC1* was cloned into pCAMBIA-cLUC to generate the fusion construct, pCAMBIA-CmRCC1-cLUC (Chen et al., 2008). *Agrobacterium tumefaciens* GV3101 was transformed with the empty vector and fusion constructs and incubated at 28°C for 16 h. Then, the *A. tumefaciens* cells were collected and resuspended at $OD_{600} = 0.3$. The tobacco leaves were then infiltrated with *Agrobacterium* strains containing the indicated constructs at a ratio of 1:1. After 3 days, the leaves were treated with luciferin, and firefly luciferase (LUC) signal was observed according to Xiong et al. (2019).

Analysis of Chlorophyll Fluorescence

Chlorophyll fluorescence was measured by pulse amplitude-modulated fluorometry (MAXI; Heinz Walz, Effeltrich, Germany) as previously described (Cheng et al., 2016). The seedlings were adapted to the dark for at least 30 min before the measurements, and the whole area of the third leaf from the bottom was used for the experiment. The intensities of actinic light and saturating light were set to 280 and 4,000 μmol m^{-2} s^{-1}, respectively. The maximum quantum yield of PS II (Fv/Fm) and the effective quantum yield of PS II (Φ_{PSII}) were measured and calculated in accordance with the following equations (van Kooten and Snel, 1990): $Fv/Fm = (Fm - Fo)/Fm$ and $\Phi_{PSII} = (F'm - Fs)/F'm$. The quantum yield of regulated energy dissipation (Φ_{NPQ}) and the quantum yield of nonregulated energy dissipation (Φ_{NO}) in PS II were calculated according to the equation (Kramer et al., 2004): $\Phi_{PSII} + \Phi_{NPQ} + \Phi_{NO} = 1$. Electron transfer rate (ETR) was measured using a rapid light-response curve.

Determination of Lipid Peroxidation

Lipid peroxidation was determined by measuring malondialdehyde (MDA) content as described by Hodges et al. (1999). Briefly, leaf samples (0.3 g) were ground in 3 ml of ice-cold 25 mmol/L HEPES buffer (pH 7.8) containing 0.2 mmol/L EDTA and 2% (w/v) polyvinylpyrrolidone. The obtained homogenates were centrifuged at 4°C for 20 min at 10,000 rpm, and the resulting supernatants were used to analyze MDA content. The samples were mixed with 10% trichloroacetic acid containing 0.65% 2-thiobarbituric acid (TBA) and heated at 95°C for 25 min. MDA content was corrected for non-MDA compounds by subtracting the absorbance at 532 nm of a TBA-less solution that contained the plant extract.

Gene Expression Analysis

We amplified the PCR products for qRT-PCR analysis in triplicate using 2 × TransStart™ TOP Green qPCR SuperMix

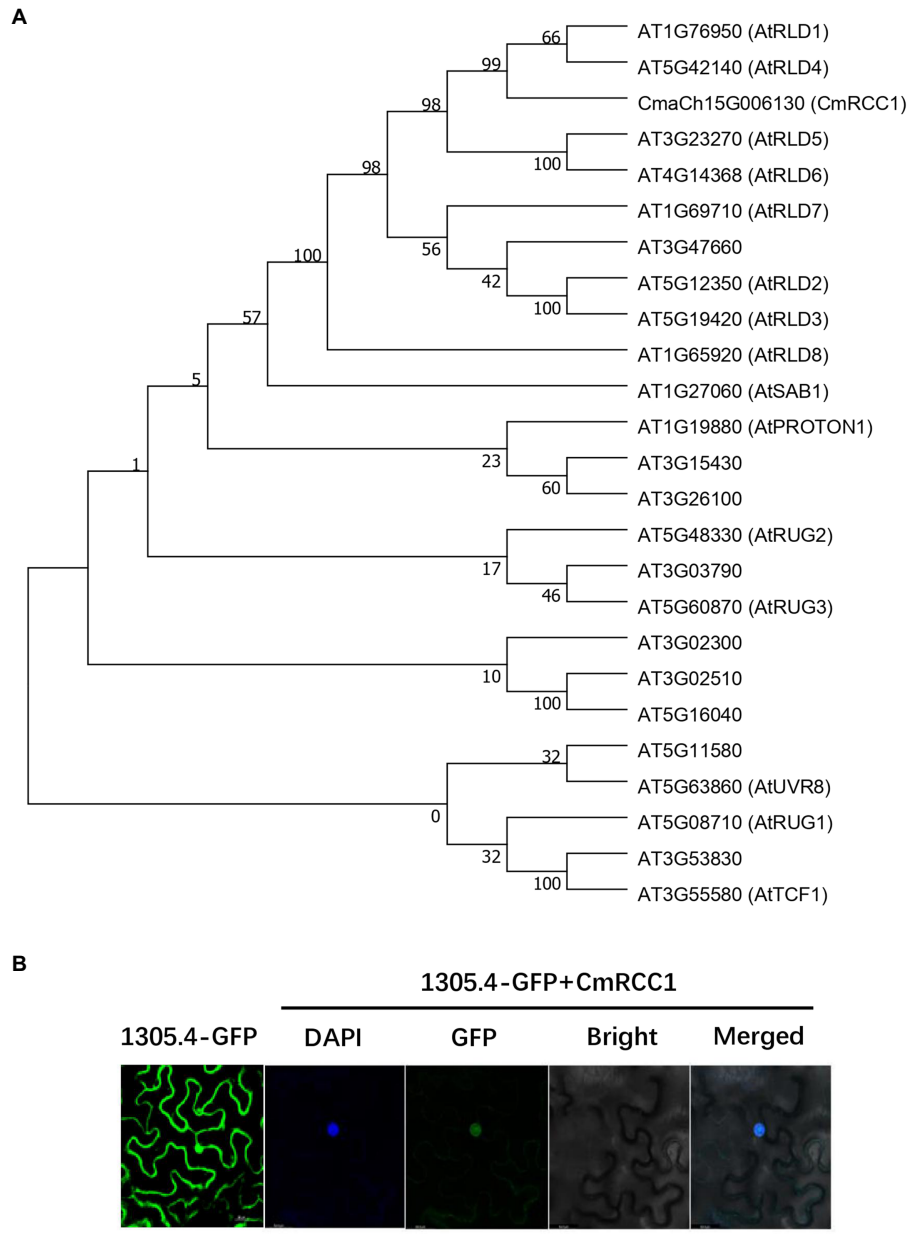

FIGURE 1 | Phylogenetic analysis of RCC1 family proteins in *Arabidopsis* and subcellular localization of CmRCC1. **(A)** Phylogenetic tree of CmRCC1 with those identified RCC1 proteins from *Arabidopsis*. The phylogenetic tree was constructed using MEGA 7 with the Neighbor–Joining method. **(B)** Subcellular localization of CmRCC1 in tobacco epidermal cells. Nucleus was stained with DAPI. Co-localization between DAPI and GFP signals in *35S*::GFP-CmRCC1 fusion protein was shown in merged picture.

(TransGen Biotech Inc., Beijing, China) in 10 µl qRT-PCR assays. PCR was performed using the QuantStudio 7 Flex Real-time PCR System (Applied Biosystems, Foster City, CA, United States). The cycling conditions consisted of denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 58°C for 15 s, and extension at 72°C for 10 s. The reference genes, *CmCAC* and *NbACTIN*, were used as the internal controls (Obrero et al., 2011; Nie et al., 2020). The gene-specific primers for *CmRCC1* and the *NbPIN* gene family are listed in **Supplementary Table S1**. Relative gene expression was determined as previously described by Livak and Schmittgen (2001).

Statistical Analysis

The experiment involved a completely randomized block design with four replicates. Statistical analysis was performed using the SAS statistical package. The differences between the treatment

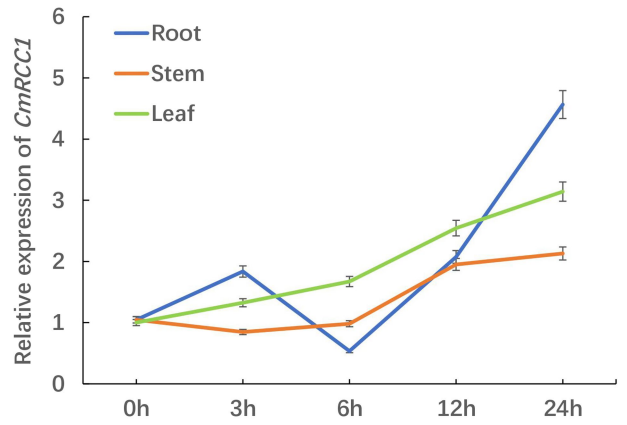

FIGURE 2 | The time-course response in *CmRCC1* gene expression to chilling stress in pumpkin. Root, stem, and leaf samples were collected at the indicated times under chilling stress. The data are the means of four replicates with SEs.

means were separated using Tukey's test at a significance level of $p < 0.05$.

RESULTS

Identification and Characterization of the *CmRCC1* Gene

CmRCC1 gene (CmaCh15G006130) was predicted to contain a 3,360 bp CDS isolated from 4,143 bp cDNA and encode the protein of 1,119 amino acids in the Cucurbit Genomics Database. A Pfam domain search was performed to characterize the pleckstrin homology (PH_12), RCC1 repeats, FYVE zinc finger, BRX N-terminal, and BRX domains of the CmRCC1 protein (**Supplementary Figure S1A**).[1] Moreover, a database (The Arabidopsis Information Resource) search indicated 24 RCC1 family proteins in *A. thaliana*, among which 15 protein members have been named and functionally annotated. The phylogenetic tree built from the alignment of CmRCC1 with the previously identified *Arabidopsis* RCC1s revealed the evolutionary distances between the sequences (**Figure 1A**). Among these sequences, CmRCC1 showed high similarity to the sequences of AtRLD1 and AtRLD4.

The GFP-CmRCC1 fusion construct and GFP control in the pCAMBIA1305.4-N-GFP vector driven by *CaMV35S* promoter were transiently expressed in tobacco epidermal cells and visualized under a laser scanning confocal microscope to determine the subcellular localization of CmRCC1. The GFP fluorescence signal of GFP-CmRCC1 fusion protein was detected in the nucleus as confirmed by DAPI staining (**Figure 1B**).

[1]http://pfam.xfam.org/search/sequence

Temporal and Spatial Responses of *CmRCC1* Expression to Cold Stress

We detected the changes in *CmRCC1* expression in the root, stem, and leaf at different time points after 24 h cold treatment to evaluate the response characteristics of *CmRCC1* to cold stress in pumpkin. The transcription levels of *CmRCC1* in the stem and leaf increased slowly with the extension of cold stress treatment, and they reached 2.13 and 3.15 times of the control (0 h) after 24 h treatment, respectively. However, the expression level of *CmRCC1* in the pumpkin root peaked at 3 h, and then reached 4.57 times at 24 h of cold treatment (**Figure 2**). These results indicate that *CmRCC1* may be involved in the response of pumpkin root to early cold stress.

Involvement of CmRCC1 in the Control of Root Architecture and the Regulation of *PIN* Gene Expression

CmRCC1 was overexpressed in tobacco under the control of *CaMV35S* promoter to analyze the role of *CmRCC1* in root development. The insertion of the *CmRCC1* cassette in 28 independent kanamycin-resistant transformants was confirmed by RT-PCR (**Supplementary Figure S2**). Three transformed lines (*OxCmRCC1-1/−3/−6*) which showed that the *CmRCC1* gene segregated in the Mendelian segregation ratio of 3:1, were subsequently selected to obtain T_2 homozygous lines (**Supplementary Table S2**). qRT-PCR analysis of the *CmRCC1* transcripts in three independent lines revealed variable levels of transgene expression (**Figure 3A**). Compared with the wild type, all the overexpressed transgenic lines showed increased gravitropic set-point angle (GSA) in lateral roots (**Figure 3B**). Moreover, *CmRCC1* overexpression increased the root diameter and volume of transgenic tobacco but not root length (**Figures 3C–E**).

In *Arabidopsis*, the characterized PIN proteins demonstrate specific expression patterns and are involved in polar auxin transport and root patterning (Paponov et al., 2005). Thus, we further measured the expression levels of four *PIN* genes in the roots of wild-type and *CmRCC1* transgenic plants. As shown in **Figure 4**, *PIN3* expression level remarkably increased in the *CmRCC1* overexpression lines than in the wild type. However, the expression of *PIN2* and *PIN6* showed no substantial differences between the transgenic lines and wild type. By contrast, the expression level of *PIN1* differentially decreased in the *CmRCC1* overexpression lines compared with the wild type.

Interaction of CmRCC1 With CmLAZY1 Protein

LAZY1 functions upstream of lateral auxin translocation in gravity signal transduction in the root and shoot of *Arabidopsis* and rice (Yoshihara and Iino, 2007; Taniguchi et al., 2017). We co-transformed pGADT7-CmLAZY1 and pGBKT7-CmRCC1 in yeast cells and found that the transformants grew on SD/−Leu/−Trp/−Ade/-His media, which was consistent with the results of the positive control yeast cells (**Figure 5A**). Furthermore, we performed luciferase

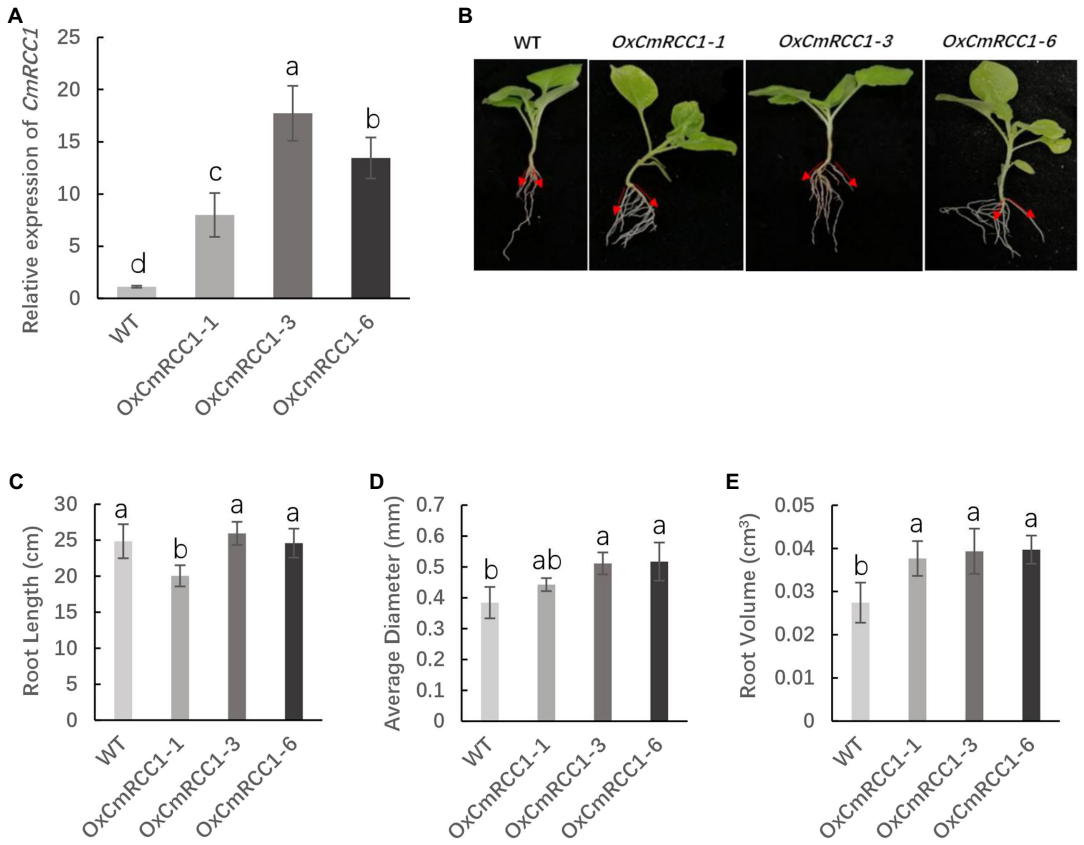

FIGURE 3 | T_2 generation phenotypes of three lines in overexpressed *CmRCC1* transgenic tobacco. **(A)** Relative expression level of *CmRCC1* in three different transgenic tobacco lines. **(B)** Gravitropic set-point angle (GSA) in lateral roots of WT and transgenic tobacco (*OxCmRCC1-1/–3/–6*). **(C)** Total root length in WT and transgenic lines. **(D)** Average root diameter in WT and transgenic lines. **(E)** Total root volume in WT and transgenic lines. WT, wild type. Samples were collected at the 4-week-old seedling stage. The data are the means of four replicates with SEs. Different letters indicate significant differences according to Tukey's test ($p < 0.05$).

complementation imaging assay to verify the interaction of CmRCC1 with CmLAZY1 *in vivo*. We were able to image LUC signals in tobacco leaves that co-infiltrated with *Agrobacterium* strains that expressed CmLAZY1-nLUC and CmRCC1-cLUC, but no signal was observed in the negative controls (CmRCC1-cLUC/nLUC and nLUC/cLUC, **Figure 5B**). Together, the results suggest that CmRCC1 interacts with CmLAZY1 protein.

Increased Cold Tolerance in Transgenic Tobacco With *CmRCC1* Overexpression

The seedlings of T_2 transgenic lines and wild type were exposed to chilling stress at 4°C for 12 h to examine the possible role of *CmRCC1* overexpression in the cold tolerance of tobacco. We observed that the leaves in the wild type completely shrank, and the plants were lodging after chilling stress treatment, whereas the transgenic tobacco plants still stood upright with flat leaves and light wilting (**Figure 6A**). We then measured the chlorophyll fluorescence of PS II in the third leaves of chilling-stressed and non-stressed plants in the wild-type and transgenic lines. The *Fv*/*Fm* and Φ_{PSII} decreased by 28.6 and 56.7%, respectively, in the wild type after chilling stress in comparison with the control. However, *Fv*/*Fm* and Φ_{PSII} decreased by 11.1–14.7 and 6.7–15.3%, respectively, in the *CmRCC1*-overexpressed lines in response to chilling stress (**Figures 6B**,**C**). A high Φ_{NO} value indicates that photochemical energy conversion and protective regulatory mechanisms are inefficient. Therefore, it indicates that the plant is already damaged or will be photodamaged upon further irradiation. Here, we found Φ_{NO} increased by 36.6% after chilling stress in wild-type plants, whereas *CmRCC1* overexpression compromised the increase in Φ_{NO} in chilling-stressed plants (**Figure 6D**). By contrast, Φ_{NPQ} showed no substantial differences between chilling-stressed and non-stressed plants in wild-type and *CmRCC1* transgenic lines, which indicates that the photoprotection ability was not affected under chilling stress (**Figure 6E**). We also analyzed the ETR versus incident photosynthetic photon flux density. Light-saturated ETR decreased by 55.0% in chilling-stressed wild-type plants. Again, the decrease in ETR was compromised in *CmRCC1*-overexpressed lines (**Figure 6F**). Moreover, increased MDA content (62.5%) was observed after 12 h of chilling stress in wild-type plants

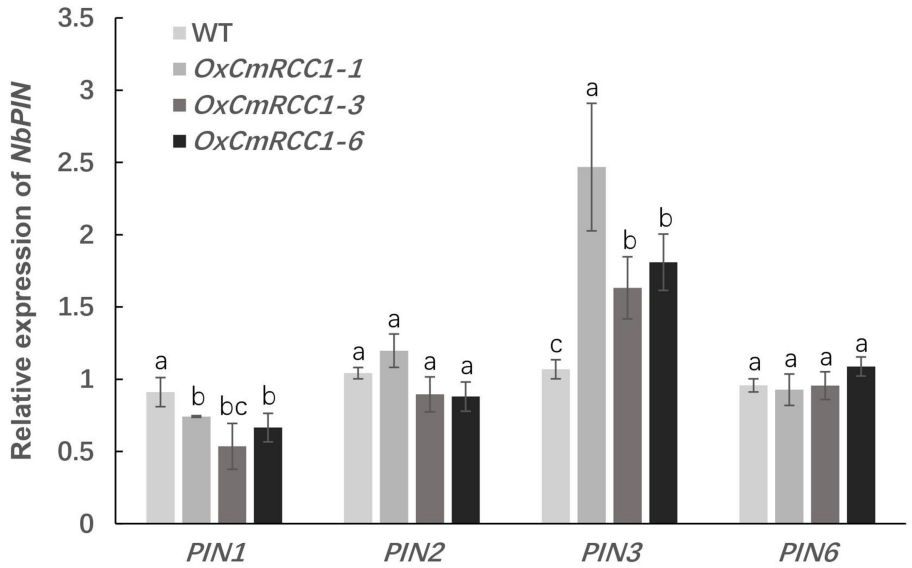

FIGURE 4 | Expression analysis of the PIN family genes in transgenic tobacco. Root samples were collected at the 4-week-old seedling stage. Data represent means and SE of four replicates. Different letters indicate significant differences according to Tukey's test ($p < 0.05$).

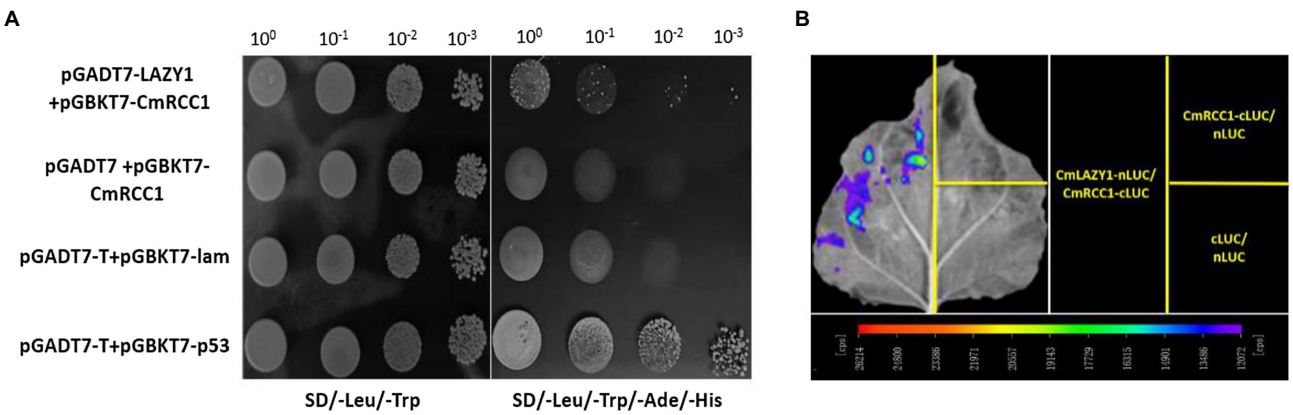

FIGURE 5 | Interactions between CmRCC1 and CmLAZY1. **(A)** Interactions between CmRCC1 and CmLAZY1 in the yeast two-hybrid system. Recombinant plasmids containing either pGADT7-T and pGBKT7-p53 or pGADT7-T and pGBKT7-lam were introduced into yeast Y2H Gold cells and used as positive and negative controls, respectively. Yeast cells were cultured on SD/−Leu/−Trp and SD/−Leu/−Trp/−Ade/-His media. **(B)** Interactions between CmRCC1 and CmLAZY1 assayed with the luciferase complementation imaging assay. Tobacco leaves were divided into three parts and infiltrated with *Agrobacterium* strains harboring CmLAZY1-nLUC and CmRCC1-cLUC. The following two pairs of constructs were used as negative controls: CmRCC1-cLUC/nLUC and cLUC/nLUC. The images were captured with a charge-coupled device camera at 3 days post-inoculation (dpi).

compared with the control. However, no remarkable differences in MDA content were observed between the control and chilling-stressed transgenic lines (**Figure 6G**). Thus, we conclude that *CmRCC1* overexpression increases the cold tolerance of transgenic tobacco.

DISCUSSION

Vegetable crops, particularly those from the Cucurbitaceae and Solanaceae families, are extensively grafted for increased yield and enhanced stress tolerance (Gaion et al., 2018). Facility cultivation producer would benefit from grafting to rootstocks that confer abiotic stress (i.e., cold) tolerance, which offer protection from soil-borne pathogens and maximize output by increasing yield (Williams et al., 2021). The characterization and identification of resistance genes can amplify the contribution of a breeding program to improve rootstock resistance.

RCC1 is a eukaryotic protein with seven repeated domains that fold into a seven-bladed propeller structure (Renault et al., 1998). RCC1-like domains (RLDs) have been identified

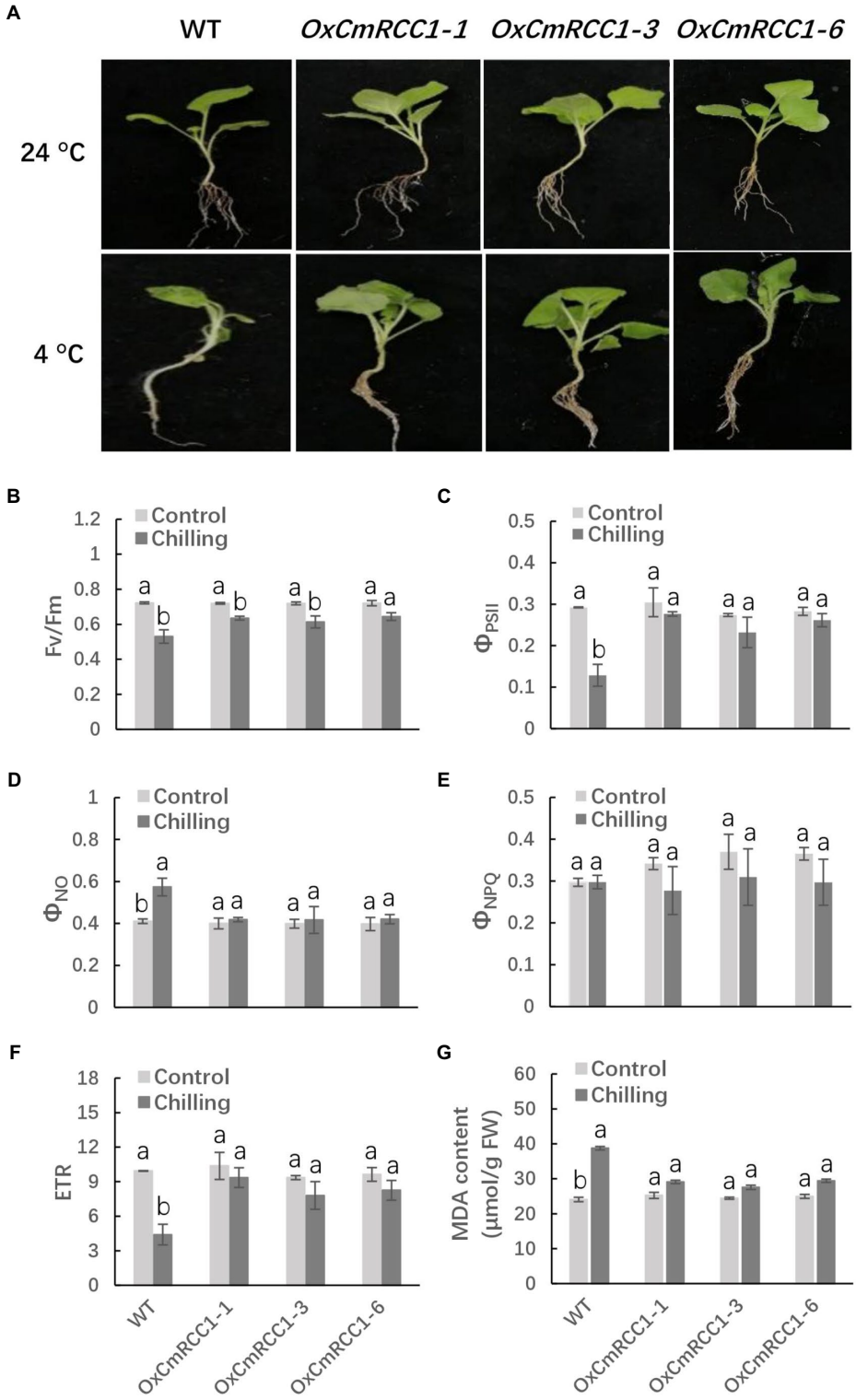

FIGURE 6 | Chilling tolerance phenotypes in wild type (WT) and *CmRCC1* transgenic plants. **(A)** Phenotypes of 4-week-old WT and transgenic plants under normal (24°C) and chilling stress (4°C) conditions. The picture of representative plants was taken after 12 h of 4°C treatment. **(B)** The maximum quantum yield of PS II (*Fv*/*Fm*). **(C)** The effective quantum yield of PS II (Φ_{PSII}). **(D)** The quantum yield of nonregulated energy dissipation in PS II (Φ_{NO}). **(E)** The quantum yield of regulated energy dissipation in PS II (Φ_{NPQ}). **(F)** The electron transfer rate (ETR) at saturated light. **(G)** Malondialdehyde (MDA) content. Leaf samples were collected after 12 h of 4°C treatment for chlorophyll fluorescence analysis. The data are the means of four replicates with SEs. Different letters indicate significant differences between the treatments according to Tukey's test ($p < 0.05$).

in a variety of proteins that mediate diverse biological processes (Hadjebi et al., 2008). Two *Arabidopsis* RCC1 family proteins, UVR8 and TCF1, mediate UV-B response and tolerance to low temperature, respectively (Brown et al., 2005; Ji et al., 2015). Here, we show that the CmRCC1 protein plays a crucial role in the cold tolerance of transgenic tobacco. CmRCC1 shares conserved RCC1 repeat domains with the characterized *Arabidopsis* RCC1 family proteins, although the proteins differ concretely in sequence (**Supplementary Figure S1**). Similar to TCF1, CmRCC1 is localized in the nucleus, and the gene expression of *CmRCC1* is responsive to cold stress (**Figures 1B, 2**), which suggest a similar role of CmRCC1 during cold tolerance.

Photosynthesis is particularly sensitive to chilling during plant growth and development (Ruelland et al., 2009). Photosynthetic light harvesting is regulated by nonphotochemical quenching (NPQ), which allows the dissipation of harmful excess energy as heat through its energy-dependent NPQ (qE) component to avoid photodamage under chilling stress (Li et al., 2009; Niyogi and Truong, 2013; Ruban, 2016; Lu et al., 2020). In the green alga *Chlamydomonas reinhardtii*, UVR8 induces the accumulation of specific members of the light-harvesting complex (LHC) superfamily, particularly LHC Stress-Related 1 and Photosystem II Subunit S, which contribute to qE and reduce photodamage to the photosynthesis machinery under UV-B (Allorent et al., 2016). Our study showed that photoinhibition and photodamage around PS II were compromised in the *CmRCC1*-overexpressed lines under chilling stress (**Figures 6B**–**D,F**), which reveals a promising role of CmRCC1-mediated photoprotective regulation of photosynthetic activity in the chloroplast during chilling stress. Interestingly, although an excessive photon flux density occurs in the cold and night (Wise, 1995), the present results showed that the wild-type and transgenic plants retained some physiological means to protect themselves against excess light intensity during chilling in the light (**Figure 6E**).

A recent study indicated that RLD proteins, identified as LZY interactors, are essential regulators of polar auxin transport and root branch angle control (Furutani et al., 2020). Phylogenetic analysis revealed closer evolutionary distances between CmRCC1 and RLD family proteins (**Figure 1A**). Our results indicated that *CmRCC1* overexpression increased the GSA in lateral roots (**Figure 3B**), and the *in vitro* and *in vivo* interactions of CmRCC1 with CmLAZY1 protein suggest a possible role of CmRCC1 in the GSA control of lateral roots (**Figure 5**). Auxin is an important internal positive regulator during lateral root development, and genes of the PIN family have an important role in adaptation to stress responses through modulation in root system (Shibasaki et al., 2009; Wang et al., 2015; Zwiewka et al., 2019). *CmRCC1* overexpression induced decreased *PIN1* expression and increased *PIN3* expression in transgenic tobacco (**Figure 4**), which imply the differential roles of PIN family genes in the gravitropism regulation of lateral roots (Rosquete et al., 2013). In addition to GSA, the length, diameter, and volume of root components determine root system architecture (RSA). The exposure of monocot and dicot plant roots to temperatures below or above their optimum temperature decreases (i) primary root length, (ii) lateral root density (numbers of lateral roots per unit primary root length), and (iii) the branching angles between primary and lateral roots, whereas the average lateral root length is unaffected (Mcmichael and Quisenberry, 1993; Seiler, 1998; Nagel et al., 2009). In the present study, transgenic tobacco lines overexpressing *CmRCC1* exhibited increased root diameter and volume (**Figures 3D**,**E**), which help improve the soil volume that roots may access for the uptake of water and nutrients and further guarantee plant cold tolerance. Several NAC-type transcription factors from *Glycine max* were recently reported to increase lateral root formation by regulating the expression of auxin signaling-related genes, and improved cold tolerance was induced in transgenic plants with *GmNAC20* overexpression (Yang et al., 2019; Yarra and Wei, 2021).

We conclude that *CmRCC1* overexpression could enhance cold tolerance by improving RSA and maintaining photosynthetic activity under cold stress. Functional evidence on the role of root plasticity will support breeders in their efforts to include root properties in their future selection pipeline for cold stress tolerance to improve crop yield and quality.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

FC and MW conceived and designed the research. MW, SZ, JL, and AX performed the experiments and analyzed the data. YH and ZB supervised the study. FC wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articless/10.3389/fpls.2021.765302/full#supplementary-material

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Genetic and Molecular Mechanisms Conferring Heat Stress Tolerance in Tomato Plants

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Climate change is a major threat to global food security. Changes in climate can directly impact food systems by reducing the production and genetic diversity of crops and their wild relatives, thereby restricting future options for breeding improved varieties and reducing the ability to adapt crops to future challenges. The global surface temperature is predicted to rise by an average of 0.3°C during the next decade, and the Paris Agreement (Paris Climate Accords) aims to limit global warming to below an average of 2°C, preferably to 1.5°C compared to pre-industrial levels. Even if the goal of the Paris Agreement can be met, the predicted rise in temperatures will increase the likelihood of extreme weather events, including heatwaves, making heat stress (HS) a major global abiotic stress factor for many crops. HS can have adverse effects on plant morphology, physiology, and biochemistry during all stages of vegetative and reproductive development. In fruiting vegetables, even moderate HS reduces fruit set and yields, and high temperatures may result in poor fruit quality. In this review, we emphasize the effects of abiotic stress, especially at high temperatures, on crop plants, such as tomatoes, touching upon key processes determining plant growth and yield. Specifically, we investigated the molecular mechanisms involved in HS tolerance and the challenges of developing heat-tolerant tomato varieties. Finally, we discuss a strategy for effectively improving the heat tolerance of vegetable crops.

Keywords: climate change, abiotic stress, heat stress, molecular mechanism, vegetable, tomato

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INTRODUCTION

Climate change, specifically a rise in ambient temperatures, is predicted to significantly affect plant growth and development, resulting in a devastating reduction in crop productivity, causing severe famine and limiting global food security (FAO-STAT, http://faostat.fao.org; Verisk Maplecroft, https://www.maplecroft.com; (Bita and Gerats, 2013). According to the report of the Intergovernmental Panel on Climate Change (IPCC), the accumulation of atmospheric concentrations of greenhouse gases (GHGs), such as CO_2, N_2O, and CH_4, which can absorb infrared radiation reflected from the Earth's surface, was caused by the combustion of fossil energy sources and the associated GHG emissions. Changes in the atmospheric concentrations of GHGs suggested an alteration in the energy balance of our climate, causing the global surface temperature to increase by 0.3°C during the next decade and is expected to reach 1.8–4.0°C by 2100

(Jones et al., 1999; Stocker et al., 2013). During the Conference of Parties 21 (COP21) conference in Paris in 2015, governments of most countries agreed to reduce the use of fossil fuels with the ambition of a complete waiver at the end of the century, thereby attempting to limit global warming to below a 2°C increase, preferably to 1.5°C compared to pre-industrial levels. However, even if the goal of the Paris Agreement is achieved, the initiated threat of heat stress (HS) is not addressed in agriculture as HS is a major global abiotic stress factor for many crops. Due to the increased frequency of extreme weather events, including heatwaves, HS remains a threat to global agricultural production and food security. With 75% of the world's poor living in rural areas and nearly 50% of people in underdeveloped countries relying on agriculture for income, these stakeholders are likely to experience the most serious effects of climate change. In addition, a population rise to 9 billion by the year 2050 and rising food demand in rapidly growing economies, such as China and India, will require a 70% increase in food production to fulfill future needs. Increasing food production while climate change is expected to lead to tremendous crop losses is a challenge that can only be solved by more sustainable agricultural production systems using crop varieties that are more tolerant to abiotic stresses than the presently used varieties. Insights into the mechanisms allowing plants to grow and yield under stressful conditions are key to breeding more stress-tolerant varieties.

Plants, as sessile organisms, are frequently affected by adverse environmental factors, such as drought and temperatures that are hotter or colder than their optimal range. Therefore, plants adapt to stressful conditions to a certain extent. In general, when the ambient temperature is 10–15°C higher than the optimum temperature range for plant cultivation, such conditions are defined as HS (Wahid et al., 2007). HS can cause negative effects on plant morphology, development, physiology, biochemistry, and molecular pathways at all vegetative and reproductive stages. Anther and pollen development at anthesis are very sensitive to temperature fluctuations, causing failure of reproduction and fertilization processes (Warrag and Hall, 1984; Monterroso and Wien, 1990; Peet et al., 1998; Erickson and Markhart, 2002). Consequently, significant adverse effects on reproduction and fertilization processes cause a reduction in fruit set and lower quality fruit and vegetable yields (Bita and Gerats, 2013; Hasanuzzaman et al., 2013). Significant efforts by researchers and breeders are dedicated to overcoming the negative effects of HS. Plants respond to temperature fluctuations and induce short-term stress avoidance or acclimatization mechanisms, including leaf re-orientation to create space, transpiration acceleration for cooling, and alteration of membrane lipid composition (Wahid et al., 2007). At the cellular level, plants adapt to HS through various mechanisms, such as transcription, post-transcription, translation, post-translation, and regulation, at different levels, for example, in calcium, phytohormone, sugar, and lipid signaling, and in primary and secondary metabolism (Bita and Gerats, 2013). Moreover, thermotolerance is regulated by a complex transcriptome network of distinct and interconnected pathways to maintain protein homeostasis and minimize cellular damage (Keller and Simm, 2018).

Tomato (*Solanum lycopersicum*), as a fruit vegetable crop, is of immense importance to the global economy and food culture and is a popular vegetable that is produced worldwide. China is the world's largest tomato producer, followed by India and Turkey (FAOSTAT, http://www.fao.org/). Tomatoes are rich in nutrients, such as vitamin C, β-carotene, and lycopene, which have positive effects on human health (Bergougnoux, 2014). Several institutions have developed tomato genetic resources for researchers and breeders studying heat tolerance and many traits of importance. The Solanaceae Genomics Network (SGN, http://solgenomics.net/) is an online genomic database that provides essential information for researchers. In the USA, the Tomato Genetics Resource Center (TGRC) at the University of California, Davis (http://tgrc.ucdavis.edu/) is an excellent source of diverse germplasm, wild species, and core collections. In Taiwan, the World Vegetable Center (http://seed.worldveg.org) curates 8,835 tomato accessions, of which 6,676 are available on request. In Japan, genetic resources of tomato plants have been collected by the National Agriculture and Food Research Organization (NARO) Genebank (https://www.gene.affrc.go.jp) and the National BioResource Project (NBRP)-Tomato (https://tomato.nbrp.jp). In the NBRP-Tomato, over 10,000 Micro-Tom mutants, created by ethyl methanesulfonate (EMS) mutagenesis and gamma-ray irradiation, have been collected (Watanabe et al., 2007; Matsukura et al., 2008). Micro-Tom is becoming a model plant for studying both fruit production and tolerance to various abiotic and biotic stresses (Ezura, 2016). Researchers can access information regarding this mutagenic line through the online database TOMATOMA (http://tomatoma.nbrp.jp/index.jsp) (Saito et al., 2011; Shikata et al., 2016).

Tomato plants are often exposed to temperature fluctuations during cultivation, and HS significantly affects reproduction and fertilization, leading to crop failure and a decrease in the quantity and quality of harvested fruit (Prasad et al., 1999; Sato et al., 2000). The morphological and physiological changes in response to HS in tomatoes are different among entries or accessions, at different development stages, and with varying HS exposure periods. These changes are not only detected in vegetative organs, such as leaves (Zhou et al., 2017), but also in reproductive organs, such as flowers and gametophytes (Firon et al., 2006). Firon et al. (2006) reported that the relationship between pollen viability and fruit set in tomatoes was detected under HS conditions. Pan et al. (2019) reported that the alteration of flower structure, such as stigma exertion, was associated with jasmonate (JA) signaling and other plant hormone pathways, resulting in low fruit setting.

We reviewed the effects of high temperatures on tomato plants to address key processes determining plant growth and yield. This review focuses on the molecular mechanisms, the morphological and physiological mechanisms contributing to HS tolerance, and the challenges in developing heat-tolerant vegetable varieties.

Abbreviations: ABA, abscisic acid; *AGL6, AGAMOUS-LIKE 6*; *AP3, APETALA3*; BRs, Brassinosteroids; DREBs, dehydration-responsive element-binding; ER, endoplasmic reticulum; ET, ethylene; GAs, gibberellins; GHG, greenhouse gas; HS, heat stress; HsfA1, heat stress transcription factor A1; HSFs, heat stress transcription factors; HSPs, heat shock proteins; JA, jasmonate; PCD, programmed cell death; *PI, PISTILLATA*; ROS, reactive oxygen species; *sHSPs*, small heat shock proteins; SA, salicylic acid; *TM6, TOMATO MADS BOX GENE6*; TSS, total soluble solids content; UPR, unfolded protein response.

MORPHOLOGICAL AND PHYSIOLOGICAL PROCESSES IN TOMATO PLANTS UNDER HS

Plant response to HS varies according to developmental stage, species, genotype, and the timing of **HS** events (Firon et al., 2006; Barnabás et al., 2008; Sakata and Higashitani, 2008; Shanmugam et al., 2013; Sharma et al., 2014) (**Figure 1**). HS resistance is genetically diverse (Ayenan et al., 2019; Bineau et al., 2021). Since their physiological mechanisms are equally diverse, we will first explain the physiological mechanisms and then explain the genetic diversity of HS resistance for breeding. Under HS, plants exhibit many physiological responses, such as abscission and senescence of leaves, growth inhibition of the shoots and roots, and fruit damage, resulting in a substantial decrease in plant productivity (**Figure 1**) (Vollenweider and Günthardt-Goerg, 2005). Extreme HS affects performance and crop quality characteristics. The productivity decrease under HS has been attributed to decreased assimilatory capacity associated with reduced photosynthesis caused by altered membrane stability, enhanced maintenance respiration costs, and a reduction in radiation use efficiency (Zhang et al., 2006; Reynolds et al., 2007; Hasanuzzaman et al., 2013). At the beginning of cultivation, reduced germination percentage, reduced plant emergence, abnormal seedlings, poor seedling vigor, and reduced radicle and plumule growth of germinated seedlings are major impacts of HS and have been documented in various cultivated plant species (Toh et al., 2008; Kumar et al., 2011; Piramila et al., 2012). When tomato plants are cultivated at 42°C, they sustain severe damage at various stages of development, including seed germination, vegetative and reproductive growth, and fruit setting (Wahid et al., 2007).

In general, the intensity, duration, and rate of temperature alteration during the growth and development of tomatoes are the main factors for evaluating the influence of HS (Wahid et al., 2007). During primary synthesis and respiration processes, leaves retain stomata machinery, which regulates gas exchange and water vapor between the atmosphere and the intracellular space, resulting in an adaptation to changes in the cultivation environment (Negi et al., 2008). An increase in CO_2 concentration inhibits the opening and closing of stomata on stomatal apertures (Medlyn et al., 2001; Hashimoto et al., 2006; Ainsworth and Rogers, 2007; Ji et al., 2015). The temperature on the leaf surface influences stomatal density and status; for example, the opening and closing of stomata form a complex network that controls gas exchange and water vapor to adapt to abiotic stresses (Valladares and Pearcy, 1997; Reynolds-Henne et al., 2010). Tomato plants of the cultivar Campbell 28, when heat treated (45°C), had increased stomatal conductance compared to plants under control conditions (25°C), indicating that stomatal closure did not control the reduction in CO_2 (Camejo et al. 2005). Increases in stomatal conductance have been reported in plants exposed to HS (Radin et al., 1994; Zhou et al., 2015), while others have found that stomatal conductance is significantly reduced (Weston and Bauerle, 2007; Neill et al., 2008; Lahr et al., 2015; von Caemmerer and Evans, 2015). It is known that salicylic acid (SA) auxin, cytokinin, ethylene (ET), brassinosteroids (BRs), and JA regulate stomatal function, while abscisic acid (ABA) is not involved (Miura and Tada, 2014).

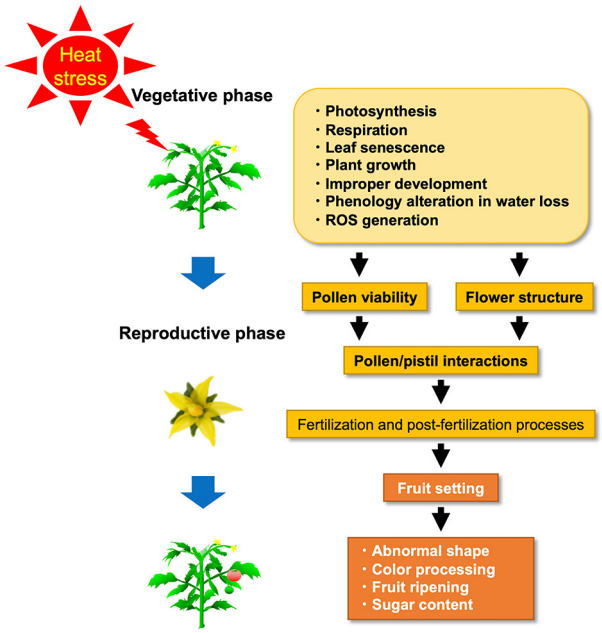

FIGURE 1 | Adverse effects of heat stress (HS) on the vegetative and reproductive phase in tomatoes.

Heat stress adversely affects respiration and photosynthesis, leading to a shortening of the life cycle and a significant decrease in plant productivity (Barnabás et al., 2008). At the beginning of HS, the response is expressed through structural alterations in chloroplast protein complexes and reduced enzyme activity (Bita and Gerats, 2013), followed by damage to the cell membrane and the organization of microtubules. The cytoskeleton can also be damaged, because HS negatively influences membrane permeability, causing alterations in cell differentiation, elongation, and expansion (Smertenko et al., 1997; Potters et al., 2009). The retention of cellular membrane function is essential for sustainable and stable photosynthetic and respiratory continuity under HS (Chen et al., 2012). Some researchers have reported that swelling and aberration of grana stacks occur on photosynthetic membranes, resulting in associated changes in energy allocation to photosystems and ion leakage from leaf cells (Wahid and Shabbir, 2005; Allakhverdiev et al., 2008).

The negative effect of HS on chlorophyll and the photosynthetic apparatus results in the overproduction of reactive oxygen species (ROS), which are involved in responses to biotic and abiotic stresses (Vara Prasad et al., 2000; Shi et al., 2015). HS reduces photosynthesis and respiratory activity by increasing chlorophyllase activity and reducing the number of photosynthetic pigments (Todorov et al., 2003; Sharkey and Zhang, 2010). An increase in the concentration of ROS was not only associated with programmed cell death (PCD) but also with various metabolic reactions, such as DNA damage,

enzyme activity impairment, lipid peroxidation in cellular membranes, carbohydrate oxidation, protein denaturation, and the breakdown of pigments (Bose et al., 2014). Hydrogen peroxide (H_2O_2) is one of the main ROS components produced by plants and fruit tissues under control and HS conditions. In plants, H_2O_2 functions not only as an essential signal that upregulates antioxidant enzyme activities but also mediates ABA-induced stomatal closure to promote stress tolerance (Hu et al., 2005). In addition, hydrogen peroxide accumulates and enhances the thermotolerance of plants when they are treated with low concentrations of SA (Horváth et al., 2007).

Ionic leakage is related to ROS accumulation under stress conditions (Demidchik et al., 2014). Drought stress has been found to result in increased ion leakage in drought-sensitive tomato entry (Thirumalaikumar et al., 2018). There are two popular methods for measuring ion leakage to estimate the heat tolerance in plants: (i) the common ion leakage measurement based on the total electrical conductivity released before and after heating and (ii) the estimation of basal heat tolerance based on the cell suspension or the gradual (linear) heating of plant segments (Ilík et al., 2018). Total ionic leakage is among the most important factors in determining plant responses to abiotic and biotic stresses, as it is associated with stress-induced injury related to PCD in plants (Zhu, 2016).

Antioxidant defense plays an important role in the response of tomato plants to various abiotic stresses. **HS** causes serious damage to antioxidant enzymes function; therefore, tomato plants are required to regulate SA and activate other biochemical pathways to enhance heat tolerance (Jahan et al., 2019). ROS acts as a transduction signal of heat tolerance; hence, superoxide dismutase (SOD) and ascorbic acid peroxidase (APX) are involved in the antioxidant defense mechanism in tomato plants in response to the negative effects of high temperature (Zhou et al., 2019) (**Table 1**).

It has been reported that leaf senescence is accelerated by HS during cultivation. Leaf senescence genes are correlated with PCD and are regulated by multiple levels of chromatin structure, transcription, post-transcription, translation, and post-translation (Woo et al., 2013) (**Table 1**). Leaf senescence genes are also interconnected with other genes responsible for responding to abiotic and biotic stresses. Senescence upregulated 3 (*SENU3)* is a ubiquitous cysteine protease (CP) that is associated with vacuolar senescence in pepper (Drake et al., 1996; Xiao et al., 2014). Another gene involved in leaf senescence is the rubisco large subunit (*RbcL*), which is in the chloroplast DNA and functions as a key enzyme for carbon assimilation and fixation (Enyedi and Pell, 1992; Wang et al., 2015). *RbcL* expression is regulated in response to environmental changes (Xu and Tabita, 1996).

MOLECULAR MECHANISM FOR THERMOTOLERANCE IN TOMATOES

Plants respond to elevated temperatures and ensure survival through various mechanisms, such as transcription, translation, and regulation of calcium, phytohormone, sugar, and lipid signaling, and of primary and secondary metabolism (Bita and Gerats, 2013). Molecular pathway-related thermotolerance has been identified in *Arabidopsis,* tomato, and other species (Qu et al., 2013; Ohama et al., 2017) (**Table 1**). The complex transcriptional pathways were reviewed by Ohama et al. (2017). The HS factor (Hsf) is a transcription factor (TF) associated with HS (**Figure 2**). Many eukaryotes have one to three Hsfs, but plants have over 20, which are classified as A, B, and C. Class A Hsfs are transcriptional activators. Ikeda et al. (2011) found that class B Hsfs of *Arabidopsis*, HsfB1, and HsfB2b, are transcriptional repressors that negatively express heat-induced Hsfs (HsfA2, HsfA7a, HsfB1, and HsfB2b) and a few heat shock protein genes. Yoshida et al. (2011) analyzed the dehydration-responsive element-binding protein 2A (DREB2A) promoter and discovered a heat shock element that functions as a cis-acting element in the expression of HS responsiveness of DREB2A. They generated multiple mutants and found that HS-responsive expression of DREB2A was abolished in the *hsfa1a/b/d* triple and *hsfa1a/b/d/e* quadruple mutants. They further showed that HsfA1a, HsfA1b, and HsfA1d function as major positive regulators of HS-responsive gene expression and that four HsfA1-type proteins are important for gene expression during normal plant growth. Therefore, HsfA1 is the master regulator of the plant's HS response (HSR). Due to HS, HsfA1 causes a transcription cascade composed of many TFs. Higashi et al. (2013) reported HsfA1d, a protein identified through full-length cDNA Over-eXpressing gene (FOX) hunting, using *Thellungiella salsuginea*, a species closely related to *Arabidopsis*. cDNAs improve heat tolerance by regulating HS-responsive gene expression. Ohama et al. (2016) reported that the central region of HsfA1d, one of several *Arabidopsis* HsfA1, is an important regulatory domain that suppresses HsfA1d transactivation activity by interacting with heat shock protein70 (HSP70) and HSP90. They designated this region as the temperature-dependent repression (TDR) domain. Overexpression of constitutively active HsfA1d, which lacks the TDR domain, induced the expression of heat shock proteins in the absence of HS, thereby conferring strong thermal stability to the overexpressors. In this manner, HsfAs control many HS-related factors, including DREB2, and the understanding of their temperature-controlled mechanism is also progressing.

Four types of HsfA1 were isolated in *Arabidopsis* (HsfA1 a, b, c, and d) (Liu et al., 2011), while four different types were identified in tomatoes (HsfA1, a, b, c, e) (El-Shershaby et al., 2019). In HsfA1 families, HsfA1a seems to have a unique function as a master regulator for acquired thermotolerance, and it cannot be replaced by other genes (Mishra et al., 2002; Scharf et al., 2012). Other members of the HsfA1families are induced in specific tissues and stages of the HSR (El-Shershaby et al., 2019). SlHsfA1a function was confirmed by the heat tolerance levels at the incorporation of two HsfA1 transgene cassettes, resulting in a 10- to 15-fold increase in the overexpression line that contained a single HsfA1a transgene cassette and co-suppression line with two cassettes of transgene tandem inverted repeat inserted, respectively (Baniwal et al., 2004). Moreover, with a low abundance of mRNA, HsfA1a was constitutively expressed (Fragkostefanakis et al., 2016). HsfA1d increases heat tolerance

TABLE 1 | Key genes related to heat stress (HS) mechanisms are introduced in this review.

Gene/locus symbol	Origin	Defined function	Related trait/phenotype	References
SOD	Tomato	Antioxidant enzyme	Antioxidant defense	Zhou et al., 2019
APX	Tomato	Antioxidant enzyme	Antioxidant defense	Zhou et al., 2019
SENU3	Tomato, Pepper	Senescence-associated cysteine proteinase Vacuolar localization protein	Leaf senescence	Drake et al., 1996; Xiao et al., 2014
RbcL	Potato	Carbon assimilation and fixation	Leaf senescence	Enyedi and Pell, 1992; Wang et al., 2015
HsfA1 a, b, c, d	*Arabidopsis*	Transcriptional activators to HS	Transcription regulatory network	Liu et al., 2011; Ohama et al., 2017
HsfA1	*Arabidopsis*	Master regulator of HSR	Transcription regulatory network	Yoshida et al., 2011
HsfB1, HsfB2b	*Arabidopsis*	Transcriptional repressors	Transcription regulatory network	Ikeda et al., 2011
HsfA1d	*Thellungiella salsuginea*	HS-responsive gene expression, Temperature-dependent repression (TDR) domain	Transcription regulatory network	Higashi et al., 2013; Ohama et al., 2017
DREB2A	*Arabidopsis*	Transcriptional activators to HS	Transcription regulatory network	Ohama et al., 2017
HsfA1, a, b, c, e	Tomato	Transcriptional activators to HS	Transcription regulatory network	El-Shershaby et al., 2019
HsfA1b	Tomato	Later response gene in transcription regulatory network	Transcription regulatory network	El-Shershaby et al., 2019
ERF.C1/F4/F5	Tomato	Ethylene-responsive transcription factors	HS regulation	Balyan et al., 2020
HSFA7, HSFA6b, HSFA4c, HSFB1, HSFB2b	Tomato	Downstream targets of HSFA	Transcription regulatory network	Rao et al., 2021
HSPs	*Arabidopsis*	Chaperone proteins regulating the folding and accumulation of proteins, localization, and degradation	Transcription regulatory network	Kotak et al., 2007; Qu et al., 2013
AP1	*Arabidopsis*	Class A activity	Flower morphology	Wellmer et al., 2014
AP3, PI	*Arabidopsis*	Class B activity	Flower morphology	Wellmer et al., 2014
AG	*Arabidopsis*	Class C activity	Flower morphology	Wellmer et al., 2014
STK, SHP1, SHP2	*Arabidopsis*	Class D activity	Flower morphology	Wellmer et al., 2014
SEP1, SEP2, SEP3, SEP4	*Arabidopsis*	Class E activity	Flower morphology	Wellmer et al., 2014
AGL6	*Arabidopsis*	MADS-box transcription factor	Flower morphology	Wellmer et al., 2014
TTS, TGL11	Tomato	Pistil-specific expression	Flower morphology	Müller et al., 2016
TAP3, TM6, PI	Tomato	Class B activity	Flower morphology	Müller et al., 2016
AGL6	Tomato	MADS-box transcription factor, fruit parthenocarpy	Flower morphology, Fruit parthenocarpy	Klap et al., 2017
CLV	Tomato	Signal peptide, shoot, and floral meristem regulation	Shoot and floral meristem	Somssich et al., 2016; Fletcher, 2018; Quinet et al., 2019
WUS	Tomato	Homeodomain transcription factor, shoot and floral meristem regulation	Shoot and floral meristem	Somssich et al., 2016; Fletcher, 2018; Quinet et al., 2019
ELF3	*Arabidopsis*	Transcriptional repressor	Auxin-dependent primordia production	Jones et al., 2021
TAG1, TAGL1	Tomato	MADS-box transcription factor	Fruit size	Gimenez et al., 2016
ZjDA3	*Ziziphus jujuba*	ubiquitin-specific protease	Fruit size	Guo et al., 2021
CCS52A, WEE1	Tomato	Cell cycle switch protein	Fruit size	Gonzalez et al., 2007; Mathieu-Rivet et al., 2010
miRNA172	Tomato Apple	miRNA	Fruit size	José Ripoll et al., 2015; Yao et al., 2016
FAS, LC	Tomato	Flattening and fruit locule number	Fruit size	Rodríguez et al., 2011
SUN, OVATE	Tomato	Fruit elongation	Fruit size	Rodríguez et al., 2011
LIN5	Tomato	Cell wall invertase	Fruit sugar	Fridman et al., 2000, 2004
SUT or SUC	*Arabidopsis*, Tomato, Potato	Sucrose transporter	Fruit sugar	Barker et al., 2000; Weise et al., 2000; Hackel et al., 2006
SlVPEs	Tomato	Vascular processing enzymes, negative regulators	Fruit sugar	Ariizumi et al., 2011

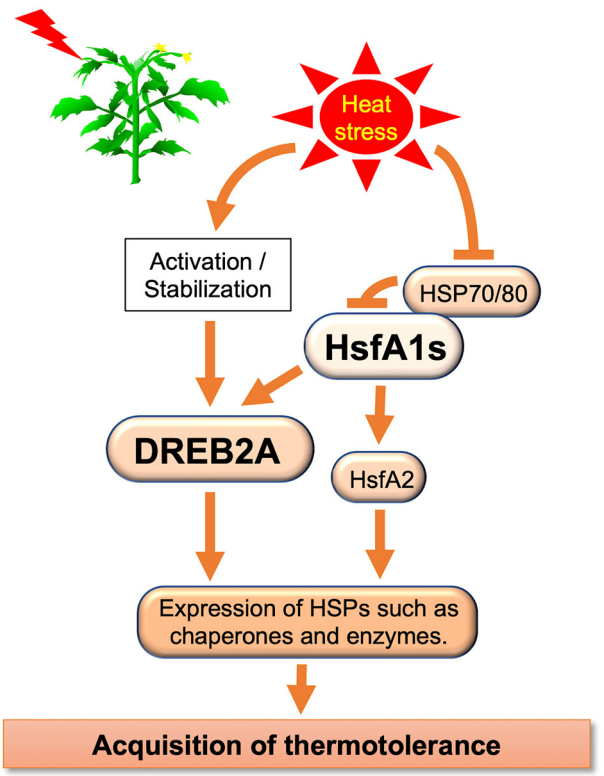

FIGURE 2 | Schematic transcriptional regulatory network in plants examined in this review.

in soybean (Ohama et al., 2017). HsfA1b is a member of the HsfA1 subfamily, which is induced under HS conditions (above 35°C). Under HS conditions, HsfA1a was stably expressed, whereas HsfA1b showed high variation in gene expression in mature green fruits and young leaves (El-Shershaby et al., 2019). In addition, the HsfA1b function is mainly controlled at the transcriptional level by HsfA1 members (Fragkostefanakis et al., 2016). HsfA1b was strongly expressed in fruits, and high fluctuation was observed among different tissues. The results suggested that HsfA1b is a later response gene under HS (El-Shershaby et al., 2019). Several tolerant genes, such as HsfA2, HsfA3, induced-heat shock protein HSPs, ET-responsive transcriptional coactivator multiprotein bridging factor ER24 (*LeMBF1*), cytosolic ascorbate peroxidase 3 *(SlAPX3)* (a ROS scavenger), and calcium-dependent protein kinase 2 (*CDPK2*), were isolated from the anthers (Frank et al., 2009; Zinn et al., 2010). Recently, Balyan et al. (2020) have reported that there is redundancy in cultivar-specific HS regulation compared to transcriptomes between resistant (CLN1621L) and susceptible (CA4) cultivars. Enzymes and proteins related to plant defense and abiotic stress are antagonistically expressed. This study suggested that three ET-responsive TFs (ERF.C1/F4/F5), as several novel HS-resistant genes, improved tomato HS resistance. Rao et al. (2021) reported HSFA7, HSFA6b, HSFA4c, HSFB1, and HSFB2b as new downstream targets of HSFA1a in tomatoes during **HS**.

Heat shock proteins are regulated by HSFs, which control protein quality (Scharf et al., 2012). HSPs are crucial chaperone proteins that are induced during HSR. The HSP family includes a number of small HSPs (sHSPs) and sub-family proteins HSP60, HSP70, HSP90, and HSP100 (Kotak et al., 2007; Qu et al., 2013). The *Hsp21* gene is related to chloroplasts and photosynthesis (Neta-Sharir et al., 2005; Zhong et al., 2013), whereas *HSP101* is among tolerant genes, such as stable Rubisco isoforms and other genes identified from anther profiling (Zinn et al., 2010). In HS, HSPs play important roles in the regulation of protein quality through protein denaturation. HSP21 is a small HSP in *Arabidopsis*, necessary for chloroplast development to protect photosynthesis (Zhong et al., 2013). HSP101 functions as a chaperone in protein degradation (Wang et al., 2004). Despite the important role it plays in sHSP thermotolerance, the underlying mechanisms are not known (Ohama et al., 2017).

Studying the expression levels of TFs and HSPs in tomatoes under HS will help understand the molecular mechanisms of mutant response to high temperatures.

INFLUENCE OF HS ON THE REPRODUCTIVE ORGANS AND REPRODUCTIVE PHASE IN TOMATO PLANTS

The reproductive stage of the plant and the reproductive organs are highly sensitive to HS, which is a major yield-reducing factor. Various reproductive phases, especially stages including meiosis in both male and female organs, pollen germination, pollen tube growth, pollen/pistil interactions, fertilization and post-fertilization processes, formation of the endosperm, and embryo development, are highly sensitive to HS (Warrag and Hall, 1984; Monterroso and Wien, 1990; Peet et al., 1998; Erickson and Markhart, 2002).

Tomato is an autogamous species with a flower structure that is compatible with self-pollination; the anther cones (stamens) cover the style (stigma or pistil). The position and maturity of the male (anther cone) and female (style) organs are markedly affected by various abiotic stresses, including HS during bud development, causing stigma (style) exertion in tomato flowers (**Figure 1**) (Saeed et al., 2007; Yan et al., 2009; Pan et al., 2019). The effects of elevated temperatures on tomato flower morphology have been previously explored. The tomato stigma under HS is exerted, preventing self-pollination, and causing fruit-setting failure (Sato et al., 2006; Giorno et al., 2013). The exertion of tomato stigmas induced by HS is associated with various factors and pathways, such as JA signaling (Pan et al., 2019). Pan et al. (2019) reported that stigma exertion induced by HS was a result of the higher susceptibility of the stamen to HS as compared to the pistil and the differences in cell morphology in both. The discrepant coregulation of pectin, sugar, expansion, and cyclin in stamens and pistils determined cell shape and number by regulating cell expansion and division under HS. Auxin is required to regulate high temperature-induced growth

inhibition in both stamens and pistils. JA plays a crucial role in protecting pistils against HS, and the JA/JA receptor CORONATINE-INSENSITIVE 1 (COI1) signaling pathway is a key hub in stigma exertion. Müller et al. (2016) reported that concurrent reduction in pollen viability and pistil-like aberrant formation of anthers under HS is caused by altered localization of two pistil-specific gene products, *TRANSMITTING TISSUE SPECIFIC (TTS)* and *TOMATO AGAMOUS LIKE11 (TGL11)*. This is accompanied by reduced expression of B-class genes, such as *TOMATO APETALA3 (TAP3), TOMATO MADS BOX GENE6 (TM6)*, and *PISTILLATA* (*PI*) in the anthers (Kramer et al., 1998; Busi et al., 2003; de Martino et al., 2006). These reports showed that the downregulation of tomato B-class genes, induced by HS, contributes to anther deformation and reduced male fertility (Müller et al., 2016). Thus, flowers exposed to HS showed negative effects at various developmental stages, such as the inhibition of pollen release from anthers due to the failure of anther dehiscence, stigma exposure due to decreased stamen length, and pistil hyperplasia (Takeoka et al., 1991; Sato et al., 2002, 2006).

Maintaining floral morphological homeostasis is important because HS has adverse effects on flower morphology (**Figure 3**). The *ABCDE* model genes include five classes, A, B, C, D, and E of floral development, which is encoded using MAD-box TFs (Rijpkema et al., 2010; Smaczniak et al., 2012), with the exception of class A gene *APETALA2* (*AP2*) (Jofuku et al., 1994). In *Arabidopsis*, *AP1* belongs to class A, *AP3* and *PI* belong to class B, *AGAMOUS* (*AG*) belongs to class C, *SEEDSTICK* (*STK*), *SHATTERPROOF1* (*SHP1*), and *SHP2* belong to class D; and *SEPALLATA1* (*SEP1*), *SEP2*, *SEP3*, and *SEP4* belong to class E (Wellmer et al., 2014). In addition, the *AGAMOUS-LIKE 6* (*AGL6*)-clade genes *AGL6* and *AGL13* play crucial roles in floral organ development, especially in ovule formation (Murai, 2013). In tomatoes, under mild HS, expression of the B-class *PI, TAP3,* and *TM6* genes is reduced in the anthers (Müller et al., 2016). TM6 was partially silenced in response to temperature elevation, resulting in a reduced frequency of pistilloid anthers, pollen viability, and pollen quantity. Müller et al. (2016) suggested that downregulation of tomato B-class genes is related to anther deformations and reduces male fertility.

The *AGL6*-clade did not belong to the conventional *ABCDE* model genes that regulate floral structure in plants but is likely to play a role in the ovary formation (Schauer et al., 2009). In tomato plants, the *AGL6* mutant is related to fruit parthenocarpy (Klap et al., 2017). In addition, *AGL6* also acts as a key regulator of the transition between the state of "ovary arrest" imposed toward anthesis and the fertilization-triggered fruit set (Klap et al., 2017). Silencing *AGL6* results in green petals and fused sepals (Yu et al., 2017). The no apical meristem (*NAM*) protein is involved in the separation between sepal boundaries and flower whorls (Hendelman et al., 2013).

The reproductive phase of tomato plants starts from the first bud formation with the development of pollen that is more HS sensitive than female gametophytes and other vegetative organs (Bokszczanin et al., 2013). There are some reports on the pollen viability of tomato plants under HS, and flower buds at 7–15 days before anthesis were the most heat-sensitive of

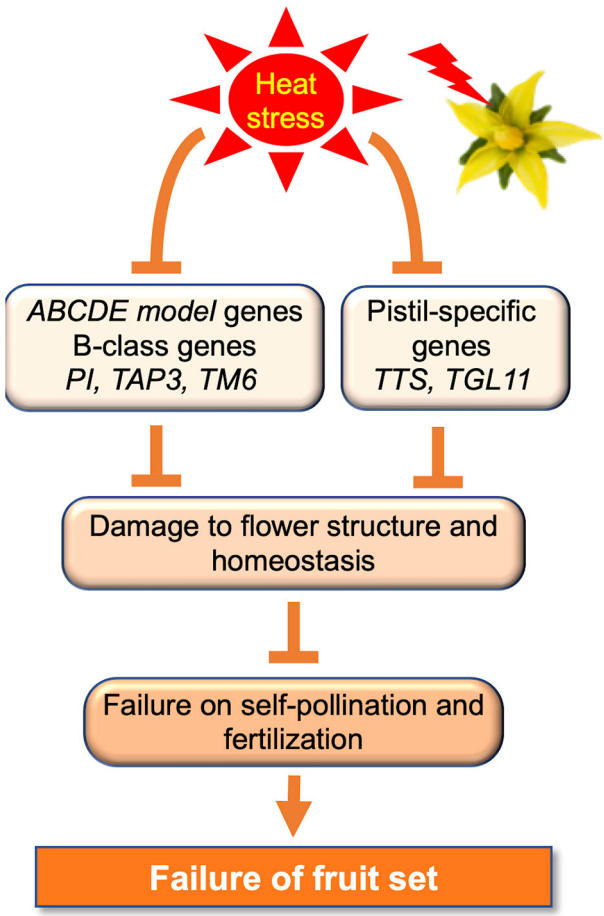

FIGURE 3 | Gene regulation to avoid adverse effects of heat stress on the reproductive phase in tomatoes.

all developmental stages in tomato plants, as spindle formation in the meiosis phase is hypersensitive to HS (Sato et al., 2006). When pollen mother cells in the meiosis phase are damaged by HS, the quality and quantity of pollen grains are markedly reduced. As a matter of fact, tomatoes grown under 32/26°C day/night temperature could not release enough pollen, resulting in diminished fruit set (Sato et al., 2000, 2006). Additionally, pollen viability in tomato plants is controlled by a series of factors that are directly or indirectly involved in pollen thermotolerance. For example, secondary metabolites, such as flavonoids, accumulated in the mature pollen, might reduce the damage caused by ROS scavengers (Paupière et al., 2017). HS negatively affected both the early and late stages of pollen development. A complex network of metabolites and plant hormones is involved in the thermotolerance machinery of tomato pollen at different stages: (i) the early stage of pollen development involves the accumulation of unfolded protein response (UPR) in the endoplasmic reticulum (ER), cytoplasm, changes in histones, alternative splicing, ROS homeostasis, metabolic reprogramming, carbohydrates, plant hormones, and

gibberellins (GAs); and (ii) the later stage of pollen development involves UPRs, ROS, amino acids (proline), carbohydrates, auxins, polyamines, flavonoids, and plant hormones (such as JAs, ETs, BRs, and ABA). Furthermore, compatible stigma and pollen also contributed to successful fruit and seed formation (Raja et al., 2019). Therefore, these factors should be considered when developing strategies to improve tomato fruit production under high-temperature conditions.

NEGATIVE EFFECTS OF HS ON FRUIT DEVELOPMENT IN TOMATO PLANTS

Heat stress suppressed tomato fruit development, resulting in abnormal fruit shapes and negative changes in color processing. HS not only decreases fruit setting but also influences fruit dehiscence and fruit morphology, resulting in dehydration, with wrinkled skin and dry, locular fruit cavities (Lin et al., 2011). Fruit quality is controlled by the cell number and cell size, sugar accumulation, traits related to fruit shape, colorimetry, total solids, texture, and flavor (Cheniclet et al., 2005; Chusreeaeom et al., 2014; Quinet et al., 2019). Fruit size is determined by the coordinated control of cell division and cell expansion. Fruit size is regulated by several molecular mechanisms, including hormonal regulation, the CLAVATA-WUSCHEL (CLV-WUS) signaling pathway, the MADS-box family, the ubiquitin-proteasome pathway, quantitative trait loci (QTLs), microRNA, and endoreduplication (Yuste-Lisbona et al., 2020; Zhao et al., 2021). The CLV-WUS signaling pathway regulates the maintenance of stem cells in shoot and floral meristem, contributing to several agronomic traits, such as flower and fruit numbers (Fletcher, 2018; Quinet et al., 2019). The CLV-WUS feedback loop regulates meristem activity and floral meristem size during the initial phase of tomato fruit development, and it determines carpel number in flowers and, thus, seed locules in fruit during the later phases (Rodríguez-Leal et al., 2017). The signaling peptide CLV3 interacts directly with CLV1 or CLV2, which are leucine-rich repeat receptor kinases, and activates a signaling cascade that negatively regulates the activation of the stem cell-promoting TF WUS (Somssich et al., 2016). A loss-of-function mutation in any *CLV* genes, such as natural mutations in *fasciated (fas)* and *locule number* (*lc*), results in increased proliferation of stem cells and consequently the development of extra floral organs and larger fruits (Barrero et al., 2006; Xu et al., 2015; Fletcher, 2018). Jones et al. (2021) reported that high temperatures bypass CLV signaling and upregulate auxin through transcriptional repressor EARLY FLOWERING 3 (ELF3) in *Arabidopsis*; therefore, high temperatures and ELF3 regulate auxin-dependent primordia production. However, it is unclear how CLV2/CRN is involved in auxin-dependent flower initiation in *Arabidopsis*. Additionally, auxin and GA signaling pathways stimulate and directly activate tomato fruit sets (de Jong et al., 2009). *TOMATO AGAMOUS1 (TAG1)* and *ARLEQUIN/TOMATO AGAMOUS LIKE1 (TAGL1)* genes, which are members of the tomato MADS-box gene family, influence fruit size in tomatoes (Gimenez et al., 2016). Tomatoes that overexpress *ZjDA3*, an ortholog of *Arabidopsis* ubiquitin-specific protease (DA3/UBP14) in Chinese jujube (*Ziziphus jujuba* Mill.), have reduced fruit size and weight (Guo et al., 2021). The level of endoreduplication in tomatoes was correlated with cell size in fruit pericarp (Cheniclet et al., 2005). The expression of *CCS52A* (*Cell cycle switch protein*) or *WEE1* (cell cycle-associated protein kinase) genes involving endoreduplication in tomatoes affects cell size and fruit size (Gonzalez et al., 2007; Mathieu-Rivet et al., 2010). Although it is known that miRNA172 influences fruit size regulation in horticultural plants, such as tomato and apple (José Ripoll et al., 2015; Yao et al., 2016), their relationship in tomatoes under **HS** is unclear. Four genes are related to fruit shape in tomatoes; *FASCIATED (FAS)* and *LOCULE NUMBER (LC)* control flattening and fruit locule number and *SUN* and *OVATE* contribute to fruit elongation (Rodríguez et al., 2011). HS increases parthenocarpic fruit production (Pan et al., 2017; Xu et al., 2017; Shinozaki et al., 2018; Pham et al., 2020). Parthenocarpic fruits are induced by several plant hormones, such as GAs and auxin (Ariizumi et al., 2013; Bita and Gerats, 2013; Shinozaki et al., 2020). Additionally, ABA plays a critical role in regulating transcript expression to induce plant defense responses under HS (Scharf et al., 2012; Paupière et al., 2017; Rieu et al., 2017).

In tomatoes, sugar content is closely linked to fruit development (Kanayama, 2017) and is controlled by the *phosphoenolpyruvate carboxykinase* gene (*PEPCK*) (Huang et al., 2015), biochemical factors (Beckles et al., 2012), vacuolar processing enzymes (Ariizumi et al., 2011), and putative sucrose sensors (Barker et al., 2000). Total soluble solid (TSS or Brix°) represents the fruit sugar content. TSS content is highly influenced by various biotic and abiotic stresses, including HS, which also damages fruit morphology and quality. Tomatoes have three different developmental stages (Ho, 1996): (i) cell division to increase cell number that contributes to mature fruit size, (ii) rapid cell expansion, and (iii) fruit ripening (Ezura, 2016). Sugar accumulation also generally consists of three steps: first, the vascular system imports the sucrose and water influx; second, starch biosynthesis and sugar metabolism; and third, the breakdown of starch into glucose while fruits soften rapidly (Carrari et al., 2006). In the TSS of tomato fruits, sugars (glucose, galactose, and fructose) contributed the largest portion (Selahle et al., 2014), and TSS commonly ranges from 4 to 6 °Brix among different genotypes.

In tomatoes, the functional amino acid polymorphism of cell wall invertase (*LIN5*) was encoded by *Brix9-2-5*, which regulates fruit sugar content (Fridman et al., 2000, 2004). Fruit sugar content and seed development are affected by the inhibition of sucrose transporters. Several sucrose transporters (*SUT* or *SUC*) that are essential membrane proteins localized in the phloem sieve element, including *LeSUT1*, are expressed in leaves; *LeSUT2* is expressed in stems, fruits, and anthers, and *LeSUT3* is expressed in ovaries and immature fruits (Barker et al., 2000; Weise et al., 2000; Hackel et al., 2006). In addition, five genes encode vascular processing enzymes *(SlVPEs)*: two seed coating type genes, *SlVPE1* and *SlVPE2*, one seed type gene *SlVPE4*, and two vegetative genes, *SlVPE3* and *SlVPE5* were reported (Ariizumi et al., 2011). *SlVPEs* are negative regulators of sugar content in tomato plants. Therefore, using transgenic RNAi lines

for single or multiple gene expression could be an approach to increase sugar accumulation in tomatoes.

BREEDING MATERIALS AND TECHNOLOGY TO MITIGATE HS INFLUENCE

The morphological and physiological traits in the vegetative and reproductive phases, that are useful for identifying heat-tolerant tomatoes, are summarized in **Figure 1**. Several breeding lines have been recently identified and evaluated by focusing on phenotypes and indicators to generate a heat-tolerant tomato (**Table 2**). Zhou et al. (2015) described the differences in the quantum efficiency of photosystem II (F*v*/F*m*) between the heat-tolerant and heat-sensitive groups of tomato entries, and they reported that F*v*/F*m* was useful as an early indicator of HS tolerance. Subsequently, Poudyal et al. (2018) evaluated some genotypes using F*v*/F*m* and identified some novel heat-tolerant entries. Paupière et al. (2017) evaluated the accessions of 17 different cultivated and wild tomato phenotypes to high temperatures, focusing on a pollen viability screening approach, and identified thermotolerant and thermosensitive entries. Some heat-tolerant tomato mutants were identified from over 4,000 lines of Micro-Tom tomato mutant collections by evaluating pollen viability, fruit yield, and fruit setting under long-term HS (Pham et al., 2020). Kugblenu et al. (2013) evaluated heat adaptation traits, such as flower drop and fruit number using commercial varieties, that are widely available to farmers in West Africa. Under HS, tomato plants carry the *procera (pro)* and *procera-2 (pro-2)* mutants, which are loss-of-function mutants of tomato *DELLA (SlDELLA)*; the hypomorphic allele showed higher fruit set efficiency, and their fruits were parthenocarpic (Shinozaki et al., 2018). In general, there are two main approaches to studying thermotolerance in tomato plants: screening the germplasm (for long-term mild heat treatment) and physiological responses (for short-term heat shock, up to 45°C). Therefore, breeding and genetic engineering strategies can be individually applied or suitably integrated to develop HS tolerant lines or mitigate the effects of HS on tomatoes. Meta-quality trait loci (meta-QTL analysis) and multi-parent advanced generation intercross (MAGIC) have been used to provide a higher mapping resolution in heat-tolerant tomato breeding programs. In addition, speed breeding and genomic selection (GS) significantly contribute to thermotolerance in tomatoes (Ayenan et al., 2019; Aleem et al., 2020; Bineau et al., 2021). There are several ways of mitigating the effects of **HS** on tomatoes, for example, applying plant growth-promoting rhizobacteria (PGPR) (Mukhtar et al., 2020), or using 6 ppm sulfur (Ali et al., 2021), or nitrate seed priming (Kumar V. et al., 2021). These breeding materials can be used to elucidate the physiological responses conferring adaptation to HS and provide a basis for further studies on the identification of heat-tolerant lines and phenotyping segregating populations.

On the other hand, genetic modification (GM) technology provides rapid and effective cultivars exhibiting tolerance to diverse abiotic stresses, including HS, compared with traditional breeding. There has been some research on the supply of heat tolerance using various genes involved in the regulatory and signaling pathways (Gerszberg et al., 2015) (**Table 3**). To date, the provision of tolerance to HS was performed using transgenic plants overexpressing TF or HSP involved in transcription regulatory networks, such as *HsfA1* (Mishra et al., 2002), *hsp21* (Neta-Sharir et al., 2005), *MasHSP24.4* (Mahesh et al., 2013), and *MT-sHSP* (Nautiyal et al., 2005). Transgenic plants expressing yeast S-adenosyl-l-methionine decarboxylase (*SAMDC*), which is a key regulatory enzyme in polyamine biosynthesis, increased the accumulation of spermidine and spermine and enhanced antioxidant enzyme activity, thereby protecting membrane lipid peroxidation. Subsequently, the plant was protected from HS by improving the efficiency of CO_2 assimilation through its enhanced activity and protection (Cheng et al., 2009). Transgenic tomato with an increased anthocyanin-associated R2R3-MYB TF, *Lycopersicon esculentum Anthocyanin 2* (*LeAN2*) overexpression, is highly tolerant to HS (Meng et al., 2015). LeCDJ1 *(Lycopersicon esculentum* chloroplast-targeted DnaJ protein) is involved in the plant response to ABA. *LeCDJ1* overexpressed plant improved growth, chlorophyll content, lower malondialdehyde accumulation, relative electrical conductivity, and less PSII photoinhibition under HS (Kong et al., 2014). Transgenic tomato plants overexpressing choline oxidase (COD), which is involved in glycine betaine (GB) synthesis, showed a high accumulation of GB. The *codA*-transgenic plants showed increased CO_2 assimilation and photosystem II photochemical activity and mitigated the accumulation of H_2O_2, superoxide anion radicals, and malondialdehyde. Zhang et al. (2020) suggested the major role of GB in HS tolerance and the importance of H_2O_2 as a signaling molecule in heat resistance.

DEVELOPING HEAT-TOLERANT TOMATOES FOR BREEDING

Genome analysis has progressed significantly and allows us to breed genomes not only in major crops, such as rice but also in vegetables, such as tomatoes. Large-scale phenotypic analysis has also seen significant development. To make the best use of these technologies, it is important to choose traits carefully and conduct the evaluation that suits the objectives, in an appropriate environment. In tropical regions, such as sub-Saharan Africa (SSA) and Southeast Asia, where rapid population growth is predicted in the future, sustainable production and supply of vegetables will contribute to food security, household improvement of farmers, nutrition improvement of residents, and health promotion. However, currently, most of the vegetables in the tropics are produced and consumed as it is. The primary varieties cultivated are developed by foreign seed companies in developed countries, and these varieties are not very resistant to high temperatures and humidity. Insufficient resistance to diseases results in instability in the yield and quality. Our research team is promoting genome breeding research to develop vegetables, such as tomatoes that are resistant to high-temperature stress, by utilizing unused genetic resources with

TABLE 2 | Heat-tolerant tomato germplasms and screening conditions.

Identified tolerant accessions or genotype	Screening environment	Screening conditions	Traits and phenotypes	References
LA1500, LA1563, LA1994, LA2093 (*S. pimpinellifolium*), LA3120, LA3183, Bush Italian Roma, Super Sweet	Controlled environment Open field (for validation)	67 genotypes, 2 heat tolerant and 2 heat sensitive for validation HS: 36/28°C for 4 d 67 genotypes HS: 40°C for 7 h	Pollen germination rate Pollen tube length Fruit set	Zhou et al., 2015
Doti Local 1, HRD 1, HRD 17, ST 10, ST 52	Controlled environment Open field	HS: 4 d at 38/28°C, 5 d for recovering 38/26°C	Photosynthesis Stomatal conductance Plant size Pollen viability Fruit yield	Poudyal et al., 2018
LA2854, LA1478, Nagcarlang, CL5915-153D4-3-3-0, CL1131-0-0-13-0-6, CLN1621F and CL5915-93D4-1-0-3 (highest pollen viability) LA1580, LA2854 (*S. pimpinellifolium*), CLN1621F, CL5915-206D4-2-2-0-4, CLN65-349D5-2-0, M-82, CL5915-93D4-1-0-3 (highest number of pollen per flower)	Controlled environment	HS: 32/26°C (day/night) Control: 25/19°C (day/night under 12-18 h of natural day light for 1 month)	Number of pollen per flower Pollen viability	Paupière et al., 2017
15 heat-tolerant tomato mutants	Controlled environment Greenhouse	HS: 35/25°C, 16 h/8 h light/dark, 60.0 μmol m^{-2} s^{-1} Control: 25°C, 16 h/8 h light/dark, 60.0 μmol m^{-2} s^{-1} Greenhouse: Over 35°C (daily maximum temperature)	Flower number Fruit number Fruit set Fruit yield Average fruit weight Seed number SPAD score Pollen viability Pollen germination	Pham et al., 2020
Nkansah (CLN2001A) (high fruit set)	Controlled environment	HS: 33.8/25.9°C (day/night)	Percentage of flower drop Number of fruits Days to flowering Fruit yield per plant Fruit weight Number of truss Number of flowers per truss Number of fruits per plant	Kugblenu et al., 2013
procera (pro), procera-2 (pro-2)	Greenhouse	Summer conditions (June-September 2014)	Fruit number Fruit set Fruit yield Average fruit weight Stem elongation Brix value	Shinozaki et al., 2018
69 genotypes (13 and 19% of the core collection and MAGIC populations, respectively)	Greenhouse	MAGIC HS: 26.9/34.4°C Control: 21.2/28.8°C Core collection HS: 27.5/35.5°C Control: 22.7/31°C Daily mean/maximal temperatures	Stem diameter Leaf length Plant height Flowering time Flower number Fruit number Fruit set Average fruit weight Fruit color Fruit pH	Bineau et al., 2021

excellent traits and making full use of marker selection based on genomic information, especially in SSA and Southeast Asia.

In vegetable breeding, in addition to genome breeding that makes full use of genome information and phenotyping technology, there is great potential for genome editing that has been developed in recent years. In countries with a product-based mindset, unlike traditional GM crops, GM technology is used to generate genome-edited crops, but null segregants do not contain transgenes, which can be suggested using Southern hybridization and PCR (El-Mounadi et al., 2020; Kumar S. et al., 2021).

TABLE 3 | Representative genes available for improving heat tolerance in tomato plants.

Gene/locus symbol	Source	Expression	Defined function	References
HsfA1	Tomato	Overexpression	Transcription regulatory network	Mishra et al., 2002
hsp21	Tomato	Overexpression	Accumulation of heat shock proteins Transcription regulatory network	Neta-Sharir et al., 2005
MasHSP24.4	*Musa acuminata*	Expression	Accumulation of heat shock proteins Transcription regulatory network	Mahesh et al., 2013
MT-sHSP	Tomato	Expression	Accumulation of heat shock proteins Transcription regulatory network	Nautiyal et al., 2005
SAMDC	Yeast	Overexpression	Polyamine biosynthesis	Cheng et al., 2009
LeAN2	Tomato	Overexpression	Anthocyanin-associated R2R3-MYB transcription factor	Meng et al., 2015
LeCDJ1	Tomato	Overexpression	Chloroplast-targeted DnaJ protein	Kong et al., 2014
codA	Tomato	Overexpression	Glycine betaine (GB) synthesis	Zhang et al., 2020
slmapk3	Tomato	CRISPR/Cas9	Mitogen-activated protein kinases (MAPKs) family	Yu et al., 2019

In this case, the deregulation process required for GM crops becomes unnecessary; thus, commercialization is relatively easy, and consumers' resistance to GM can be excepted. The University of Tsukuba leads the development and sale of the tomato variety Sicilian Rouge High GABA (Sanatechssed; https://sanatech-seed.com/en/), which has improved GABA contents (a component that has the effect of suppressing blood pressure rise) through genome editing (Nonaka et al., 2017; Lee et al., 2018; Yamamoto et al., 2018; Gramazio et al., 2020). To date, many genes involved in HS resistance have been identified. It is expected that the improved genome editing technology will be used to improve HS resistance by inducing mutations in the negative regulatory genes, which are the key to HS resistance in vegetables, such as tomatoes. Genome editing has already been used to improve stress resistance in tomatoes. For example, the *mitogen-activated protein kinase* (*mapK*) *3* gene, *slmapK3* gene, branched-amino acid (ALS1), cytidine base editor (CBE*)* genes, and LATERAL ORGAN BOUNDARIES DOMAIN—LBD TF gene—*SlLBD40* increased resistance to HS, sulfonylurea herbicide chlorsulfuron, and drought, respectively (Ayenan et al., 2019; Yu et al., 2019; Salava et al., 2021; Xia et al., 2021). Both the achievements of genome editing technology with regard to tomatoes and the identification of key genes, such as *HsfA2, HsfB1, JA/COI1, SlAGL6,* and *SlIAA9*, are related to the thermotolerant acquires mechanism in tomatoes, positively contribute to the breeding of heat-tolerant tomato. On the other hand, considering the future movement of products across countries, harmonization between countries that handle genome editing on a product basis and countries that handle genome editing on a process basis is a future concern. Genome editing is an epoch-making technique that can easily cause mutations that occur in nature for the chosen study species. However, there is a concern that consumers will reject it simply because it is a new technology that manipulates the genome. Scientists need to obtain scientific evidence and communicate it to society by communicating closely with governments, producers, consumers, the media, and other stakeholders.

It is necessary to elucidate the physiological mechanisms underlying heat tolerance and facilitate breeding research to improve both tolerance and recovery ability with respect to resilience. Interdisciplinary approaches that go beyond genetic breeding, such as improving HS resistance by utilizing plant-microbial interactions by elucidating the relationship between the microbiome and HS, may also be effective. By utilizing Digital Transformation (DX), which has developed remarkably in recent years, from the viewpoint of Genotype x Environment x Management (G x E x M), to elucidate the appropriate combination of excellent varieties (genotype), cultivation environment and method (management), model and recommend it to agricultural sites. Utilizing these various innovations may improve climate change adaptation in vegetables, such as tomatoes, by improving HS tolerance in a broad sense.

CONCLUSION

The latest IPCC report clearly indicates that climate change is currently occurring and will threaten food security in the future. The increasing vulnerability of future food systems is a point of concern. **HS**, resulting from climate change-induced temperature increase, has a negative impact on all stages of crop growth. For fruiting vegetables, such as tomatoes, even moderate HS reduces fruit set and quality; therefore, enhancing crop HS tolerance is among the best ways to adapt to climate change. In this review, we discuss the important processes that affect the growth and yield of tomatoes, especially HS. This review examines the molecular, morphological, and physiological mechanisms that contribute to HS tolerance and the challenges of developing thermostable vegetable varieties. HS has several complex adverse effects on a wide range of plant growth stages in tomatoes. To understand plant tolerance mechanisms against HS, it is necessary to investigate molecular tolerance mechanisms at each growth stage and type of HS (short or long term). There are several reports on gene regulation networks with respect to short-term HS, but there are few regarding long-term HS. Considering the need to produce heat-tolerant tomato plants, it is crucial to determine how HS occurs in each target area, select germplasm for screening heat tolerance materials, and design molecular pathways to adjust to the target. The nutritional and functional properties of vegetables, including tomatoes, are valuable in terms of global food and nutritional safety assurance. Studies investigating the rapidly increasing HS tolerance and the development of heat-resistant vegetable

varieties will contribute toward climate change adaptation and the construction of sustainable and resilient food systems to achieve sustainable development goals.

AUTHOR CONTRIBUTIONS

KH and KN wrote the draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Germplasm, Breeding, and Genomics in Potato Improvement of Biotic and Abiotic Stresses Tolerance

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Potato is one of the most important food crops in the world. Late blight, viruses, soil and tuber-borne diseases, insect-pests mainly aphids, whiteflies, and potato tuber moths are the major biotic stresses affecting potato production. Potato is an irrigated and highly fertilizer-responsive crop, and therefore, heat, drought, and nutrient stresses are the key abiotic stresses. The genus *Solanum* is a reservoir of genetic diversity, however, a little fraction of total diversity has been utilized in potato breeding. The conventional breeding has contributed significantly to the development of potato varieties. In recent years, a tremendous progress has been achieved in the sequencing technologies from short-reads to long-reads sequence data, genomes of *Solanum* species (i.e., pan-genomics), bioinformatics and multi-omics platforms such as genomics, transcriptomics, proteomics, metabolomics, ionomics, and phenomics. As such, genome editing has been extensively explored as a next-generation breeding tool. With the available high-throughput genotyping facilities and tetraploid allele calling softwares, genomic selection would be a reality in potato in the near future. This mini-review covers an update on germplasm, breeding, and genomics in potato improvement for biotic and abiotic stress tolerance.

Keywords: biotic, abiotic, breeding, potato, genomics, omics approaches

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the third most important food crop of the world after rice and wheat. Potato suffers from various biotic and abiotic stresses, which may cause crop failure and yield loss depending on their severity. The key factors affecting potato cultivation are (a) biotic stresses including diseases like late blight, viruses, bacterial wilt, soil and tuber-borne diseases, insect-pests like aphids, whiteflies, thrips, mites, hoppers, potato tuber moths, and potato cyst nematodes (Singh et al., 2020); and (b) abiotic stresses like heat, drought, nutrient deficiency, salinity, and cold/frost

stress (Handayani et al., 2019). Late blight is still the most serious disease of potato, however, in the current climate change scenario, viruses are becoming new threats especially for healthy seed production. Similarly, heat and drought stresses are major challenges in potato due to rising temperature, erratic rainfall, and drought conditions (Singh et al., 2020). Hence, management of these problems is very critical for developing climate resilient varieties through an accelerated breeding approach. Although conventional breeding has made significant progress, it is relatively slow and harnessed the limited potential of the *Solanum* gene pool (Hardigan et al., 2017). Now, the potato genome sequences (Potato Genome Sequencing Consortium, 2011) and resequence of wild/cultivated species are available publicly, such as *de novo* sequencing of two wild species namely *S. commersonii* (Aversano et al., 2015) and *S. chacoense* "M6" (Leisner et al., 2018); and resequencing of over 100 *Solanum* species (Hardigan et al., 2017; Kyriakidou et al., 2020; Tiwari et al., 2021). The rapid advancements in sequencing technologies, multi-omics approaches, genome editing, and genomic selection coupled with softwares/bioinformatics allow discovery of SNP markers, genes, and regulatory elements for breeding and also to enhance understanding of potato biology (Aksoy et al., 2015). This mini-review highlights the prospects of germplasm, breeding, and genomics in potato improvement for biotic and abiotic stresses tolerance.

BIOTIC STRESSES IN POTATO

Late Blight and Viruses

Late blight, caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, is the most devastating disease of potato crop worldwide. In the year 1845, this disease caused a complete loss of potato crops in the European countries mainly Ireland, and known as "Irish Famine." More than 30 viruses are reported to infect potato crop, of which major viruses are *Potato virus X* (PVX), *Potato virus Y* (PVY), and *Potato leaf roll virus* (PLRV) in the world; and *Tomato leaf curl New Delhi virus-potato* (ToLCNDV) is a new problem in India. Potato viruses are transmitted by contact/mechanical (e.g., PVX) and insect vectors (e.g., PVY/PLRV), and cause mosaic or leaf curl and mixed symptoms (Singh et al., 2020).

Soil and Tuber-Borne Diseases

Soil and tuber-borne diseases like dry rot (*Fusarium oxysporum*), charcoal rot (*Macrophomina phaseolina*) and bacterial soft rot (*Pectobacterium atrosepticum*) are the main problems involved in the post-harvest, storage, and transport of potato. Black scurf (*Rhizoctonia solani*) and common scab (*Streptomyces scabies*) deteriorate tuber appearance. Bacterial wilt (*Ralstonia solanacearum*) is also a serious disease, while wart caused by *Synchytrium endobioticum* is a problem of hilly regions like Darjeeling hills in India. These diseases are managed by using healthy seeds, disinfection by boric acid treatment, cultural practices, and crop rotation (Singh et al., 2020).

Insect-Pests

Insect-pests such as aphids, whiteflies, thrips, white grubs, cutworms, leaf hopper, potato tuber moths, and mites infest potato crop. Aphids (*Myzus persicae*) transmit viruses in two ways i.e., persistent and circulative (PLRV), and non-persistent (PVY). Whiteflies (*Bemisia tabaci*) transmit ToLCNDV-potato virus. Thrips (*Thrips palmi*) transmit *Groundnut bud necrosis virus* and cause stem necrosis disease. Importantly, potato cyst nematodes (PCN) (*Globodera rostochiensis* and *G. pallida*) are key problems in temperate regions. Besides, other insect-pests are potato leaf hopper (*Amrasca biguttula biguttula*), white grub (*Brahmina coriacea*), cutworm (*Agrotis segetum*), potato tuber moth (*Phthorimaea operculella*), and mites (*Polyphagotarsonemus latus*) (Singh et al., 2020).

ABIOTIC STRESS IN POTATO

Heat and Drought Stress

Heat stress is a great problem for potato crop, particularly in early planted crop and after the harvest of the main *rabi* crop under sub-tropical Indian conditions. A minimum night temperature below 20°C (day 25°C) is essential for tuber growth and development (Singh et al., 2015). Potato is mostly an irrigated crop, except in rain fed hilly regions. Therefore, all growth stages are sensitive to water availability such as germination, foliage, and root/stolon/tuber growth. Thus drought i.e., moisture deficit plays a very crucial role in determining potato yield (Dahal et al., 2019).

Nutrient Deficiency, Salinity, and Frost/Cold stress

Nutrients are very essential for plant growth, yield, and quality of potato. Potato is a heavily fertilized crop especially for nitrogen (N), and therefore reduction of N fertilizers is necessary to save the environment and reduce the production cost (Zhang et al., 2020). Nutrient deficiency drastically affects crop growth and reduces yield. Besides, salinity is another problem due to soil or irrigation water, which causes nutrient imbalance and restricts plant growth. Frost/cold is also another issue of temperate climates, where temperatures below −2°C can result in a partial or complete loss of crop (Ahmed et al., 2020).

GERMPLASM, MAPPING, AND BREEDING

Potato Genetic Resources

Potato belongs to the genus *Solanum* (family: Solanaceae), which contains over 2,000 species, of which nearly 235 are tuber bearing potato species, where 73% are diploids (2*x*), 4% triploids (3*x*), 15% tetraploids (4*x*), and 8% pentaploids (5*x*)/hexaploids (6*x*) (Hawkes, 1990). The cultivated potato (*S. tuberosum* ssp. *tuberosum*) is a tetraploid ($2n = 4x = 48$). Potatoes are classified into four major groups (i) *S. tuberosum* group Andigenum of upland Andean genotypes (2*x*/3*x*/4*x*), and

S. tuberosum group Chilotanum of lowland Chilean landraces (4*x*), (ii) *S. ajanhuiri* (2*x*), (iii) *S. juzepczukii* (3*x*), and (iv) *S. curtilobum* (5*x*) (Spooner et al., 2014). These species belong to different endosperm balance numbers (EBNs) like 1EBN (2*x*), 2EBN (2*x*/4*x*), and 4EBN (4*x*/6*x*), where hybridization within the same EBN species is successful but not with different EBN species (Hawkes, 1990). Over 98,000 potato accessions are conserved *ex situ* (*in vitro*), of which 80% are maintained in 30 key collections worldwide (FAO, 2010). To harness the potential of diverse species, a wide range of genetic variation has been recorded and deployed through breeding and ploidy manipulation techniques for potato improvement.

Linkage and Association Mapping

Gene mapping is important for molecular breeding. The complex tetrasomic inheritance, acute inbreeding depression, and high heterozygosity of potato complicate its genetic mapping. Linkage mapping is the genetic association of traits with segregating alleles of molecular markers in a defined mapping population. The first linkage map was reported in 1988 using tomato RFLP (restriction fragment length polymorphism) markers in diploid species (*S. tuberosum* group Phureja/Tuberosum) (Bonierbale et al., 1988). Then uncounted PCR-based molecular markers like simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP), and diversity array technology (DArT) were applied for mapping. Over 10,000 AFLP markers were used to create an ultra-high-density (UHD) genetic and physical map of potato (Van Os et al., 2006), which was used in the potato genome sequencing. Sharma et al. (2013) constructed a dense genetic and physical map for a diploid backcross progeny using 2,469 markers (SSR/AFLP/DArT/SNP). Numerous genes/QTLs have been mapped in potato for various traits like late blight resistance (Hein et al., 2009) and drought stress (Anithakumari et al., 2012). On the contrary to linkage mapping, association mapping identifies genes/QTLs associated with phenotypic variation in a natural population based on the historical recombination and linkage disequilibrium (Flint-Garcia et al., 2003). In potato, diploid/tetraploid clones have been utilized in association mapping for several agronomic traits (D'hoop et al., 2014), particularly resistance to late blight (Gebhardt et al., 2004) and *Verticillium* wilt (Simko et al., 2004) to name a few.

Marker-Assisted Selection

Over 40 traits are considered to be important in potato breeding. Conventional breeding is a time consuming process mainly due to several years of field evaluation and clonal selection. Hence, identification of tightly linked markers with a target gene for a trait is considered to be ideal for MAS. MAS allows a significant decrease in field exposures by selection in the early stage, and thereby reduces field exposures and breeding cycles. In potato, a considerable number of linked markers have been developed and deployed mainly for simply inherited traits like late blight, viruses, and potato cyst nematode resistance (Ramakrishnan et al., 2015). However, meager information is available on MAS for complex traits like yield, nutrient use efficiency, heat, drought, and cold stress.

PROGRESS IN GENOMICS-LED POTATO IMPROVEMENT

Potato Genome Sequencing/Resequencing

In 2011, the Potato Genome Sequencing Consortium (PGSC),—formed by 26 international institutes belonging to 14 countries—successfully deciphered the potato genome (840 Mb) containing 39,031 protein-coding genes using a homozygous doubled monoploid (DM 1-3 516 R44) of *S. tuberosum* group Phureja ($2n = 2x = 24$) (Potato Genome Sequencing Consortium, 2011)[1]. Later Sharma et al. (2013) improved the DM potato assembly with a more accurate arrangement of scaffolds and pseudomolecules. Recently, a chromosome-scale long-read reference assembly has been constructed (Pham et al., 2020). By now over 100 potato species have been sequenced/re-sequenced mostly using Illumina platforms like wild *S. commersonii* (Aversano et al., 2015), tuber-bearing *Solanum* species (Hardigan et al., 2017), *S. chacoense* "M6" (Leisner et al., 2018), *S. pinnatisectum*-derived somatic hybrid (Tiwari et al., 2021), and cultivated potato taxa using Illumina and long-read (PacBio) technologies (Kyriakidou et al., 2020; **Table 1**). The rapid advancement in sequencing and bioinformatics has spurred innovation in discovery of new genes/markers/haplotypes to enable better understanding of potato biology (Zhou et al., 2020). **Figure 1** illustrates different approaches used in potato germplasm, breeding, and genomics-led improvement for biotic and abiotic stresses tolerance.

Multi-Omics Approaches

Functional genomics allows the mining of genes for trait of interest through transcriptome analysis like RNA sequencing and microarray. Besides structural genomics, other omics approaches are transcriptomics (genes), proteomics (proteins), metabolomics (metabolites), phenomics (high-throughput phenotyping), and ionomics (mineral ions). The aims of multi-omics approaches are to acquire comprehensive and integrated understanding of biological processes (system biology) to identify various biological players/genes/regulatory elements underlying the traits like heat and drought stress (Aksoy et al., 2015). Numerous studies have been performed on transcriptomics in potato such as heat (Tang et al., 2020), drought (Moon et al., 2018; Chen et al., 2019), salinity (Li et al., 2020), and nitrogen deficiency (Tiwari et al., 2020a,b) but limited work has been carried out on proteomics, metabolomics, and ionomics (Hong et al., 2016; Boguszewska-Mańkowska et al., 2020). A few recent research works on multi-omics on biotic/abiotic stresses are mentioned in **Table 1**.

Genome-Wide Genetic Diversity and Association Studies Using High-Throughput Genotyping

High-Throughput Genotyping (HTG) is an essential requirement for genome-wide research on genetic diversity and association studies. First, genotyping-by-Sequencing (GBS) is a now popular

[1]http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml

TABLE 1 | A few recent examples of application sequencing and multi-omics technologies in potato for biotic and abiotic stress resistance/tolerance.

Application	System	Traits/objectives	References
Genome sequencing	Illumina HiSeq (and PacBio in some species)	Genome sequencing and structural variation in many *Solanum* species	Hardigan et al., 2017; Kyriakidou et al., 2020
		S. checoense "M6" genome	Leisner et al., 2018
		S. commersonii genome	Aversano et al., 2015
Genome-wide genetic diversity and GWAS	22K SNP array	Construction of core collection	Pandey et al., 2021
	20 K SNP array	Population structure, LD and SNP/haplotypes	Vos et al., 2017
	12K SNP array	Population structure of CIP accessions	Ellis et al., 2018
	8.3 K SNP array	Population structure and LD	Berdugo-Cely et al., 2017
	RenSeq/GenSeq	Late blight and nematode resistance	Strachan et al., 2019
	20K SNP array	Wart disease resistance	Prodhomme et al., 2020
	12 K SNP array	Common scab resistance	Yuan et al., 2020
	8.3K SNP array	Late blight resistance	Mosquera et al., 2016
Genomic selection	8.3k SNP array	Late blight resistance	Stich and Inghelandt, 2018
	8.3k SNP array	Late blight and common scab resistance	Enciso-Rodriguez et al., 2018
Transcriptomics	Illumina HiSeqTM2500	Late blight, bacterial wilt, and PVY resistance	Cao et al., 2020
	Illumina HiSeq2500	Common scab resistance	Fofana et al., 2020
	Ion torrent	Colorado potato beetle resistance	Bastarache et al., 2020
	Illumina NextSeq500	Potato cyst nematode resistance	Kochetov et al., 2020
	Illumina HiSeq × Ten	Salt stress	Li et al., 2020
	Illumina NextSeq	Drought stress	Moon et al., 2018
	Illumina HiSeq 4000	Drought stress	Chen et al., 2019
	Illumina HiSeq-2000	Heat stress	Tang et al., 2020
	Illumina	Nitrogen stress	Tiwari et al., 2020b; Zhang et al., 2020
	NextSeq500	Nitrogen stress	
	Illumina HiSeq 4000		
Proteomics	iTRAQ	Late blight resistance	Xiao et al., 2020
	iTRAQ	Bacterial wilt resistance	Wang et al., 2021
Metabolomics	LC-MS/MS	Potato virus A resistance	Rajamaki et al., 2020
	GC-MS	Salt stress	Hamooh et al., 2021
	LC-ESI-Q-TOF-MS/MS	Nitrogen stress	Jozefowicz et al., 2017
Transcriptomics and metabolomics	Illumina HiSeq 4000, LC-MS	Heat stress	Liu et al., 2021
Proteomics and metabolomics	2-DE	Cold stress	Li et al., 2021
	LC-ESI-MS/MS		
Phenomics (HTP)	X-ray computed tomography (CT)	Heat and drought stress	Harsselaar et al., 2021
	RGB camera and LED light system	Drought stress	Musse et al., 2021
	Unmanned aerial vehicle	Plant height and canopy cover	Colwell et al., 2021
Genome editing	CRISPR/Cas13a	PVY resistance	Makhotenko et al., 2019; Zhan et al., 2019
	CRISPR/Cas9		

LD, linkage disequilibrium; CIP, International Potato Center; GWAS, Genome-Wide Association Studies; htp, high-throughput phenotyping.

method of HTG in crops including potato (Uitdewilligen et al., 2013; Bastien et al., 2018). GBS has been applied effectively in genome-wide studies in potato on genetic diversity and population structure (Pandey et al., 2021), QTL mapping (Schönhals et al., 2017), and SNP discovery (Caruana et al., 2019). Secondly, the SNP array-based HTG system has already been developed and applied in potato for population structure and SNP discovery using 20K SNP array (Vos et al., 2015, 2017), 22K SNP array for starch phosphorylation (Khlestkin et al., 2019), and 12K SNP array (Illumina) for genetic diversity in the genbank of the International Potato Centre, Peru (Ellis et al., 2018). Moreover, 8.3K SNP potato array has

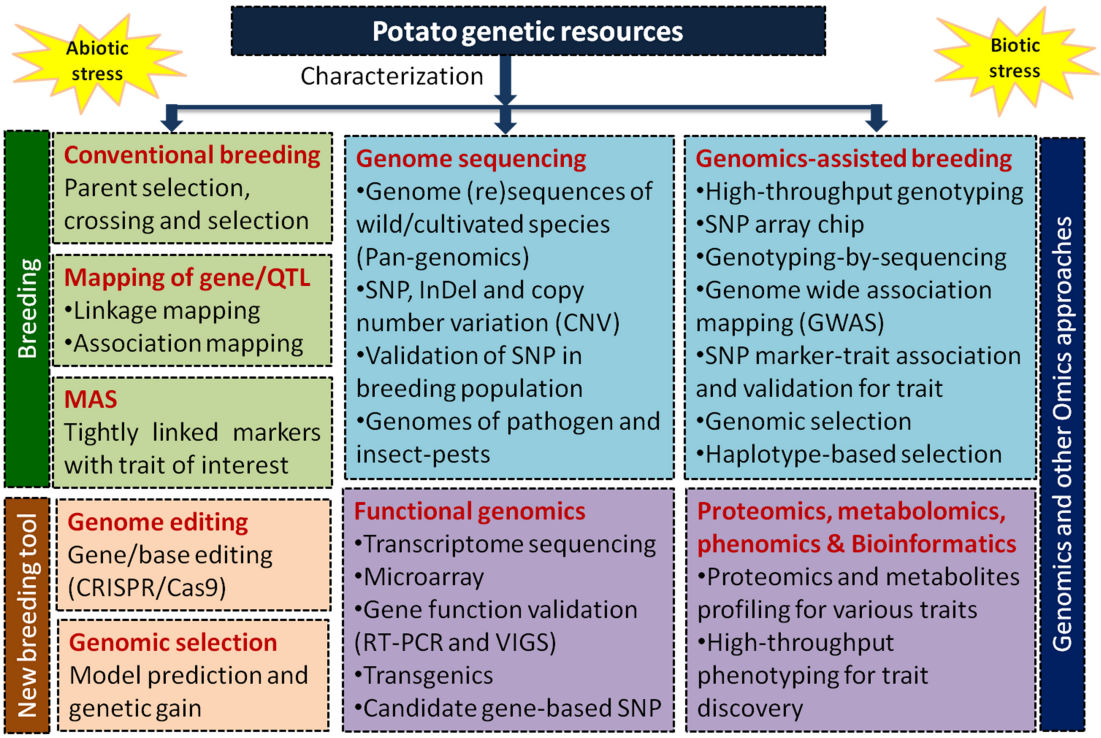

FIGURE 1 | A schematic presentation of different approaches used for genetic enhancement and improvement of potato under various biotic and abiotic stresses applying breeding and modern genomics approaches like genome sequencing, functional genomics, genomics-assisted breeding through high-throughput genotyping by SNP markers, omics (transcriptomics, proteomics, metabolomics, and ionomics), high-throughput phenotyping, genome editing, and genomic selection.

been demonstrated in studies on *Synchytrium endobioticum* resistance (Obidiegwu et al., 2015), genetic diversity (Berdugo-Cely et al., 2017), and physical mapping of yield and quality traits (Schönhals et al., 2017).

Genome-Wide Association Studies (GWAS) or linkage disequilibrium (LD) mapping is a family-based mapping approach to identify linked markers with the trait of interest in a diverse population structure. GWAS is more useful in a diverse germplasm which offers new perspectives toward the discovery of new genes and alleles especially for complex traits. The software STRUCTURE is very popular among scientific communities, and GWASpoly has been developed for tetraploid potato (Rosyara et al., 2016). GWAS has been applied in potato for QTLs/genes via LD mapping using 20K SNP array (Vos et al., 2017), wart resistance using 20K SNP array (Prodhomme et al., 2020) and common scab resistance using 12K SNP array (Yuan et al., 2020). Likewise, 8.3K SNP array has been used in LD mapping for phenotype, yield, and quality traits (Sharma et al., 2018), late blight resistance (Mosquera et al., 2016), and genetic diversity in 809 andigenum Colombian accessions (Berdugo-Cely et al., 2017). Applications of SNP array in potato for biotic and abiotic stress traits are summarized in **Table 1**.

Genomic Selection

Genomic selection (GS) or genome-wide selection or genomics-assisted breeding is a strategy to predict breeding model at whole-genome level for both simple and complex inherited traits. Therefore, partitioning of genetic variance and genome wide prediction with allele doses is very important in tetraploid potato (Endelman et al., 2018). GS allows the integration of phenotyping and HTG data of a training population (both genotyped and phenotyped) with a targeted breeding population (genotyped only) for the prediction of genomic models to select superior clones based on the genomic estimated breeding value (GEBV). GS accelerates the breeding cycle with an increase in genetic gain per unit time. Unlike animals and cereals, the application of GS is very limited in tetraploid potato (Caruana et al., 2019) and has been demonstrated recently for late blight and common scab resistance (Enciso-Rodriguez et al., 2018; Stich and Inghelandt, 2018). The advancement in sequencing, softwares, HTG, HTP, and marker-trait association can reduce the breeding cycle from over 10 to as few as 4 years to increase the genetic gain in potato (Slater et al., 2014; **Table 1**).

High-Throughput Phenotyping

Conventional phenotyping is often slow, has limited phenotyping capability, and mostly relies upon destructive sampling. Hence, modern High-Throughput Phenotyping (HTP) or phenomics is an automated precision phenotyping system allowing identification of key traits associated with phenotypic variation under different growth conditions. HTP is usually based on automation, sensors, high resolution imaging cameras (RGB,

multi/hyperspectral and thermal sensors), unmanned aerial vehicle (UAV) and robotics to record real-time images and hardwares/softwares to analyze data from field or controlled growth chamber[2]. HTP enables measurement of phenotype, yield and its contributing traits, and physiological processes under stress such as photosynthesis, nutrient uptake and transport with precision and accuracy in a large set of genotypes with non-destructive sampling, for example the LemnaTec Scanalyzer 3D platform (LemnaTech GmbH, Germany). HTP has been applied in potato for phenology study in field (Prashar and Jones, 2014), heat and drought (Harsselaar et al., 2021), drought (Musse et al., 2021), and canopy cover using UAV (Colwell et al., 2021). Examples of HTP in heat and drought stress in potato are mentioned in **Table 1**.

Genome Editing

Genome editing is a powerful technology to create new variation in the genome with desirable gene combinations. Earlier sequence-specific nucleases (SSNs) methods like Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs) were used. Now, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) is the most widely used genome editing tool, which is an RNA-guided approach to target DNA/RNA sequences. CRISPR/Cas9 has revolutionized the plant research for multiple traits due to its ease in use, multiplexing capability, cost-effectiveness, and high efficiency. Although, in potato highly heterozygous and tetrasomic inheritance have complicated its deployment (Butler et al., 2015; Andersson et al., 2017) but found effective for PVY resistance using CRISPR/Cas9 (Makhotenko et al., 2019) and CRISPR/Cas13a (Zhan et al., 2019). Additionally, CRISPR/Cas9 has been demonstrated in potato for various other traits like cold-induced sweetening, glycoalkaloid content, homozygous mutants generation, *acetochalactate synthase 1* and *granule bound starch synthase* genes (Nadakuduti et al., 2018; Dangol et al., 2019; **Table 1**).

CONCLUDING REMARKS

Biotic and abiotic stresses are major limiting factors of yield reduction in potato. Management of these stresses are more challenging under the climate change scenario due to emergence of new strains of pathogens and insect-pests, and erratic nature of environmental factors. Potato improvement through genomics-aided methods is essential to shorten the breeding cycle to develop new varieties. Earlier, conventional breeding, bi-parental linkage mapping, and MAS have been successfully demonstrated in potato. The potato genome sequencing and resequencing of *Solanum* species allow discovery of genes, markers and other regulatory elements to provide better understanding of the crop. Now, with the unprecedented advancement in sequencing technologies, genomes of *Solanum* species (pan-genomics), multi-omics for system biology approach (transcriptomics, proteomics, metabolomics, and ionomics), HTG by GBS and SNP array, HTP for precision phenotyping, GWAS and genomic selection would play crucial roles in genomics-led improvement of potato in the near future. There is an immense potential of genome editing for rapid breeding of climate resilient varieties resistant/tolerant to biotic and abiotic stresses. Nonetheless, the availability of an efficient CRISPR/Cas system, target gene selection, plant transformation, and off target mutants would be some challenges in tetraploid crop. Overall, designs of potato that apply genomics, particularly genome editing and genomic selection, and other omics are inevitable in the future.

[2] http:/www.plantaccelerator.org.au/

AUTHOR CONTRUBITIONS

JT conceived idea and wrote manuscript. JT, TB, RZ, NB, DD, SN, HK, CC, SL, and VK performed research and literature collection. RS, AP, and MK edited the manuscript. All authors approved the manuscript.

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REVIEW
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Approaches Involved in the Vegetable Crops Salt Stress Tolerance Improvement: Present Status and Way Ahead

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Salt stress is one of the most important abiotic stresses as it persists throughout the plant life cycle. The productivity of crops is prominently affected by soil salinization due to faulty agricultural practices, increasing human activities, and natural processes. Approximately 10% of the total land area (950 Mha) and 50% of the total irrigated area (230 Mha) in the world are under salt stress. As a consequence, an annual loss of 12 billion US$ is estimated because of reduction in agriculture production inflicted by salt stress. The severity of salt stress will increase in the upcoming years with the increasing world population, and hence the forced use of poor-quality soil and irrigation water. Unfortunately, majority of the vegetable crops, such as bean, carrot, celery, eggplant, lettuce, muskmelon, okra, pea, pepper, potato, spinach, and tomato, have very low salinity threshold (EC_t, which ranged from 1 to 2.5 dS m^{-1} in saturated soil). These crops used almost every part of the world and lakes' novel salt tolerance gene within their gene pool. Salt stress severely affects the yield and quality of these crops. To resolve this issue, novel genes governing salt tolerance under extreme salt stress were identified and transferred to the vegetable crops. The vegetable improvement for salt tolerance will require not only the yield influencing trait but also target those characters or traits that directly influence the salt stress to the crop developmental stage. Genetic engineering and grafting is the potential tool which can improve salt tolerance in vegetable crop regardless of species barriers. In the present review, an updated detail of the various physio-biochemical and molecular aspects involved in salt stress have been explored.

Keywords: oxidative stress, physio-biochemical responses, antioxidant, transgenic crops, gene regulation, yield loss

INTRODUCTION

Nearly, three thousand species of plants are being utilized for the food by human; anyhow, presently, the total global population mainly depends mostly upon 20 species of crops for its major calorie needs from which 50% is contributed by eight cereal crop species (Krishna et al., 2019). The insufficient availability of vegetables is mainly due to increasing population, abiotic

(drought, salt, heat, water logging, etc.) and biotic (virus, viroids, bacteria, fungi, nematodes, and insects) stresses, which potentially reduce the production and quality of the vegetable crops (Ingram, 2011; Prasanna et al., 2015; Karkute et al., 2019; Krishna et al., 2021a,b; Singh et al., 2021; Soumia et al., 2021). The key challenge of modern agriculture is to fulfill the nutritional and food security of the global growing population. Among abiotic stresses, salt stress is the second most destructive stress as it persists throughout the crop life cycle. Salinity stress is one of the most important environmental constraints that limits the economic productivity of vegetable crops (Hong et al., 2021). Salinity in soil is influenced by a regular fluctuation in climatic conditions, irrigation of crops with low quality water, excessive use of ground water, and massive introduction of irrigation associated intensive farming (Tiwari et al., 2010). Further, prolonged water stress conditions in soil could result in increased salinity in soil profile due to lack of leaching rain, increased bore water salinity, and evaporation from irrigation dams. Vegetable crops are more prone to climatic changes compared with other horticultural crops (Giordano et al., 2021), and particularly, salinity stress influences the growth and development throughout their ontogeny. Salinity-induced oxidative stressed in vegetables could affect the qualitative and quantitative value of vegetables as this oxidative stress could lead to a plethora of biochemical and physiological changes in plants (Kashyap et al., 2020, 2021). The most common of them include membrane damage, leakage of substances causing water imbalance and plasmolysis, disturbance in ROS detoxification system, changes in nutrient flux and dynamics, and photosynthetic attributes. These changes ultimately affect the physiological activities like respiration, photosynthesis, transpiration, hormonal regulation, water use efficiency, germination, production of antioxidants, and plasma membrane permeability (Chourasia et al., 2021). The most common approach adopted by plants during such extreme conditions is transcriptional reprogramming of stress responsive genes (Aamir et al., 2017; Tolosa and Zhang, 2020), although the conventional breeding approaches have helped a lot in developing stress-tolerant breeds of vegetables. However, we do not still have developed optimum solutions to prevent the economic losses of vegetables from salt stress, particularly, in intensively irrigated areas (Machado and Serralheiro, 2017). Transgenic technology for salt stress tolerance has been reported as one of the most crucial tool in developing the stress-tolerant vegetable crops (Kumar et al., 2017). For example, to avoid salt tolerance in plants, genes encoding for proteins like Na^+ "exclusion" (PM-ATPases with SOS1 antiporter, and HKT1 transporter), vacuolar compartmentalization of Na^+ V-H^+-ATPase and V-H^+-PPase with NHX antiporter, and also other genes encoding proteins such as aquaporins and dehydrins that are involved in mitigation of water stress during salinity have been transferred and/or overexpressed in tomato or *Arabidopsis* through transgenic technology (Kotula et al., 2020). Since tomato is one of the most important vegetable crop and experimental model for molecular biology studies, most of the research done so far with respect to abiotic and biotic stresses have been done in tomato (Meena et al., 2016, 2018; Zehra et al., 2017a,b). For example, overexpression of *LeNHX2* and *SlSOS2* proteins resulted in salinity tolerance in tomato transgenic lines (Maach et al., 2021). The stress-responsive genes expressed during salinity stress and their fine-tuning could be an eminent tool for developing stress-resistant varieties.

On an average every year approximately 12 billion USD are lost worldwide due to the salinity stress which greatly affects the agriculture production (Zahedi et al., 2019). Almost 10% of the world's entire land area (950 Mha), 20% of the world's cultivated land (300 Mha), and approximately 50% of the total irrigated land (230 Mha) are consequently distressed with extreme salinity (Abiala et al., 2018).

SALT STRESS RESPONSES IN PLANTS

Important physiological and biochemical processes in plants are adversely affected by salinity in various ways through an intense concentration of salts and unavoidably leading to a gradual reduction in plant growth. High salt concentration in rhizosphere of plant cell causes osmotic effect, which remains as a chief contributor to growth reduction during the preliminary stages of a plant life cycle. Amendment in K^+/Na^+ ratio arises when ions reach the plant cell through saline water, leading to augmented Na^+ and Cl^- ion, inflicting extensive damage of numerous physiological processes like protein metabolism and enzyme activities (Tester and Davenport, 2003). The interactions between salts and essential mineral nutrients may consequently result in significant nutrient deficiencies and disproportion. Ionic imbalances may also result in decreased uptake of various significant minerals like potassium, manganese, and calcium to the plants. However, in response to ionic and nutrient imbalances, salt-tolerant plants have uniquely developed the capability of accumulation and compartmentalization of Na^+ and Cl^- in their matured leaves, but sensitive species at absurdly high salinity stage cannot manage to compartmentalize the ions or Na^+ transport, leading to the ionic or osmotic effect. Considerable reduction in plant height has been documented under different abiotic stresses. Due to salinity, plants are exposed to serious water deficit conditions that reduces the leaf growth and leaf areas in several species such as wheat (Sacks et al., 1997), poplar (Wullschleger et al., 2005), and cowpea (Manivannan et al., 2007). One example of the physiological changes in response to salt is shedding of the older leaves of plants (Shao et al., 2008). The upsurge in root to shoot ratio due to salinity conditions was found to be associated with the ABA content of plants (Sharp and LeNoble, 2002). Plant productivity under salinity is strongly correlated with biomass distribution.

MECHANISM OF SALINITY TOLERANCE

Salinity tolerance is related to a list of morphological, biochemical, molecular, and physiological traits that govern the plant growth and productivity (Alexieva et al., 2001). Morphological and physiological adaptation toward tolerance to the salt-induced osmotic stress is also facilitated by

reducing water loss from cuticle and stomata and maximized uptake of water by root to maintain the osmotic adjustment (Rai et al., 2021). Tolerance and adaptation to salt stress are governed by a cascade of molecular networks, which trigger response processes like production of stress proteins, upregulation of antioxidants, and accumulation of compatible solutes (Nahakpam and Shah, 2011) to provide homeostatic reestablishment of cells and to repair and protect the damaged membranes and proteins. On the basis of responses to salinity, plants are categorized as either halophytes or glycophytes (Flowers and Flowers, 2005). Under high saline conditions, glycophytes are unable to survive, whereas halophytes can easily grow and reproduce. Tissue tolerance and salt avoidance are two main approaches implemented by plants to overcome the salt stress. Plants also execute the compartmentalization of ions in the plant tissues. To regulate their osmotic pressure, plants continuously generate water-soluble and low molecular-weight compatible solutes like sugars, glycinebetaine, and proline and the metabolic processes of plants are not disturbed. Plants also produce many enzymatic and non-enzymatic antioxidants to minimize the adverse effect of salinity. In the procedure of plant tissue tolerance, ions compartmentalization occurs in the vacuole, resulting in sustained salt concentration in cytosol, and thus the cytoplasm of the plant cell can be protected from water stress and ion toxicity (Chinnusamy et al., 2005).

To cope with salt stress many strategies have been evolved and developed to secure the vegetable yield under salt stress like transgenic development, regulation of transcription factors (TFs), and grafting. In this review, we have presented updated information on biotechnological interventions in vegetable crops for salt stress.

TRANSGENIC VEGETABLES FOR SALT STRESS TOLERANCE

Vegetables are the cheapest source of minerals, vitamins, antioxidative phytochemicals, and consumed all over the world in raw, semicooked, cooked, and/or in processed forms. Salt stress does not affect vegetable yield but it also affects the nutritional quality of the vegetables. Due to lack of novel salt tolerance in the gene in many vegetable crops gene pools, the transgene has been transferred from the non-parent sources like bacteria, fungi, plant, and animals. For developing transgenic vegetable crops, firstly gene is being identified, characterized, then transferred in the desired vegetables for salt stress. The transgene is being induced under salt stress and the upregulation or downregulation of transgenes initiates a cascade of stress regulatory phenomenon, which ultimately results in salt tolerance (**Figure 1**). Among vegetable crops, Solanaceous crops like potato, tomato, capsicum, and chili constitute major group of vegetables consumed all over the world, and out of these, potato is the most important and ranks third in the world in terms of economic importance and a key agriculture crop for food and nutritional security. Potato is cultivated globally and very sensitive to salt stress, and more than 60% crop loss is caused abiotic stresses including salt (Upadhyaya et al., 2011; Xu et al., 2014; Shafi et al., 2017). To improve potato yield and quality under salt stress condition, many transgenic potato plants have been developed using different genes with different modes of action (Shafi et al., 2017; Wang et al., 2019; Ali et al., 2020). Many osmoprotectant genes like *P_5CS, mtlD,* and *AtBADH* have been transferred to potato, which significantly improves the salt tolerance under salt stress (Karthikeyan et al., 2011; Rahnama et al., 2011; Zhang et al., 2011). Like potato, tomato is the second most important vegetable fruit crop that belongs to the Solanaceae family and it is the highest processed crop in the world. Tomato is a rich source of proteins, minerals, carbohydrates, and vitamins, especially vitamin C. Tomato also contains many phytochemicals like carotenes and lycopenes which have anticancer properties and other health benefits (Krishna et al., 2021a,b; Rai et al., 2021). In tomato far salt stress tolerance, osmoprotectants genes like *BADH-1, ToOsmotin, Ectoine (ectA, ectB, and ectC),* and *coda* gene have been transformed in tomato, which reduces the impact of salt stress by encoding osmoprotectant solutes (Moghaieb et al., 2000, 2011; Goel et al., 2010; Wei et al., 2017). To maintain the cellular acidity under salt stress many Na^+/H^+ antiporter genes also have been transformed like *NHX1, TaNHX2,* and *LeNHX4* which regulate the Na^+/H^+ to maintain cellular homeostasis (Zhang and Blumwald, 2001; Yarra et al., 2012; García-Abellan et al., 2014). Transgenes like *cAPX, MdSOS2L1, AnnSp2, LeNHX2 and SlSOS2, At FeSOD,* and *BcZAT12* have been also transformed and works with different modes of action, details of the gene transformed in tomato and their mode of action are summarized in **Table 1**. Like potato and tomato, other important Solanaceous crops like brinjal and chili face the salt stress; in these crops also transgenics have been developed for salt stress, and details of the transgenic crop in Solanaceae family are given in **Table 1**. In vine crops group like cucumber, cucurbits, and bottle gourds are also a very popular in vegetable crops and play an important role in food and nutritional security, salt significantly reduced the yield and quality of vine crops also (Park et al., 2014; Kim et al., 2015; Sun et al., 2018; Li et al., 2020). In water melon *HAL1* transferred which encodes for 32 kDa water soluble proteins which protects from salt induced osmotic stress (Bordas et al., 1997). Park et al. (2014), Han et al. (2015), and Kim et al. (2015) transformed bottle gourds with *AVP1* which encodes vacuolar H^+-pyrophosphatase, which regulate the proton pump and ultimately maintains the cellular acidity to avoid salt stress (**Table 2**). Cole crops like cabbage, cauliflower, mustard green, rape seed, and Chinese cabbage constitute a major group of leafy vegetable, which are considered as cheapest and richest sources of mineral, vitamins, and oils and they play an important role in nutrition and food security (Wang et al., 2010; Kim et al., 2016; Ahmed et al., 2017; Luo et al., 2017). The cole crops are very sensitive to the salt stress, and different genes like *CodA*, *PgNHX1*, *OsNASI, BnSIP1-1, APX, SOD, and LEA4-1* have been transferred to sustain salt stress (Park et al., 2005; Wang et al., 2010; Kong et al., 2011). Details of the gene transferred and their mechanism of action in cole crops is given in **Table 3**.

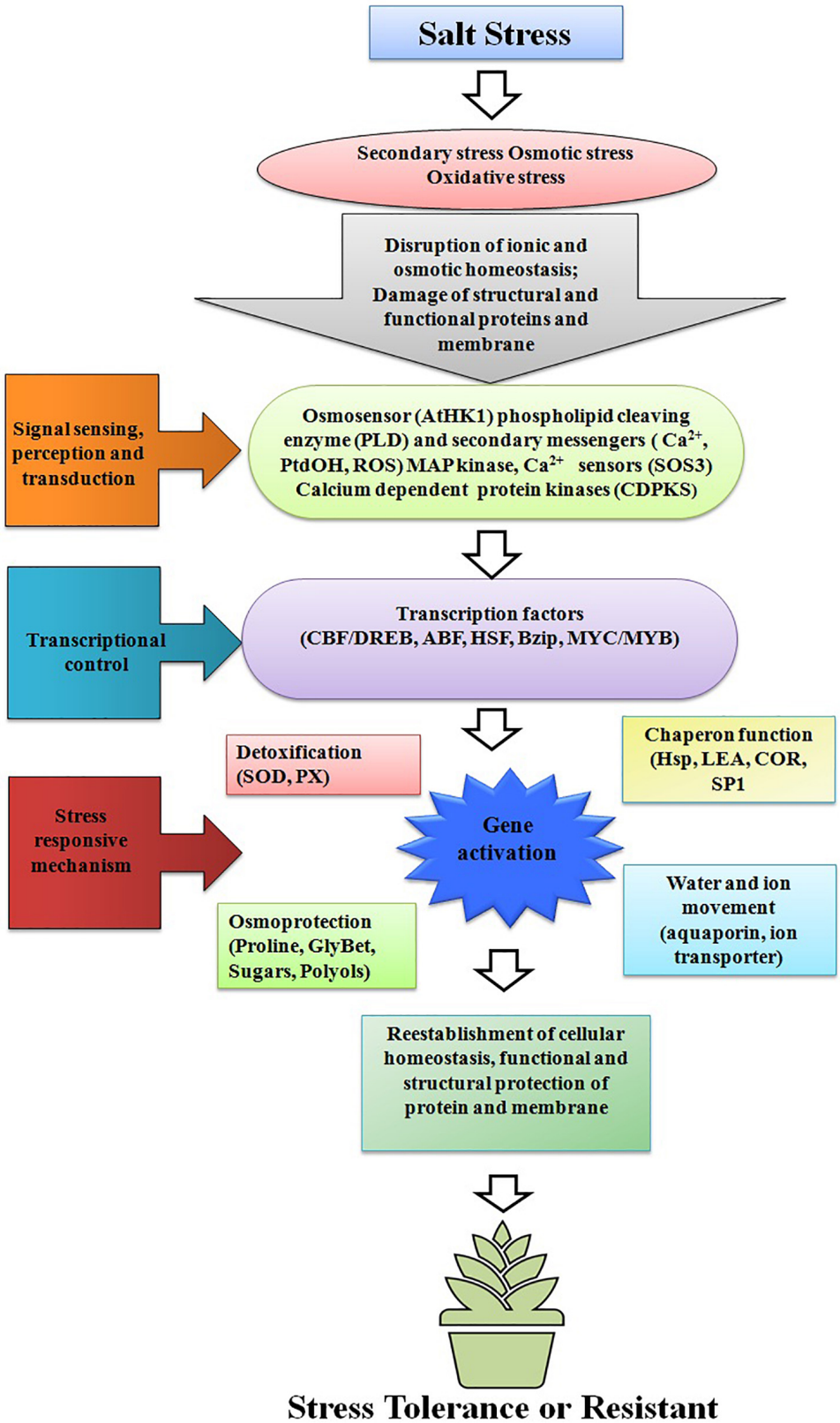

FIGURE 1 | Mechanisms of transgene action in transgenic plants; downstream signaling process and transcription controls that stimulates stress-responsive mechanisms to reestablish cellular homeostasis and damage repair.

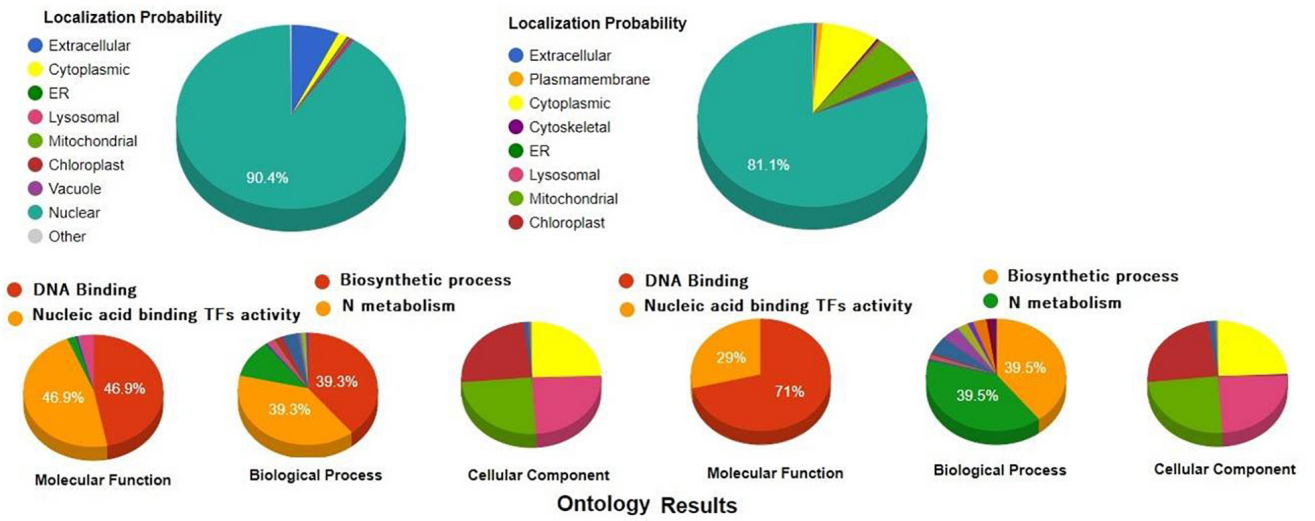

FIGURE 2 | Functional annotation of *WRKY* and NAC proteins. These functional annotation were retrieved from Cello2GO server and were structured around gene ontologies like biological process, molecular function, and cellular component along with their localization probability. It is to be noted that both WRKY and NAC proteins have diversified function with more specifically targeted to DNA binding or Nucleic acid binding transcriptional factor activity.

TRANSCRIPTIONAL REGULATION OF SALINITY STRESS SIGNALING IN VEGETABLES

Recently, it was demonstrated that during salt stress, the expression level of multiple TFs increases much more compared with their basal trends, which reflects their crucial role in regulating the function mechanism and stress-dynamics of stress-tolerance (Franzoni et al., 2019, 2020). TFs are key regulators that play important roles in various stress responses (Debbarma et al., 2019). In fact, TFs are the key players that actually bind with the *cis* acting elements to regulate the spatial and temporal expression of specific genes, or genes regulating the functional activities of signal transduction and/or other genes regulating the transcriptional efficiency of stress-responsive genes under environmental stresses (Liu et al., 2014). Therefore, transcriptomic characterization for identification of stress-responsive TFs in plants or vegetable crops could be useful as a prominent tool or may provide a genetic resource for transgenic technology to improve the stress-responsive traits in different crops (Hong et al., 2021). The TFs belonging to WRKY, NAC, bZIP, MYB, and AP2/ERF play a crucial role in modifying and fine-tuning of the different stress-responsive genes involved in stress avoidance (Golldack et al., 2014). We have provided a comparative pie chart showing the functional annotation of two different transcriptional factors WRKY and NAC having WRKY and NAC domain. Based on functional annotation and gene ontology structured around three ontological terms, biological processes, molecular function, and cellular component, we reported and confirmed the function of WRKY and NAC TFs as to bind with DNA and also playing an important role in metabolism, stress response, and nucleic acid binding transcriptional factor activity (2) (**Figure 2**). During the last few years, many TFs have been deployed for transgenic overexpression of different TFs to mitigate various abiotic stresses (Tran et al., 2010). For example, the overexpression of moso bamboo *WRKY* (*Phyllostachys edulis) in Arabidopsis* uncovered the importance of *PeWRKY83* in imparting salinity tolerance in transgenic *Arabidopsis* (Wu et al., 2017). SlAREB1, a bZIP transcriptional activator that belongs to ABA-responsive element binding protein (AREB)/ABA-responsive element binding factor (ABF) subfamily overexpression in tomato lines, reported enhanced salt and drought tolerance (Orellana et al., 2010). Furthermore, with the help of CRISPR/Cas9 genome editing technology it has now become possible to edit specific transcriptional factors that could be directly or indirectly fine-tune the expression and regulation of stress-responsive genes against salinity tolerance in plants (Debbarma et al., 2019). For example, CRISPR/Cas9 mediated genome editing of *SlMAPK3* gene in tomato affected the expression level of other drought stress-responsive genes, particularly, *SlDREB, SlLOX*, and *SlGST* in tomato. The downregulation of these genes indirectly affected the salinity response and provided tolerance to salinity. Moreover, genetic engineering, gene silencing, CRISPR/Cas9 mediated-genome editing, transgenic overexpression, gene complementation and genetic transformation, mutant analysis studies done so far for engineering better salt-tolerance strategies in various vegetable and horticultural crops have of course identified novel signaling pathways, interconnected networks, transcriptional activators in mitigating salinity as well as other environmental stresses. Recently, CRISPR/Cas9 technology has provided a novel platform for precise editing of alleles that could assist in providing stress tolerance in plants. Further, the latest advancement in CRISPR-Cas system has sparked the genome editing revolution in plant genetics and breeding. We have discussed the role of

TABLE 1 | Transgene used for development of salt stress tolerance, their function and mechanism of action.

S. N.	Genes	Function	Mechanism of action	References
Potato (*Solanum tuberosum*)				
1	*P_5CS*	Encodes for pyrroline-5-carboxylate synthetase (P_5CS)	*P_5CS* gene expression enhances proline content in the cells, proline is a potential osmolyte and tolerate to salt stress	Karthikeyan et al., 2011
2	*StCYS1*	Encodes for, Cysteine protease inhibitors (CPI)	Cysteine protease inhibitors (CPI) is a cystatin protein superfamily and facilities biological activities by cysteine protease inhibition	Liu et al., 2020
3	*Glycinebetaine*	Glycinebetaine (GB) synthesizing enzymes	Glycinebetaine GB is a osmolytes and potent compatible compound, its accumulation does not hamper plants normal activities and help in salt tolerance	Ahmad et al., 2014
4	*StDREB1*	Transcription factors	Regulate differential gene expressions in the different signaling pathways due to their different DNA-binding specificity	Bouaziz et al., 2013
5	*AcBADH*	Encodes for *betaine aldehyde dehydrogenase*	Betaine aldehyde dehydrogenase converts betaine aldehyde to glycine betaine which predominantly accumulate in the leaves and stems in dicot and monocot and enhance salt tolerance	Ali et al., 2020
6	*AtNHX1*	Na^+/H^+ antiporters	*AtNHX1* gene improves the absorption and transportation of the Na^+ of the host plant species and enhances salt stress tolerance	Wang et al., 2013
7	*mtlD*	Encodes for mannitol 1-phosphate dehydrogenase	Mannitol accumulation increases in plants in response to osmotic stresses like salt	Rahnama et al., 2011
8	*AtHKT1*	Facilitates high-affinity potassium transporter	HKTs actively involve at the plasma membrane level, HKT transporters exclude Na^+ from the leaves while increasing K^+ transportation to resist salt stress	Wang et al., 2019
9	*PaSOD* and *RaAPX*	Encodes for superoxide dismutase (SOD) and ascorbate peroxide (APX) enzymes	SOD and APX enzyme system converts superoxide radical to hydrogen peroxide (H_2O_2), followed by conversion of H_2O_2 to water and oxygen, respectively	Shafi et al., 2017
10	Rat *GLOase*	Over-expressing L-gulono-c-lactone oxidase	Enhanced ascorbic acid accumulation have been reported to have salt/osmotic stress	Upadhyaya et al., 2010
11	*GalUR*	L-gulono-1,4-lactone conversion to AsA	D galacturonic acid reductase (GalUR over-expression enhances AsA production enhances salt tolerance)	Upadhyaya et al., 2009
12	*GalUR*	Encodes for D galacturonic acid reductase	Overexpression of GalUR, an ascorbate pathway enzyme enhances its ascorbic acid content (L-AsA) and enhances salt tolerance	
13	*StNAC2*	Regulates NAC transcription factors	NAC proteins are plant-specific TFs and to play important roles in abiotic biotic stresses	Xu et al., 2014
14	*AtBADH*	Encodes for betaine aldehyde dehydrogenase	Converts betaine aldehyde to glycine betaine, the elevated glycine betaine level enhances cellular buffering capacity and stress tolerance	Zhang et al., 2011
Tomato (*Solanum lycopersicum*)				
1	*BADH-1*	Over expression of betaine aldehyde dehydrogenase	Betaine aldehyde dehydrogenase catalyzes conversion of betaine aldehyde into glycine betaine which improves abiotic stresses tolerance	Moghaieb et al., 2000
2	*NHX1*	Over expression the *NHX1* antiporter	Over expressed *NHX1* vacuolar Na^+/H^+ antiporter helps in maintaining cellular integrity and improve salt stress tolerance	Zhang and Blumwald, 2001
3	*cAPX*	Over expression of *APX*	Enhanced activity of ascorbate peroxidase activity reduces cellular damage by scavenging the superoxides under salt stress	Wang et al., 2005
4	*CaKR1*	Over expression of *LeSOD2, LeAPX2,* and *LeAPX3*	High transcript level of antioxidative enzyme machinery scavenge the ROS under abiotic stresses	Seong et al., 2007
5	*ToOsmotin*	Osmotic adjustment	Over expression leads accumulation or compartmentalization of solutes and also protect proteins denaturation under salt stress	Goel et al., 2010
6	*Ectoine (ectA, ectB and ectC)*	Compatible solute	Enhance peroxidase activity and decrease MDA contents by ectoine accumulation	Moghaieb et al., 2011
7	*AtSlSOS2 (AtSlSOS2)*	Homeostasis of Na^+ and K^+	Upregulation of the plasma membrane Na^+/H^+ (*SlSOS1*) and endosomal-vacuolar K^+, Na^+/H^+ (*LeNHX2* and *LeNHX4*) antiporters, responsible for Na^+ extrusion out of the root, active loading of Na^+ into the xylem, and Na^+ and K^+ compartmentalization	Huertas et al., 2012
8	*TaNHX2*	Na^+/H^+ antiporter	Na^+/H^+ antiporters are involved in intracellular ion (Na^+), pH regulation and K^+ homeostasis in plants under salt stress	Yarra et al., 2012
9	*HAL5*	Maintaining Na^+/K^+ homeostasis	Maintenance of Na^+ and K^+ transporters like SlHKT1;2 and SlHAK5 improve homeostasis	García-Abellan et al., 2014
10	*MdSOS2L1*	Codes for *MdSOS2L1* protein kinase	MdSOS2L1 protein kinase physically interacts with MdCBL1, MdCBL4, and MdCBL10 proteins to increase tolerance against salt	Hu et al., 2016
11	*coda*	Encode for glycine betaine	Glycine betaine enhanced NaCl-induced expression of genes encoding the K^+ transporter, Na^+/H^+ antiporter, and H^+-ATPase	Wei et al., 2017

(Continued)

TABLE 1 | (Continued)

S. N.	Genes	Function	Mechanism of action	References
	AnnSp2	Encodes annexins proteins	AnnSp2 alleviated ABA sensitivity in tomato in the germination and seedling stages under salt stress	Ijaz et al., 2017
12	*SlCMO*	Choline monooxygenase (CMO)	Is a key enzyme involved in the synthesis of glycine betaine, which is a osmoprotectant that plays an important role in plant salt tolerance	Li Q. L. et al., 2018
13	*LeNHX2 and SlSOS2*	Homeostasis of Na^+ and K^+ and Na^+/H^+ antiporter	Involves Na^+ and/or K^+ intracellular accumulation mediated by NHX transporters	Baghour et al., 2019
14	*SlMYB102*	Decrease the transcripts of ABA-dependent genes	Suppress the expression of PP2Cs or protein phosphatases of PP2Cs to help plants adapt to higher salt concentrations	Zhang et al., 2020
15	*At FeSOD*	Encodes for super oxide dismutase enzyme	The main function of these enzymes is the enzymatic conversion of such a highly toxic molecule for cells as superoxide into hydrogen peroxide (H_2O_2)	Bogoutdinova et al., 2020
16	*LeNHX4*	K^+, Na^+/H^+ antiporter	An important mechanism to overcome salt stress is the exclusion of Na^+ from the cytoplasm, by the operation of Na^+/H^+ antiporters at the plasma membrane or tonoplast. Plant NHX antiporters play a key role in NaCl tolerance by the extrusion of Na^+ out of cytosol	Maach et al., 2020
17	*COMT1*	Promote the synthesis of melatonin	*SlCOMT1* overexpression could maintain the balance of Na^+/K^+ and decrease ion damage by activating salt overly sensitive (SOS) pathway under salt treatment	Sun et al., 2020
18	*BcZAT12*	Encodes for C_2H_2 type zinc finger protein	The C_2H_2 type zinc finger protein is known to confer tolerance to dehydration, heat stress, salt and/or cold stresses	Rai et al., 2021
Brinjal (*Solanum melongena*)				
1	*Yeast HAL1*	Encodes a water-soluble protein	*HAL1* and *HAL3*, which were involved in the regulation of K^+ and Na^+ transport, respectively, and considerably enhanced salt tolerance in egg plants	Kumar et al., 2014
2	*TaNHX2*	Vacuolar Na^+/H^+ antiporter	Na^+/H^+ antiporters are involved in intracellular ion (Na^+), pH regulation, and K^+ homeostasis in plants	Yarra and Kirti, 2019
3	*adc*	Biosynthetic of polyamine by arginine decarboxylase	Accumulation of higher polyamine in cells works as a osmoprotectants	Prabhavathi and Rajam, 2007
4	*mtlD*	Mannitol-1-phosphate dehydrogenase	The accumulation of mannitol in the cytoplasm and increased tolerance to salt stress	Prabhavathi et al., 2002
Chili pepper (*Capsicum annuum* L.)				
1	*TaNHX2*	Vacuolar Na^+/H^+ antiporter	Na^+/H^+ antiporters are involved in intracellular ion (Na^+), pH regulation, and K^+ homeostasis in plants	Bulle et al., 2016
2	*PDH45*	Encodes for Pea DNA Helicase 45	DNA and RNA helicases have proved their translational efficacy in multiple crops by improving tolerance to salinity and drought stress. DNA and RNA helicases, also known as molecular motors, are involved in myriad cellular processes of protein turnover and protection	Shivakumara et al., 2017
3	*Osmotin*	Encodes for Osmotin is a stress-responsive protein	Osmotin is a stress-responsive protein adapted to salinity and desiccation and accumulates in saltadapted cells. Osmotin is an abundant cationic 26-kDa protein that belongs to the family of PR-5 type proteins. Osmotin provides osmotolerance to plants probably by facilitating the compartmentation of solutes	Subramanyam et al., 2011

some of the important TFs that regulate the stress-tolerance mechanism in plants.

WRKY Gene Family in Salt Tolerance in Vegetables

WRKY gene family is one of the most important transcriptional regulators that regulates stress tolerance mechanism in plants. Many studies done till so far have highlighted the functional role of WRKY gene signaling against various abiotic and biotic stress response in plants (Aamir et al., 2017, 2018, 2019; Hichri et al., 2017; Bai et al., 2018; Li et al., 2020). It has been well documented that *WRKYs* gene-mediated plant defense is controlled by both crossregulation and autoregulation, and extensive signaling involving multiple protein partners like histone acetylases, MAP kinases, MAP kinases kinases (MAPKK), calmodulin, 14-3-3 proteins, and other associated WRKYs partners in a complex network and dynamic web with built in redundancy to fine tune the transcriptional reprogramming, genetic-expression, and stress-tolerance (Rushton et al., 2010). WRKYs prominent role in both abiotic as well as biotic stress tolerance is well-documented (Rushton et al., 2010; Phukan et al., 2016; Bai et al., 2018). WRKYs role in salinity tolerance against various horticultural crops and other plants is well reported (**Table 4**). For example, Kashyap et al. (2020) reported the relevance of tomato *WRKY1*, *WRKY3,* and *WRKY72* in mitigating salt stress in wild tomato *Solanum chilense* as the expression of these *WRKYs* was more prominent and increased the expression in

TABLE 2 | Transgenic crops developed in vine crops for salt stress tolerance.

S. N.	Genes	Function	Mechanism of action	References
Cucumber (*Cucumis sativus* L.)				
1	*LOS5*	Encodes a molybdenum cofactor (MoCo) sulfurase	Molybdenum cofactor (MoCo) sulfurase catalyzes the last step of ABA biosynthesis in plants	Liu Z. et al., 2013
2	*HAL1*	*HAL1* encodes a water soluble protein (32 kDa)	Water soluble protein (32 kDa) that may modulate monovalent ion channels, by affecting the set point of intracellular potassium determined by the feedback regulation of the uptake system	Bordas et al., 1997
3	*CsbHLH041*	Encodes Basic helix-loop-helix (bHLH) transcription factors	The bHLH genes are involved in processes such as metabolic regulation, plant growth and development, and response to environmental signals	Li et al., 2020
	CmHKT1;1	Encodes a Na^+ preferential transporter	(HKT1) encodes a Na^+ preferential transporter that principally controls root-to-shoot Na^+ delivery via the withdrawal of Na^+ from the xylem sap	Sun et al., 2018
4	Bottle gourds			
5	*AVP1*	Encodes vacuolar H^+-pyrophosphatase	A vacuolar H^+-pyrophosphatase encoded by the *AVP1* gene is one of the proton pumps in *Arabidopsis* and generates an H^+ electrochemical gradient across the tonoplast	Kim et al., 2015
6	*AVP1*	Encodes vacuolar H^+-pyrophosphatase	A vacuolar H^+-pyrophosphatase encoded by the *AVP1* gene is one of the proton pumps in *Arabidopsis* and generates an H^+ electrochemical gradient across the tonoplast	Park et al., 2014
	AVP1	Encodes vacuolar H^+-pyrophosphatase	A vacuolar H^+-pyrophosphatase encoded by the *AVP1* gene is one of the proton pumps in *Arabidopsis* and generates an H^+ electrochemical gradient across the tonoplast	Han et al., 2015
Watermelon [*Citrullus lanatus (Thunb.*) Matsum. & Nakai]				
	HAL1	A vacuolar Na^+/H^+ antiport	Water soluble protein (32 kDa) that may modulate monovalent ion channels, by affecting the set point of intracellular potassium determined by the feedback regulation of the uptake system	Ellul et al., 2003

wild genotype compared with domestic and cultivated genotype DVRT1. Villano et al. (2020) characterized the list of putative WRKYs involved in various abiotic and biotic stresses in two wild relatives of potato *Solanum commersonii* and *Solanum chacoense*, and revealed the ScWRKY23 as multiple stress regulator WRKY in wild potato (Villano et al., 2020). Likewise, Hichri et al. (2016) also reported the expression of tomato *WRKY3* in alleviating salt stress (Hichri et al., 2016). Transcriptomic characterization and function validation through qRT-PCR analysis unraveled the expression profiling and importance of sweet potato WRKYs after treatment with 150 mM salt stress (Qin et al., 2020). In one work, Yue et al. (2019) provided the genome-wide identification and characterization of 92 *WRKY* genes in *Chenopodium quinoa*, and reported the importance of 25 WRKYs in both development and stress tolerance. Similarly, genome-wide identification and characterization of WRKYs in *Cucumis sativus* (cucumber) unraveled the importance of *CsWRKY9*, *CsWRKY18*, *CsWRKY48,* and *CsWRKY57*, in both heat and salt stress tolerance (Chen et al., 2020). In another study, based on Illumina RNA-seq transcriptomic studies, Tang et al. (2014) reported the tissue-specific and differential expression profiles of the *Brassica rapa ssp. pekinensis* (Chinese cabbage) and further validated their role in different abiotic and biotic stresses (Tang et al., 2014). Karanja et al. (2017a) reported 126 WRKYs in *Raphanus sativus* out of which 35 WRKYs had differential expression in various abiotic stresses. Further, the relevance of *WRKY3* in salt tolerance could be better understood as CRISPR/Cas9-mediated *WRKY3* and *WRKY4* mutagenesis in *Arabidopsis,* decreasing both MeJA stress as well as decreased salt tolerance in *Arabidopsis* (Li et al., 2020).

Ethylene Response Factors

The APETALA2/ethylene responsive factor (AP2/ERF) family of transcription factor is one of the prominent groups of transcriptional activator/regulator during various abiotic and biotic stress responses in plants. ERF group has been further classified or subdivided into the dehydration-responsive element-binding proteins (DREBs). The AP2/ERF families in different plants have been further subdivided based on the presence of double AP2 domain, single AP2 domain, and/or single AP2 domain along with presence of a B3-DNA binding domain (Nakano et al., 2006). The interaction of ERF proteins with DRE/CRT motif and *cis*-acting elements is generally associated with stress-responsive genes and plays a crucial role in mitigation of various environmental stresses. For example, transgenic overexpression of *ERF1-V* (*Haynaldia villosa*) in wheat provided salt tolerance (Xing et al., 2017). Similarly, a ERF gene from wheat (*TaERF3)* overexpression had significant results against salt stress compared with control counterparts in wheat (Rong et al., 2014). In one work, Yang et al. (2018) performed the microarray analysis on salt-tolerant genes in wild tomato lines *Solanum pimpinellifolium* PI365967' under the effect of salt treatment and reported the increased expression of five ERF genes *(SpERF*). Sequence analysis and transcriptomic characterization of these five *SpERFs* uncovered the crucial seven amino acid residues that were involved in binding with GCC box in the promoter of

TABLE 3 | Transgenic crops developed in cole crops for salt stress tolerance.

S. N.	Genes	Function	References
Mustard green (*Brassica juncea*)			
1	*CodA*	Choline oxidase	Prasad et al., 2000
2	*PgNHX1*	Vacuolar Na^+/H^+ antiporter	Rajagopal et al., 2007
3	*AtLEA4-1*	LEA4 protein	Saha et al., 2016
4	*Lectin*	Induced fungal resistance	Kumar et al., 2015
5	*Gly I*	Detoxification of methylglyoxal	Rajwanshi et al., 2016
6	*Gly II*	Detoxification of methylglyoxal	Saxena et al., 2011
	AnnBj2	Upregulated expression of ABA-dependent (RAB18) and ABA independent (DREB2B) genes	Ahmed et al., 2017
Rape seed (*Brassica napus*)			
	CodA	Choline oxidase	Huang et al., 2000
	AtNHX1	Vacuolar Na^+/H^+ antiporter	Zhang et al., 2001
	YHem1	Accelerate endogenous 5-ALA metabolism	Sun et al., 2015
	LEA4-1	LEA4 protein	Dalal et al., 2009
	OsNASI	11 proteins upregulated including dehydrogenase, GST, POD and Rubisco	Kong et al., 2011
	DREB	Expression of many stress-inducible genes	Qamarunnisa et al., 2015
	BnSIP1-1	Regulates BnABI5, BnNAC485 or other stress-related genes	Luo et al., 2017
	PR10	Pathogenesis related	Srivastava et al., 2004
Chinese cabbage (*Brassica campestris* L. spp. Chinensis)			
	CodA	Choline oxidase	Wang et al., 2010
Cabbage (*Brassica campestris*)			
	LEA4-1	LEA protein	Park et al., 2005
Cauliflower (*B. oleracea* var. botrytis)			
	APX, SOD	Antioxidants	
Napa cabbage (*Brassica rapa* ssp. Pekinensis)			
	BrGI	Reduced expression of GI, enhanced salt tolerance	Kim et al., 2016

ethylene responsive genes and were shown to be conserved in all the reported ERFs (Yang et al., 2018). In recent years, ERF TFs have been investigated in depth in various horticultural as well as vegetable crops to enhance the breeding program as well as crop improvement, with respect to various environmental stresses in plants (**Table 4**). For example, transgenic overexpression of tomato ERF84 (*SlERF84*) in *Arabidopsis* provided resistance against salt and drought. Further, tomato ERFTF (*Sl-ERF.B.3*) expression was found to be decreased/downregulated under salinity stress and drought conditions, whereas it has been found to be upregulated/increased following the exposure to cold, flood, and heat response.

NAC Transcription Family

NAC (NAM, ATAF, and CUC) TFs have been considered an important group of transcriptional activators that play an important role in developmental programming as well as to encounter challenges against various environmental constraints (Tran et al., 2010; Puranik et al., 2012). The DNA binding property of NAC TFs lie at their N-terminal end (**Figure 3**). The expression of NAC proteins is highly dependent on the promoter region as each and every NAC gene is characterized by the presence of at least one unique *cis*-element type in their promoter (Li et al., 2016). NAC TFs role in various abiotic and biotic stress responses is well-documented (**Table 4**). For example, salt-tolerance in tomato is well regulated by NAC1 transcription factor as the enhanced expression of SlNAC1 in root, flower, seeds, and green fruits following the salt stress are well known (Yang et al., 2011). Transcriptomic characterization unraveled the importance of 10 *NAC* genes in tomato against abiotic stresses (Song et al., 2015). In this context, Liu et al. (2014) reported the relevance of tomato NAC transcription factor *SlSRN1* in mediating the positive defense response against biotic stresses while regulating negatively to abiotic stress signaling (Liu et al., 2014). Yang et al. (2011) reported the expression profiling of tomato *NAC1* (*SlNAC1*), an ATAF subfamily transcription factor in different tissues (root, leaves, seeds, and fruit) under salt stress (Yang et al., 2011). Likewise, potato NAC genes StNAC072 and StNAC101 that have been reported as orthologs of known stress-responsive *Arabidopsis* responsive to dehydration 26 (RD26) were found to play a crucial role in mitigating abiotic stress response (Singh et al., 2013). Wei et al. (2016) performed the

TABLE 4 | Transcriptional regulation and their mode of action in vegetable crops for salt stress tolerance.

S. No.	Transcription Factor/Gene/Protein	Vegetable	Functional aspects	References
1.	*JUNGBRUNNEN1* (*JUB1*), a NAC transcription factor	*Solanum lycopersicum* (tomato)	Overexpression of *JUNGBRUNNEN1* (*JUB1*) increases salinity tolerance in tomato	Alshareef et al., 2019
2.	BZR/BES transcription factor	*S. lycopersicum* (tomato)	BRASSINAZOLE RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR 1 (BES1) homologs in have potential role in salt tolerance. SlBZR1D played positive role in salt stress tolerance	Jia et al., 2021
3.	Wild tomato WRKY1, WRKY3, and WRKY72	*S. chilense* (wild tomato)	Salinity stress tolerance	Kashyap et al., 2020
4.	Transgenic overexpression of tomato ERF84 in Arabidopsis	*Arabidopsis thaliana*	Salt and Drought	Li Q. L. et al., 2018
5.	Transgenic overexpression of tomato ERF1	*S. lycopersicum*	Salt Stress	Lu et al., 2011
6.	Microarray analysis for salt-tolerant genes in wild tomato uncovered putative 5ERFs in alleviating salt stress	*S. pimpinellifolium*	Salt stress tolerance	Yang et al., 2018
7.	*ScbZIP* and *SlbZIP*	*S. chilense* (wild tomato) and *S. lycopersicum*	Salinity stress tolerance	Zhu et al., 2018; Kashyap et al., 2020
8.	Tomato SRN1 (*Solanum lycopersicum* stress-related NAC1) plasma membrane-localized protein with transactivation activity in yeast	*S. lycopersicum*	Positively regulates defense response against biotic stress but negatively regulates abiotic stress response	Liu et al., 2014
9.	Tomato NAC35	*S. lycopersicum*	Induced by drought stress, salt stress, bacterial pathogen, and signaling molecules	Wang et al., 2016
10.	Tomato SlAREB1, a bZIP transcription factor, member of the ABA-responsive element binding protein (AREB)/ABA-responsive element binding factor (ABF) subfamily	*S. lycopersicum*	Salt stress and Drought stress tolerance	Orellana et al., 2010
11.	Tomato NAC4 and NAC35	*S. lycopersicum*	Salt, Drought tolerance Biotic stress	Zhu et al., 2014; Wang et al., 2016
12.	ZFP179, a salt responsive gene encoding a Cys2/His2 zinc finger protein	*Oryza sativa*	Overexpression of ZFP179 provided salt tolerance	Sun et al., 2010
13.	BnaABF2, a bZIP transcription factor	*Brassica napus*	Salt tolerance in Transgenic Arabidopsis	Zhao et al., 2016
14.	Chili N*AC46*	*Capsicum annum*	Salt tolerance in transgenic Arabidopsis	Ma et al., 2021
15.	Tomato *DREB2*	*S. lycopersicum* and *A. thaliana*	Salt tolerance	Hichri et al., 2016
16.	Tomato *ERF84, ERF5*	*S. lycopersicum*	Positive regulation for Salt and drought tolerance; negative regulation for biotic stress	Pan et al., 2012; Li Q. L. et al., 2018
17.	Chenopodium *WRKY*	*Chenopodium quinoa*	Stress tolerance and development	Yue et al., 2019
18.	*CsWRKY9, CsWRKY18, CsWRKY48 and CsWRKY57*	*Cucumis sativus*	Heat and salt stress tolerance	Ling et al., 2011; Chen et al., 2020
19.	Radish WRKY	*Raphanus sativus*	Abiotic Stress tolerance	Karanja et al., 2017a
20.	Carrot WRKY20	*Daucus carota*	*DcWRKY20* made interaction with DcMAPK1 and *DcMAPK4* Abiotic and biotic stress tolerance	Li et al., 2016
21.	Tomato NAC1; NAC3	*S. lycopersicum*	Salt stress tolerance; NAC3 suppressed by salt stress	Yang et al., 2011; Han et al., 2012, 2014
22.	Illumina RNA-seq transcriptomic studies of root, stem and leaves in Chinese cabbage	*Brassica rapa ssp. pekinensis*	Abiotic and biotic stress tolerance	Tang et al., 2014
23.	Carrot WRKYs in hormonal regulation and mechanical injuries	*Daucus carota*	Hormone and mechanical injuries	Nan and Gao, 2019
24.	Transcriptomic studies of sweet potato under salt stress	*Ipomoea batatas*	Salt stress tolerance	Qin et al., 2020
25.	Genome-wide identification and characterization of tomato WRKYs under drought, salt and biotic stress	*S. lycopersicum*	Drought, Salt, and Biotic stress	Huang et al., 2012
26.	Genome-wide identification and characterization of WRKYs in wild potato	*S. commersonii* and *S. chacoense*	ScWRKY045 as multiple stress-responsive regulator	Villano et al., 2020
27.	Identification of biotic-stress responsive WRKY from *Brassica oleracea* var. *italica*	*B. oleracea* var. *italica*	Increased expression of BoWRKy6 against biotic stress	Jiang et al., 2016

(Continued)

TABLE 4 | (Continued)

S. No.	Transcription Factor/Gene/Protein	Vegetable	Functional aspects	References
28.	Genome-wide characterization of potato WRKYs and expression analysis of potato 22 WRKYs under different stresses	*S. tuberosum*	Increased upregulation of *StWRKY01* and *StWRKY39* under different abiotic stresses. *StWRKY58* had highest expression profile under drought and salt stress	Zhang et al., 2017
29.	Genome-wide identification and characterization of WRKYs in brinjal and Turkey berry	*S. melongena L. S. torvum Sw.*)	Biotic stress response	Yang et al., 2015
30	Tomato SR/CAMTA transcription factors SlSR1 and SlSR3L	*S. lycopersicum*	Negatively regulate disease resistance response and SlSR1L positively modulates drought stress tolerance	Li et al., 2014
31	Radish NAC145	*Raphanus sativus*	Salt, heat and drought stresses	Karanja et al., 2017b
32	Melon NAC14	*Cucumis melo*	Overexpression of *CmNAC14* increased the sensitivity of transgenic *Arabidopsis* lines to salt stress	Wei et al., 2016
33.	Potato NAC proteins StNAC072 and StNAC101; StNAC2	*S. tuberosum*	StNAC072 and StNAC101 are orthologs of known stress-responsive *Arabidopsis* RESPONSIVE TO DEHYDRATION 26 (RD26) involved in abiotic stress tolerance; Overexpression of *StNAC2* in transgenic potato increased salt tolerance	Singh et al., 2013; Xu et al., 2014
34.	Watermelon *WRKY ClWRKYs*	*Citrullus lanatus*	Growth, Development, Biotic and Abiotic stress response	Yang et al., 2018
35.	Wild turnip WRKY (BsWRKYs)	*Brassica rapa*	Biotic and abiotic stress response	Kayum et al., 2015
36.	*BjABR1*, an AP2/ERF superfamily gene, from tuber mustard	*Brassica juncea var. tumida Tsen et Lee*	Abscisic acid and abiotic stress responses	Xiang et al., 2018
37.	*Arabidopsis* NAC2	*A. thaliana*	Stress response and lateral root development	He et al., 2005
38.	Comparative transcriptome and proteome analysis of salt-tolerant and salt-sensitive genotypes of sweet potato and expression profiling of IbNAC07	*Ipomoea batatas*	Salinity stress tolerance	Meng et al., 2020
39.	Genome wide characterization of WRKY genes in summer squash	*Cucurbita pepo*	Water and salt stress tolerance	Bankaji et al., 2019
40.	Genome-Wide Identification of AP2/ERF transcription Factors in cauliflower	*Brassica oleracea L. var. botrytis*	Salt and drought stress tolerance	Li et al., 2017
41	Genome wide characterization of NAC family in celery and further transcriptomic characterization under salt stress AgNAC47 and AgNAC63 were key player	*Apium graveolens*	Heat, salinity, cold stress	Duan et al., 2020
42	Genome-wide characterization of homeobox-leucine zipper gene family in tomato (*Solanum lycopersicum*)	*S. lycopersicum*	Functional analysis of *SlHDZ34* (III sub-family member)under salinity stress revealed salt stress tolerance	Hong et al., 2021
43	SlMYB02, a R2R3-type MYB transcription factor	*S. lycopersicum*	Salt tolerance	Zhang et al., 2020
44.	*CabZIP25*	*Capsicum annum*	Salt tolerance	Gai et al., 2021
45.	Sweet potato *bZIP IbbZIP1; IbABF4*	*A. thaliana*	Transgenic overexpression of *IbbZIP1* in *Arabidopsis* provided salt tolerance; *IbABF4 imparted* multiple stress tolerance	Wang et al., 2019
46.	Tomato bZIP transcription factor SlAREB	*S. lycopersicum*	Salt tolerance	Hsieh et al., 2010
47.	*SlbZIP38* tomato bZIP transcription factor	*S. lycopersicum*	Negative regulator of drought and Salt Stress Tolerance	Pan et al., 2017
48.	*SlbHLH22* a Basic Helix-Loop-Helix (bHLH) transcription factor in tomato	*S. lycopersicum*	Transgenic over expression imparted high tolerance to both salinity and drought	Waseem et al., 2019
49.	*AtMYB20 Arabidopsis* R2R3-MYB transcription factor	*A. thaliana*	Negatively regulated type 2C serine/threonine protein phosphatases to positively regulate salt tolerance	Xu et al., 2014
50.	*SlMYB102,* R2R3-type MYB gene	*S. lycopersicum*	Transgenic overexpression provided salt tolerance	Zhang et al., 2020

genome-wide characterization of NAC transcription factor family in melon (*Cucumis melo L.*) and evaluated their expression profile during salt stress. Further, transgenic overexpression of *CmNAC14* in *Arabidopsis* resulted in increased salt-tolerance (Wei et al., 2016). Karanja et al. (2017b) reported the tissue-specific expression profiling of radish NAC TFs and reported

the positive regulation of RsNAC023 and RsNAC080 toward all types to abiotic stresses. Further, RsNAC145 had much more active expression profile under salt, heat, and drought stresses when compared with other genes that were expressed under different abiotic stresses (Karanja et al., 2017b). Li et al. (2016) investigated and characterized the list of putative NAC TFs in water melon (*Citruluslanatus*) across the genome and also checked the expression profile and potential function of several NAC TFs in different stresses. Overall, transgene overexpression of *IbNAC7* in *Arabidopsis* provided salt tolerance. Recently, Duan et al. (2020) provided the genome-wide characterization of NAC gene family in leafy vegetable *Apium graveolens* and studied the characterized WRKYs for their stress-tolerance attribute. It was found that a total 111 NAC member were present based on genomic studies. Further, transcriptomic characterization under various abiotic stresses uncovered the *AgNAC63* (ortholog of *ANAC072*/*RD26* role in mitigating salt, cold, and heat stresses). However, the study reported tissue-specific higher expression profiles *AgNAC63* and *AgNAC47* in leaves under the different treatments (Duan et al., 2020).

Basic Leucine Zipper

The basic leucine (Leu) zipper (bZIP) family also includes one of the most important group of transcriptional activator against abiotic and biotic stress response (Pan et al., 2017). bZIP family also plays an essential role in growth and development of plants (Sornaraj et al., 2016). The bZIP name was based on the presence of the bZIP domain. The bZIP domain is characterized by some specific structural features that is located on an alpha-helix. It has been reported that the first 18 amino acid residues constitute the basic group followed by an invariant N-x7-R/K-x9 motifs for nuclear localization and sequence-specific DNA binding. However, for the second part, the Leu zipper region composed of several heptad repeats of Leu amino acid or other bulky amino acids, such as isoleucine, valine, phenyl nine, tryptophan, or methionine, positioned exactly nine amino acids toward the C-terminus, creating an amphipathic helix (Jakoby et al., 2002; Nijhawan et al., 2008). It has been reported that in bZIP transcriptional proteins, apart from the bZIP domain, some other transcriptional active domains are found and play an essential role in bZIP functioning. The most common site that function as transcriptional activator include phosphorylation site [R/KxxS/T (Furihata et al., 2006; Liao et al., 2008)] and a string of glutamine rich motif. The basic part of the Leu zipper interact with ACGT core region of the B-DNA sequences, particularly, at A-box (TACGTA), G-box (CACGTG), and C-box (GACGTC) (Izawa et al., 1993; Foster et al., 1994). In fact, during the DNA–protein interaction of bZIP proteins with DNA motifs, the half N-terminal of the bZIP domain interacts with DNA major grove region, whereas the other C-terminal end of the Leu zipper constitutes dimer formation for a coiled superimposed structure, defined as zipper superimposed coiled structure, the so-called zipper (Landschulz et al., 1988; Ellenberger et al., 1992). bZIP proteins also play an essential role in imparting tolerance against several abiotic as well as biotic stresses (**Table 4**). For example, Gai et al. (2021) characterize the list of different bZIP TFs in pepper and reported the relevance of *CabZIP25* in imparting salt tolerance as overexpression done in transgenic *Arabidopsis*. Wang et al. (2018) identified the list of 54 and 50 *bZIP* proteins from whole-genome sequences of *Vigna radiata* and *Vigna angularis*, respectively.

GRAFTING STRATEGIES IN VEGETABLE CROPS FOR SALT STRESS TOLERANCE

Improving the productivity of vegetable crops is a challenge under salt-affected soil or water. Hence, increasing salt tolerance in vegetable crops will have a greater impact in nutritional and economic security, particularly of (semi) arid regions, where salinity in soil and water are widespread (Singh et al., 2020). Traditional breeding programs have been attempted to improve salt tolerance in crop plants (Borsani et al., 2003), but the commercial success is limited due to the trait's complexity. Currently, major efforts are being directed toward genetic transformation in plants to increase their tolerance, and despite the trait's complexity, the transfer of a single gene or a few genes has resulted in claims of improved salt tolerance, such as the expression of genes involved in the control of Na^+ transport (Gaxiola et al., 2001). But, the genetically complicated mechanisms of abiotic stress tolerance, as well as the possibility for adverse side effects make this a challenging task (Flowers, 2004). However, unless a full proof practical and faster breeding tool comes in vogue, a well-proven fast and eco-friendly technique "vegetable grafting" can be deployed to increase tolerance to stresses in vegetables. Vegetable grafting, in fact, has emerged as an efficient tool to sustainably increase vigor and yield of commercial cultivars under challenged growth environment by mechanically attaching with resistant root genotypes.

Grafting Alleviates Salt Stress

Salinity disturbs dry mass partitioning between vegetative and reproductive organs, whereas grafted plants exhibited less alteration (Parthasarathi et al., 2021). In grafting, some rootstocks may have better performance than the others, though their response may change depending on level of salt concentration in the growth medium (Singh et al., 2020; Bayoumi et al., 2021). Numerous reports have demonstrated the ameliorative response of grafting to salinity stress in cucurbitaceous crops (e.g., melon, watermelon, and cucumber) involving the *Cucurbita* interspecific hybrid rootstocks (Goreta et al., 2008; Rouphael et al., 2012). The agronomic performance of pepper cv. "Adige" under natural salinity condition was clearly evident with 75% higher yield and with 31% lesser fruit damage (blossom end rot) when it was grafted onto a salt-tolerant accession "A 25" as rootstock in comparison with non-grafted control plants (Penella et al., 2016). Eggplant ("SuqiQie") grafting onto the rootstock of wild eggplant (*Solanum torvum* cv. "Torvum vigor") provided salinity tolerance by minimizing the yield reduction under saline stress (Wei et al., 2009). In contrary, Chen et al. (2003) found that scion genotypes had a significant impact on the growth of grafted tomato plants, regardless of the salinity of the growing environment, but rootstock had no impact.

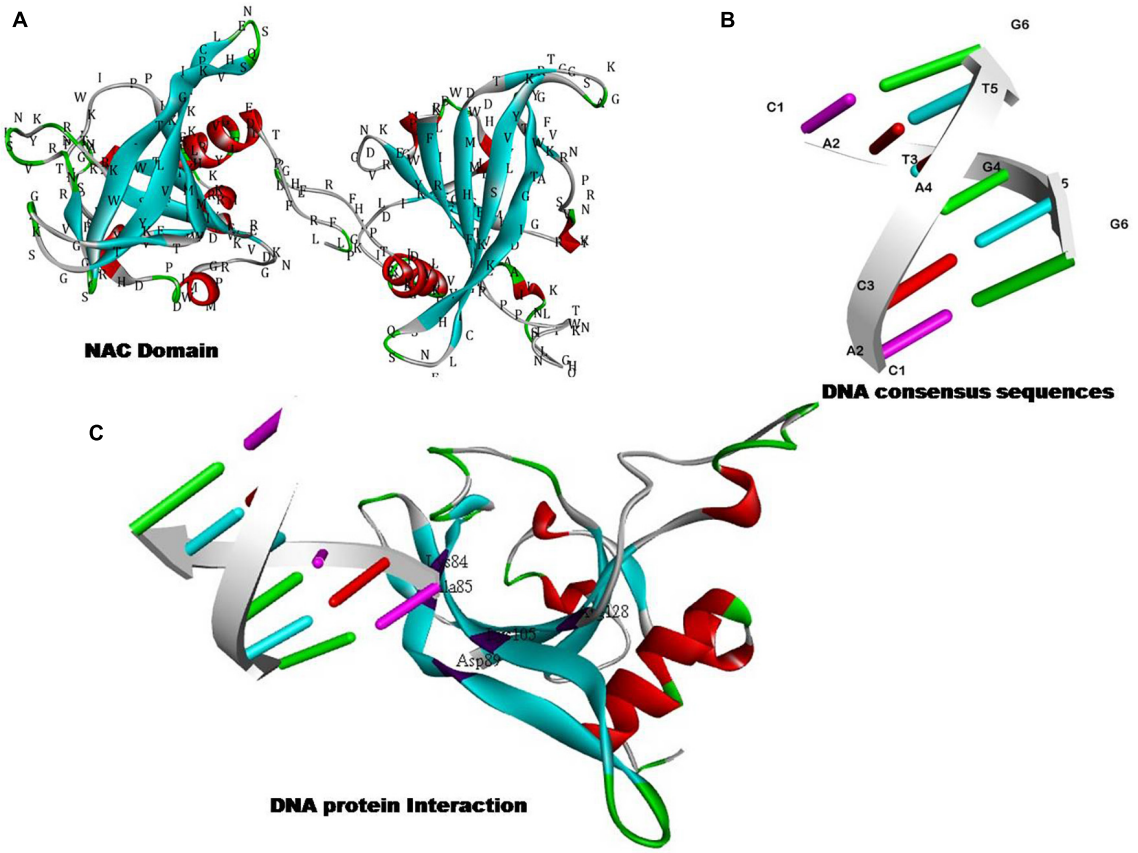

FIGURE 3 | Hypothetical models showing the DNA binding and/or nucleic acid binding transcription factor activity of NAC proteins. **(A)** The structure of NAC domain. **(B)** Structure of NAC binding DNA consensus sequences. **(C)** DNA–protein interaction of NAC proteins with relevant DNA sequences to fine tune the gene regulatory aspects.

Mechanism of Salt Tolerance in Grafted Plants

Grafting is a reciprocal integrative process; the salt tolerance of grafted plants is influenced by both scion and rootstock (Etehadnia et al., 2008). The positive response of grafting can be attributed to more vigorous and robust root systems, greater efficiency of roots for water and nutrient uptake with efficiency to exclude salt-ions, higher photosynthesis, and better oxidative defense system, hormonal regulations, and osmotic adjustment of the grafted plants as compared with the non-grafted plants (Amaro et al., 2014; Rouphael et al., 2017; Singh et al., 2020).

Root Characteristics

Root, besides providing physical support and anchor to the plants, plays a crucial role in water and ion uptake and their supply to aerial part that help regulate various plant processes (Kumar et al., 2017). However, the alteration in root characteristics is expected to occur since roots being the foremost plant organ exposed to saline growth medium (Singh et al., 2020), consequently the subsequent effects on water and mineral uptake (Rouphael et al., 2017). The numerous reports claim that grafting onto genetically strong root system can effectively mitigate the effect of salinity on the performance of salt-sensitive scion cultivars (Colla et al., 2013); hence it prompts the emphasis of selecting the vigorous root stock for increasing salt tolerance (Colla et al., 2013; Singh et al., 2020). Salinity depressed shoot and root parameters, but grafted plants of tomato onto potato rootstocks were able to avoid the changes in their growths with balanced partitioning between vegetative and reproductive dry masses (Parthasarathi et al., 2021). Furthermore, the tolerance ability of grafting provided by the rootstocks is often associated with the root morphological characteristics to exclude Na^+ and/or Cl^- under saline medium.

Regulation of Salt and Mineral Ions

Grafted plants tend to restrain Na^+ and Cl^- ions in their root tissues, preventing them from being translocated to the shoots and leaves in high concentrations. The diverse agronomic responses of grafted plants to salinity in numerous studies were resulted by the differential abilities of root genotypes to regulate the uptake and/or translocation of ions of the salts, and of nutrients, due to their competitive interactions (Rouphael et al., 2017). The ability of rootstocks to minimize toxicity of Na^+ and/or Cl^- by exclusion and/or reduction of Cl^- absorption by

the roots, as well as the replacement or substitution of K^+ by total Na^+ in the foliage has been related to the enhancement of salt tolerance by grafting (Martinez-Rodriguez et al., 2008). In pepper, salt-tolerant wild pepper rootstocks "ECU-973" (*Capsicum chinense*) and "BOL-58" (*Capsicum baccatum* var. *pendulum*) provided salinity tolerance in pepper ("Adige") plants by controlling Na^+ and Cl^- ions accumulation in shoots (Penella et al., 2015). In spite of maintaining better control over Na^+ and Cl^- accumulation in their shoots, grafted plants were able to maintain higher ratio of K^+/Na^+ in grafted cucumber on pumpkin rootstock (Usanmaz and Abak, 2019), and higher K^+ and Ca^{++} in grafted eggplant (Talhouni et al., 2019). Salinity tolerance in a salt-sensitive cucumber ("Jinchun No. 2") was enhanced by grafting onto a salt-tolerant pumpkin rootstock ("Chaojiquanwang"); this tolerance mechanism shows the better ability of pumpkin rootstock to exclude Na^+, and thus lesser amount of Na^+ ions (−69%) reaches the cucumber shoots (Huang et al., 2013).

Physio-Biochemical Alterations

The tolerance response of grafting on vigorous rootstock with efficiency to control Na^+ ion accumulation in shoots has been associated with the efficiency of rootstocks to modulate water uptake by roots and losses by transpiration. Grafting onto some rootstock was useful to maintain better leaf water status than the others. Grafting tomato ("Ikram") on potato rootstock ("Charlotte") was found promising to increase salinity tolerance of 5.0 dS/m in grafted tomato with enhanced water productivity (+56.8%) (Parthasarathi et al., 2021). Grafting onto certain rootstocks was able to mitigate salt induced photoinhibition of photosynthesis and consequently growth of grafted plants (Liu Z. et al., 2013). As a coping mechanism of oxidative damage, plants activate enzymatic (i.e., ascorbate peroxidase, catalase, superoxide dismutase, monodehydroascorbate reductase, dehydro ascorbate reductase, and glutathione reductase) as well as non-enzymatic (i.e., reduced glutathione, reduced ascorbate, carotenoids, and tocopherols) antioxidant systems (Rouphael et al., 2017; Singh et al., 2020). Grafting studies demonstrated that some of the graft combinations have a better ability to mitigate salinity stress by regulating antioxidative defense system than the others. Salt-stressed eggplants experienced oxidative stress (higher malondialdehyde, MDA), whereas grafted eggplants were capable of mitigating ROS induced by oxidative stress as a result of increased level of antioxidant enzymes (SOD, CAT, and APX) (Talhouni et al., 2019). Polyamines increase under salinity stress and hence increases plants tolerance; the increased level of polyamines (free, soluble, and conjugated polyamines) provided better tolerance to salinity (i.e., excess calcium nitrate) in grafted seedlings than the non-grafted tomatoes (Wei et al., 2009). Stegemann and Bock (2009) reported that plant grafting can result in the exchange of genetic information *via* either large DNA pieces or entire plastid genomes. However, gene transfer is restricted to the contact zone between scion and rootstock. Thus, the use of rootstock cannot change the sensitivity of scion itself to salt stress. Working with model plant *Arabidopsis*, Shi et al. (2002) reported that SOS1 (salt excessively sensitive) gene is expected to play a role in the loading of Na^+ into the xylem tracheids from xylem parenchyma cells. Further reports suggest that in *Arabidopsis*, high affinity K^+ transporters (HKTs) were involved in the removal of Na^+ from the xylem (Rus et al., 2001, 2006; Sunarpi et al., 2005; Davenport et al., 2007), and hence the leaves were safe from Na^+ ion toxicity. Furthermore, it was recently discovered that expressing the Na^+ transporter HKT1;1 in the mature root stele of *Arabidopsis thaliana* utilizing an enhancer trap expression system reduced Na^+ build up in the shoot by 37–64%, and hence increased salinity tolerance (Møller et al., 2009). Using grafting experiments, it was discovered that HKT1;1 expressed in the root rather than the shoot regulates Na^+ accumulation in *Arabidopsis* shoots (Rus et al., 2006), implying that the SOS1 analogous gene and HKTs are likely involved in Na^+ transport in the pumpkin rootstock, allowing it to limit Na^+ transport from the root to the shoot.

Grafting onto some rootstocks has shown to also increase scion's tolerance to salinity by modulating the hormonal balance namely of ABA, cytokinins, and polyamines (Rouphael et al., 2017). The reduced transpiration with maintained leaf water relations by elevated level of shoot ABA concentration under salt stress have been reported (Singh et al., 2020). The enhanced salinity tolerance in tomato was related to increased level of ABA content in scion shoots, irrespective of the rootstocks raised under saline condition (Chen et al., 2003). Likewise, increased root-to-shoot cytokine transport by rootstock that overexpressed cytokinin biosynthesis genes (e.g., isopentenyl transferase) was associated with the increased salinity tolerance in tomato presented by maintained stomatal conductance and photosystem II efficiency accompanied with lesser accumulation of toxic ions, consequently producing higher shoot and fruit growths (Ghanem et al., 2011).

CONCLUSION

Abiotic stresses like salt stress which persists throughout plant's whole life cycle negatively affects plant yield and nutritional quality. To ensure vegetable production under salt stress many transgenes have been transferred to the vegetables. Transforming vegetables are the one of the most reliable technique to cope salt stress as most of the vegetable gene pool lack novel gene for salt stress in its gene pool. To manage salt stress at molecular level, the native TFs in the vegetable crops are also being regulated to sustain yield and quality under salt stress. Grafting has shown potential to alleviate salinity stress (water or soil) on the selected vigorous and tolerant rootstocks. Certain wild accessions which possess resistance to salinity but are difficult to introgress these traits into commercial cultivars through traditional breeding tools, can be utilized as rootstock to increase grafted scion's efficiency or tolerance to salinity. Plant biologists around the world are grappling with the dilemma of exponential population growth and rising food demand. Abiotic stress, such as salt, is a major threat to agricultural productivity and has been linked to worsening food security trends since the beginning. Soil salinity and degradation of soil quality are linked to lower agricultural

yields. The production of salinity-tolerant crops is the only way to ensure global food. The actual yield produced by saline soils is more than half of what was originally predicted for normal soils. Organic matter and biodiversity are quite low in these soils.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Molecular Bases of Heat Stress Responses in Vegetable Crops With Focusing on Heat Shock Factors and Heat Shock Proteins

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The effects of the climate change including an increase in the average global temperatures, and abnormal weather events such as frequent and severe heatwaves are emerging as a worldwide ecological concern due to their impacts on plant vegetation and crop productivity. In this review, the molecular processes of plants in response to heat stress—from the sensing of heat stress, the subsequent molecular cascades associated with the activation of heat shock factors and their primary targets (heat shock proteins), to the cellular responses—have been summarized with an emphasis on the classification and functions of heat shock proteins. Vegetables contain many essential vitamins, minerals, antioxidants, and fibers that provide many critical health benefits to humans. The adverse effects of heat stress on vegetable growth can be alleviated by developing vegetable crops with enhanced thermotolerance with the aid of various genetic tools. To achieve this goal, a solid understanding of the molecular and/or cellular mechanisms underlying various responses of vegetables to high temperature is imperative. Therefore, efforts to identify heat stress-responsive genes including those that code for heat shock factors and heat shock proteins, their functional roles in vegetable crops, and also their application to developing vegetables tolerant to heat stress are discussed.

Keywords: global warming, heat shock factor, heat shock protein, heat stress, thermotolerance, vegetables

INTRODUCTION

Vegetable crops mainly comprise sessile organisms. They routinely experience detrimental conditions including biotic and abiotic stresses in natural fields. The current climate changes including frequent extreme temperatures, strong storms, heavy rainfall, and harsh droughts directly threaten normal vegetable development during the entire period of vegetative and reproductive growth (Driedonks et al., 2016; Hansen et al., 2016; Bhutia et al., 2018). Global warming is one of the main issues related to global climate change and is caused by increases of greenhouse gases such as CO_2, CH_4, N_2O, and hydrofluorocarbons (HFCs) that have been produced by urbanization and industrialization (Bhutia et al., 2018; Zandalinas et al., 2021). According to climate models

(Driedonks et al., 2016) and the report from the Intergovernmental Panel on Climate Change (IPCC[1]), the world mean temperature will rise by 0.5 to 4°C in the twenty-first century (Hansen et al., 2016; Zandalinas et al., 2021). The changes in weather/climatic events such as temperature and rainfall are found to reduce the yield of crops. Statistical evidence shows that the temperature affects rice production in Africa. It was also found that irrigated rice yields in West Africa in the dry season would decrease by ∼45% due to reduced photosynthesis at extremely high temperatures (van Oort and Zwart, 2018). This indicates that the elevated temperature brought by climate change will result in significant losses in crop yields and production (Ortiz et al., 2008; Hansen et al., 2016). Plants have evolved to acquire the ability to induce defense mechanisms against the adverse effects of high ambient temperature on their growth (Ahuja et al., 2010; Bourgine and Guihur, 2021; Tian et al., 2021). The tolerance of plants to high ambient temperatures with no prior heat experience is known as basal thermotolerance (BTT), whereas the ability to overcome extremely high temperatures (HT) with pre-exposure to mild HT (i.e., sub-lethal temperatures) is known as acquired thermotolerance (ATT) (Ahuja et al., 2010; Bourgine and Guihur, 2021; Tian et al., 2021). The defense mechanisms against elevated temperatures in plants are tightly associated with rapid changes in gene expression in both BTT and ATT (Morimoto, 1998; Feder and Hofmann, 1999). Indeed, high ambient temperatures trigger a drastic cellular remodeling at the physiological and molecular levels in plants to maintain homeostasis, thereby allowing them to survive under adverse HT (Wang et al., 2004; Ohama et al., 2017; Tian et al., 2021). Within these mechanisms, how plants recognize HT and relay HT-induced signaling downstream to modulate transcription is a central question that plant researchers have been pondering for a long time. It has recently been reported that Ca^{2+} plays important roles in the perception, response, and adaptation of plants to heat stress (HS) (Mittler et al., 2012; Ohama et al., 2017; Lee and Seo, 2021). The alteration of fluidity in the plasma membrane (PM) in plants in response to HS can open cyclic nucleotide-gated calcium channels (CNGCs) controlled by nucleotide cyclases, thereby having Ca^{2+} move into the cytosol from the PM (Saidi et al., 2009; Finka et al., 2012; Gao et al., 2012; Mittler et al., 2012; Ohama et al., 2017). The Ca^{2+} ions are associated with protein calmodulin 3 (CaM3) during HS and the complex of Ca^{2+}-CaM3 interacts with calcium/calmodulin-binding protein kinase 3 (CBK3) and phosphatase PP7 to transduce cytosol heat-stress response (HSR) signals into the nucleus by modulating phosphorytion and dephosphorylation of HSFA1, respectively (Liu et al., 2007, 2008; Mittler et al., 2012; Ohama et al., 2017). Also, the increased levels of Inositol-1,4,5-triphosphate (IP_3) via the phosophoinositide-signaling pathway result in the influx of Ca^{2+} into cytoplasm from intracellular Ca^{2+} pools such as the endoplasmic reticulum (ER) and vacuole during HS (Zhang et al., 2009; Zhou et al., 2009; Mittler et al., 2012; Ohama et al., 2017). In addition, reactive oxygen species (ROS) produced by respiratory burst oxidase homolog B (RbohB), RbohD, and NADPH oxidases are other candidate sensors of HS (Königshofer et al., 2008; Miller et al., 2009; Suzuki et al., 2012). It has also been demonstrated that the ROS causes accumulation of nitric oxide (NO), which induces the activation of CaM3. The signaling cascade of CaM3 ultimately influences the association of DNA and heat shock factors (HSFs) in nucleus via the potential involvement of HSFA1 activity (Xuan et al., 2010; Wang et al., 2014; Ohama et al., 2017). Although Ca^{2+} and ROS are evaluated as predicted signal transducers during HS, the full activation of HSR in response of plants to HT cannot be exclusively explained by them. This indicates that there may be other signal transducers and multiple layers of signaling pathways including salicylic acid (SA), ethylene (ET), abscisic acid (ABA), and jasmonic acid (JA) signals (Fujita et al., 2006; Frank et al., 2009; Zhou et al., 2009).

The effect of HS on plants leads to diverse changes in plant cells including the state of cellular membranes, structural alterations in DNA and RNA species, and conformational changes of proteins, cytoskeleton structures, and metabolites (Ruelland and Zachowski, 2010; Mittler et al., 2012). For instance, high ambient temperature influences fluidity of cellular membranes containing primarily phospholipids, proteins, and carbohydrates with the modification of membrane rigidification (Ruelland and Zachowski, 2010). Also, high ambient temperature affects the accessibility of nucleic acids, and it has been determined that elevated temperatures induce the dissociation of the histone protein H2A.Z from nucleosomes, which promotes the chromatin accessibility to RNA polymerase II for the expression of genes for heat-shock proteins (HSP) and HSF, thus showing highly inductive and responsive gene expression dynamics (Kumar and Wigge, 2010; Zhang H. et al., 2021). RNA secondary structures can be affected by HS. It has been revealed that HT leads to a change in translation rate, resulting from the altered association of mRNAs with ribosomes (Matsuura et al., 2010). Since structured nucleic acid molecules melt as the temperature increases, it can be easily conceived that temperature changes affect the conformation of regulatory RNAs (Narberhaus et al., 2006). Indeed, the RNA secondary structure of internal ribosome entry sites (IRESs), which are translation regulatory elements of mRNAs, can be modified by HS to initiate translation in a cap-independent manner (Dinkova et al., 2005; Ruelland and Zachowski, 2010). Conversely, RNA secondary structures that mask ribosomal binding sites at optimal temperature can be modified by HS, allowing the conversion of non-functional RNA to the competent RNA species with ribosomal recruitment (Narberhaus et al., 2006). Heat stress also influences the conformational changes of proteins that act as signaling effectors in response to HT in plants (Ruelland and Zachowski, 2010). In *Arabidopsis*, the oligomerization of thioredoxin and/or thioredoxin-like proteins is induced by HS, causing concomitant functional switching from a disulfide reductase and foldase chaperone to a holdase chaperone (Lee et al., 2009; Park et al., 2009). It has also been reported that the elevated temperatures from 27 to 42°C in tobacco, and from 20 to 42°C in *Arabidopsis* cause severe damage to cytoskeletones including microtubules (Smertenko et al., 1997; Müller et al., 2007). Furthermore, tobacco BY-2 cells exposed to heat (50°C, for 5 min) exhibited depolymerization of actin microfilaments (Malerba et al., 2010),

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and such a defective phenotype was also observed in *Arabidopsis* roots (Müller et al., 2007). Based on a report demonstrating that heat triggers the accumulation of HSP70 and the heat-activated MAP kinase (HAMK), both HSP70 and HAMK are likely to be necessary to disassemble the cytoskeleton under HS (Suri and Dhindsa, 2008). Altered enzymatic activities such as the catalytic rate, and the un- or mis-folding of enzymes can also be affected by HS, resulting in the imbalance of cellular metabolism in plants (McClung and Davis, 2010; Ruelland and Zachowski, 2010; Suzuki et al., 2012). The steady-state efflux and influx of metabolites such as sucrose, prolines, glycine-betaine, ascorbate, glutathione, and ROS play an important role in heat response and tolerance (Wang et al., 2004; Al-Whaibi, 2011; Mittler et al., 2012). Reactive oxygen species were initially regarded as a toxic by-product of aerobic metabolism. However, it is now apparent that ROS such as superoxide and hydrogen peroxide are able to function as signal molecules to induce the HSR (Miller et al., 2007, 2009; McClung and Davis, 2010; Ruelland and Zachowski, 2010; Suzuki et al., 2012). In particular, the levels of ROS are influenced by the participation of ROS-generating enzymes in plant response to HT (Königshofer et al., 2008). The acquisition of plant heat tolerance is closely associated with the synthesis of chaperone proteins and the levels of non-enzymatic antioxidants in response to HT (Kotak et al., 2007; Wahid et al., 2007; Frank et al., 2009; Rampino et al., 2009). Many reports have been published showing that HS influences protein conformation which can drive a protein to be denatured, aggregated, and un- or mis-folded, thereby being directly recognized by several

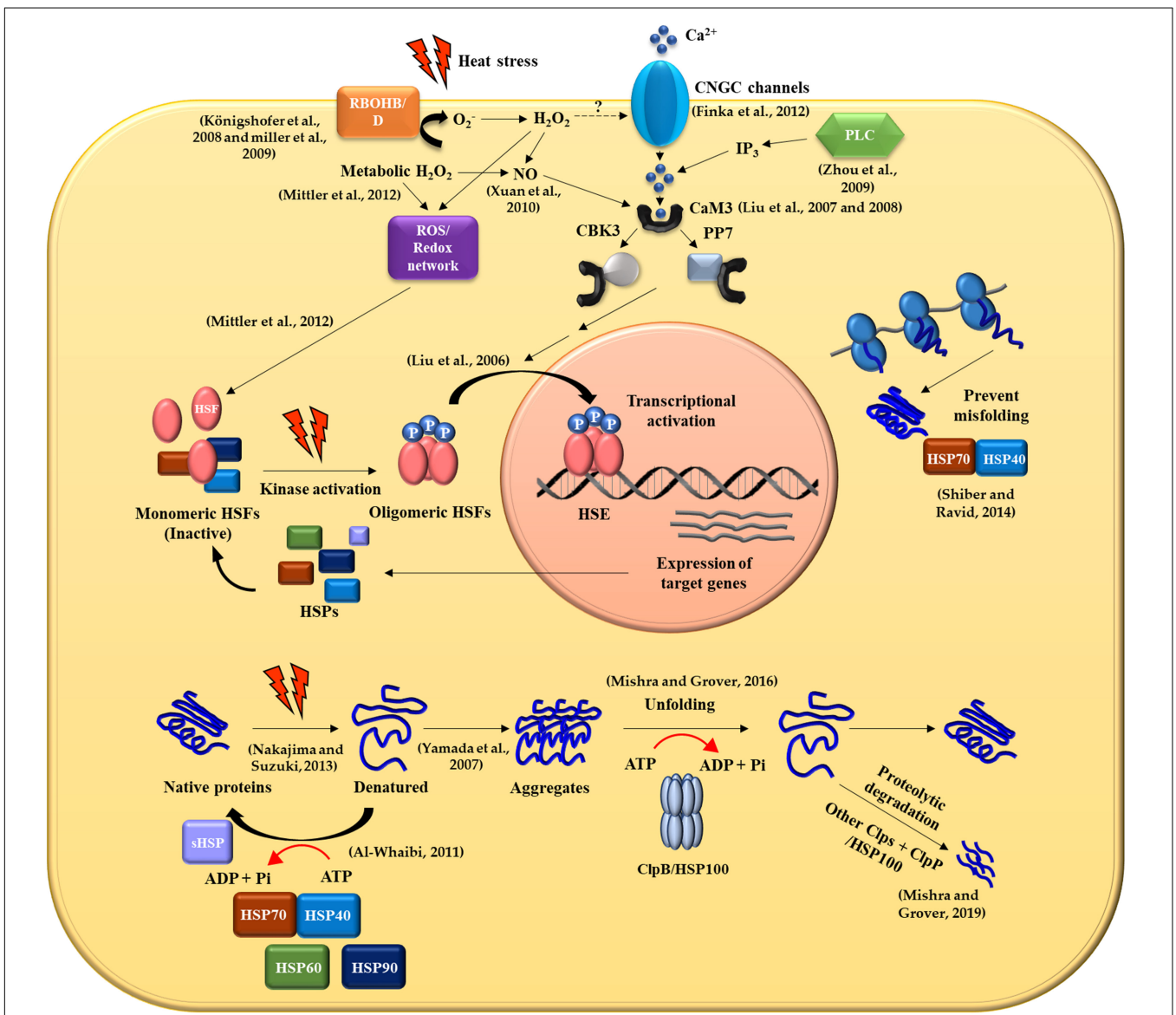

FIGURE 1 | General molecular mechanism of heat shock protein production and transcriptional regulation in response to heat stress in plant cells.

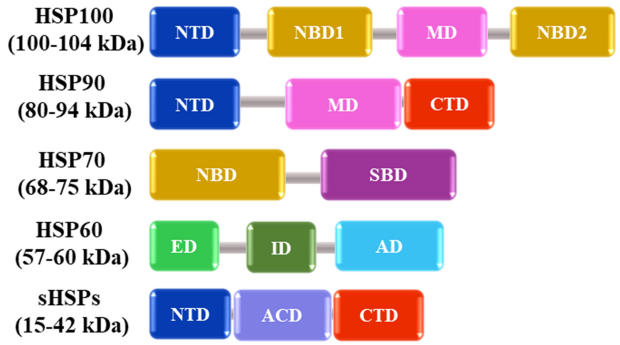

FIGURE 2 | Schematic representation of available domains in the five major families of heat shock proteins. NTD (N-terminal domain), NBD (Nucleotide binding domain), MD (Middle domain), CTD (C-terminal domain), SBD (Substrate binding domain), ED (Equatorial domain), ID (Intermediate domain), AD (Apical domain), and ACD (Alpha-crystallin domain) are shown as boxes with different colors based on their functions. Numbers in parenthesis indicate the molecular weight distribution of each HSP family.

HSPs (Yamada et al., 2007; Scharf et al., 2012; Ohama et al., 2017). Notably, plant HSPs play a crucial role in conferring plant tolerance to HS, and they help facilitate proper folding of target proteins by hindering denaturation and aggregation of the proteins as molecular chaperones (Ahuja et al., 2010; Jacob et al., 2017). For instance, under normal temperature conditions, HSFs regulate the HSR and form inactive multiprotein complexes with HSPs. On the other hand, under HS, HSFs dissociate from the complex and form phosphorylated trimers, thereby allowing their nuclear translocation and binding to heat-shock element (HSE) to induce transcription of target genes (Kotak et al., 2007; Ahuja et al., 2010; Scharf et al., 2012; Jacob et al., 2017; Ohama et al., 2017). Indeed, transcriptomic and proteomic analyses revealed that the abrupt changes in gene expression in response to high ambient temperatures enhance a selected regulatory response and synthesis of proteins linked to HSPs, HSFs, and HSR (Al-Whaibi, 2011; Jacob et al., 2017; Zandalinas et al., 2021). However, the players and their mode of action in heat perception, HS-signaling pathways and HSR still remain elusive in vegetable crops.

In this review, we give an overview of the HSPs with focus on vegetable crops. Heat shock proteins play an essential role in the regulation of HSFs and subsequently, the expression of heat responsive genes. Moreover, a better understanding of HSPs will enable us to widen our knowledge of interconnected mechanisms underlying the complex regulatory networks of HSFs and heat responsive genes at the physiological and molecular levels during the adaptation of plants against HS. We also discuss the potential applications of biotechnology for efficient development of crops with enhanced thermotolerance to cope with climate change.

HEAT SHOCK PROTEINS INVOLVED IN HEAT STRESS

In nature, plants are often exposed to various kinds of abiotic stresses including low or high temperature, deficiency or excess of water, high salinity, heavy metals and ultraviolet radiation (Rucińska-Sobkowiak, 2010; Bita and Gerats, 2013; Osakabe et al., 2013; He et al., 2018). Among these, HS has significant effects on plant growth, metabolism, and productivity (Rodríguez et al., 2015). HS causes protein misfolding and/or denaturation, leading to protein aggregation in plant cells by interactions between exposed hydrophobic amino acid residues of affected proteins (Nakajima and Suzuki, 2013). In response to HS, plants synthesize molecular chaperones including HSPs that recognize hydrophobic amino acid residues of non-native proteins and promote folding and refolding of denatured proteins (**Figure 1**). They are also responsible for assembling of multi-protein complexes, transporting, and sorting of proteins into correct compartments, controlling cell cycle and signal-transduction under various stress conditions. The different classes of HSPs play complementary and sometimes overlapping roles in protein stabilization under thermal stress. The HSPs are generally grouped into five major families based on their molecular weight: HSP100, 90, 70, 60 and the small HSPs (sHSPs) (**Figure 2** and **Table 1**).

Heat stress (HS) influences the alteration of membrane fluidity in plasma membrane (PM) *in planta* and activates the cyclic nucleotide-gated calcium channels (CNGCs), resulting in the movement of Ca^{2+} into the cytoplasm from the apoplastic space. The Ca^{2+} ions are associated with protein calmodulin 3 (CaM3) during HS and the Ca^{2+}-CaM3 complex binds to either calcium/calmodulin-binding protein kinase 3 (CBK3) or phosphatase PP7 to transduce cytosol heat-stress response (HSR) signals into the nucleus by modulating phosphorylation and dephosphorylation of the heat shock transcription factors (HSFs), respectively. The elevated levels of inositol-1,4,5-triphosphate (IP_3) via the phosophoinositide-signaling pathway (PLC) lead to an influx of Ca^{2+} into the cytoplasm from the pool of intracellular Ca^{2+} ions including the ER and vacuoles in response to HS and induce the same CaM3 signaling pathway. ROS are generated by respiratory burst oxidase homolog B (RbohB) and D (RbohD) during HS. RbohB/D-produced O_2^- is converted into H_2O_2, which depolarizes PM as well as inducing the ROS/Redox signaling network which is involved in the activation of HSFs. Also, H_2O_2 is possibly increased in plant cells due to metabolic imbalances and the production of ROS, resulting in the accumulation of nitric oxide (NO) and the activation of calcium-channels that subsequently trigger the activity of CaM3 as illustrated in the (**Figure 1**). Upon HS stimuli, HSP interacts with unfolded and aggregated proteins, thereby releasing HSF monomer. Heat shock factor monomers trimerize and bind to HSEs within promoter regions of heat shock genes. Heat shock factors undergo several post transcriptional modifications (PTMs) such as phosphorylation, which regulate the transactivation capacity of HSF. Under normal conditions, HSPs directly bind to HSF and provide negative feedback required to deactivate HSF. HSP70 and HSP40 together function as ATP-driven machines that prevent aggregation of misfolded polypeptides and participate in protein refolding. When denatured or misfolded proteins form aggregates, ClpB/HSP100 is crucial for protein disaggregation, refolding or degradation by protease especially during HS.

TABLE 1 | Five major families of heat shock proteins and their major function under heat stress conditions.

HSP family/ MW (kDa)	Subcellular location	Major functions under heat stress conditions	Major domain
HSP100/ 100-104	Cytosol Mitochondria Chloroplasts	Disaggregation of proteins and involvement in protein degradation (Mishra and Grover, 2016).	NTD (N-terminal domain) NBD (Nucleotide binding domain) MD (Middle domain)
HSP90/ 80-94	Cytosol ER Nucleus Mitochondria Chloroplasts	Protein folding, signal transduction (most of the substrates of HSP90s are kinases and transcription factors) (Kadota and Shirasu, 2012).	NTD MD CTD (C-terminal domain)
HSP70/ 68-75	Cytosol ER Mitochondria Chloroplasts	Assisting folding and refolding of non-native proteins to block protein degradation in the ER and protein import and translocation (Shiber and Ravid, 2014).	NBD SBD (Substrate binding domain)
HSP60/ 57-60	Mitochondria Cytosol ER Nucleus Chloroplasts	Assisting folding and refolding of unfolded polypeptides in the mitochondrial matrix (Martin et al., 1992; Caruso Bavisotto et al., 2020).	ED (Equatorial domain) AD (Apical domain) ID (Intermediate domain)
sHSPs/ 15-42	Cytosol ER Mitochondria Chloroplasts Membrane	Preventing aggregation and refolding of unfolded polypeptides (Waters and Vierling, 2020).	NTD ACD (Alpha-crystallin domain) CTD

Consequently, HSPs as chaperones play a pivotal role in conferring thermotolerance in plants. The dashed line indicates an unknown pathway.

Heat Shock Protein 100 Family

The caseinolytic proteinase/heat shock protein 100 (Clp/HSP100) proteins are members of the AAA+ protein group (ATPases associated with various cellular activities) that act in protein disassembly and/or protein degradation using the energy from adenosine triphosphate (ATP) hydrolysis (Sauer et al., 2004; Burton and Baker, 2005; Gul et al., 2021). In contrast to the typical molecular chaperones which function in protecting proteins from misfolding and aggregation, the Clp/Hsp100 proteins play a wide variety of functional roles in eliminating non-functional proteins and/or assisting the reassembly of denatured proteins from the aggregated protein complexes. As such, the Clp/Hsp100 proteins contribute to the maintenance of protein homeostasis in cells (Schirmer et al., 1996; Latterich and Patel, 1998; Agarwal et al., 2001; Mishra and Grover, 2019). The Clp/Hsp100 proteins consist of hexameric rings and the structural features are determined by nucleotide binding domains (NBD), spacer (linker) region, the middle domain (MD), N-terminal domain (NTD) and C-terminal domain (CTD) among diverse living organisms from prokaryotes to eukaryotes (Dougan et al., 2003; Schlieker et al., 2005; Butler et al., 2006). On the basis of the number of NBD domains, the Clp/Hsp100 family is classified into two major subclasses (class I and class II). The first class ClpA, ClpB, ClpC, and ClpD proteins that harbor two nucleotide binding domains (called ATP-binding domains) separated by spacers are clustered as large Clp proteins ranging from molecular weights of 68 to 110 kDa (Wang et al., 2004), whereas the second class including ClpM, ClpN, ClpX, and ClpY proteins that possess one NBD are grouped based on their low molecular weights ranging from 40 to 50 kDa (Wang et al., 2004; Mogk et al., 2008; Mishra and Grover, 2016). It was initially reported that the system of Clp ATPase proteins are able to hydrolyze casein *in vitro* (Hwang et al., 1987; Katayama-Fujimura et al., 1987). Later, further investigations on two-component protease systems revealed that the complexes of ClpA regulatory machine with an AAA+ ATPase module and a proteolytic component ClpP (Schelin et al., 2002) together with Lon protease complex serve as protein choppers for the degradation of toxic protein aggregates in cells (Wang et al., 2007). Moreover, the ClpAP complex recognizes target aggregated proteins via the guidance of the ClpS adapter that assists ClpAP to specifically bind and chop the aggregated proteins (Dougan et al., 2002). In addition to this, ClpB was initially found in bacteria and yeast, and it was later reported that plant HSPs were identified with high molecular weights of 100–110 kDa (Schirmer et al., 1994). Since plants harbor semi-autonomous organelles such as chloroplasts and mitochondria, plant ClpBs are classified into three different forms ClpB-C (cytoplasmic), ClpB-P (chloroplastic), and ClpB-M (mitochondrial) (Mishra and Grover, 2014). Although ClpB is considered to be a functional ortholog of ClpA with high similarity between the two proteins (Gottesman et al., 1990; Sanchez and Lindquist, 1990), it has been experimentally shown that ClpB could not replace the function of ClpA in protein degradation due to the lack of the LIV-GFL motif required for the interaction with ClpP (Weibezahn et al., 2004; Zolkiewski, 2006; Tessarz et al., 2008). Moreover, it was demonstrated that ClpB plays an essential role in the denaturing and/or renaturing pathway to release the native proteins from the aggregates rather than the degradation pathway as other Clps do. Of note, it has been displayed that ClpB is induced by HS in contrast to other

Clps (Singh et al., 2010; Kim et al., 2012), indicating that ClpB is crucial for the protein renaturation/denaturation from aggregates especially during HS. Interestingly, the possible mechanism for assisting protein folding toward native and functional form from aggregates would be collaborated with the Hsp70 member, which is another ATP-dependent chaperone that is involved in refolding of liberated proteins by ClpB/HSP100 (Glover and Lindquist, 1998; Goloubinoff et al., 1999). However, when the aggregated proteins are interacted with other Clps and the peptidase (ClpP) system, the proteins move to the degradation pathway (Wang et al., 2004). The cellular roles of ClpB have been widely studied from prokaryotes to eukaryotes such as bacteria, yeast, and plants (Lindquist, 1986; Vierling, 1991; Wang et al., 2004). Remarkably, it has been determined that the fine-tuned expression of ClpB genes within cells is required for normal growth, development, and adaptation to environmental stresses including cold, heat, drought, and high salt (Yang et al., 2006). In particular, it has been shown that ClpB proteins are essential for rendering thermotolerance to organisms in response to HS. The loss-of-function mutant of *ClpB* in *E. coli* remarkably affected cell viability in response to abrupt HT (50 ℃) with a slow growth rate at 44 ℃ (Squires et al., 1991). Also, *ScHSP104* in *Saccharomyces cerevisiae* is one of the *ClpB* genes involved in acquiring thermotolerance: *ScHSP104* deficient yeast cells grew and died at the same rate as the wild-type cells did when exposed directly to HT although the mutant cells could not acquire tolerance to heat after a mild pre-heat treatment (Sanchez and Lindquist, 1990). Plant ClpB/HSP100 proteins have been evaluated in diverse plant species including *Arabidopsis* (Lee et al., 2007), wheat (Campbell et al., 2001), soybean (Lee et al., 1994), maize (Nieto-Sotelo et al., 1999; Young et al., 2001), and rice (Agarwal et al., 2003). Analyses of ClpB/HSP100 proteins have been also conducted in vegetable crops such as pea, tomato, pepper, carrot, spinach, potato, banana, rapeseed, and mustard greens in response to heat and cold stresses.

Heat Shock Protein 90 Family

Heat shock protein 90 (HSP90; known as GroEL in *E. coli*) is one of the most abundant heat-related proteins expressed in cells accounting for 1–2% of total protein levels (Taipale et al., 2010). Heat shock protein 90 is a highly conserved molecular chaperone involved in the assembly, maturation, stabilization and activation of key signaling proteins including regulatory kinases, steroid hormone receptors and transcription factors in plant cells (Kadota and Shirasu, 2012; Chen et al., 2019). Most plants have several isoforms of HSP90 classified by their subcellular localization in the cytoplasm (HSP90.1), nucleus (HSP90.4), chloroplast (HSP90.5), mitochondria (HSP90.6), and endoplasmic reticulum (ER; HSP90.7) (Milioni and Hatzopoulos, 1997; Krishna and Gloor, 2001; Xu et al., 2012). HSP90 exists in the form of a dimer consisting of three main structural domains: NTD, which binds ATP; MD, which is important for ATP hydrolysis and client protein binding; and CTD, which mediates HSP90 dimerization and client protein binding. ATP binding to the NTD and its hydrolysis induce conformational change which is essential for chaperone activity (Krishna and Gloor, 2001; Pearl and Prodromou, 2006). HSP90 proteins play a major role in assisting the proper folding of other proteins together with HSP70s (Picard, 2002) by acting as molecular chaperones, signaling for the cellular quality control, trafficking of other HSP proteins (Pratt and Toft, 2003) and stabilizing proteins against HS (Marcu et al., 2002; Wang R. et al., 2016). Also, HSP90 proteins along with their co-chaperone HSP70s contribute to the maintenance of cellular protein homeostasis by inactivating HSF during attenuation/recovery of HSR (Hahn et al., 2011). In *Arabidopsis*, HSP90 and the co-chaperone SUPPRESSOR OF G2 ALLELE SKP1 (SGT1) positively regulate plant growth by stabilizing the auxin co-receptor F-box protein TIR1 under higher ambient temperature conditions (Wang R. et al., 2016), showing that HSP90 participates in plant growth control under changing thermal conditions.

Heat Shock Protein 70 Family

The heat shock protein 70 (HSP70) family (known as DnaK in *E. coli*), one of the most ubiquitous classes of chaperones, is highly conserved in all organisms, and also found in different cellular compartments such as the cytosol, chloroplasts, ER and mitochondria (Amir-Shapira et al., 1990; Radons, 2016; Usman et al., 2017). The HSP70 family is the central hub of the protein homeostasis network that prevents protein aggregation and uses the energy of ATP hydrolysis to solubilize, translocate and mediate the proper refolding and unfolding of proteins (Ben-Zvi et al., 2004; Imamoglu et al., 2020). Heat shock protein 70 contains two major domains: one is the N-terminal nucleotide binding domain for hydrolyzing ATP to ADP (Adenosine diphosphate) and the other is the C-terminal substrate binding domain (SBD) (Mayer, 2010). Under abiotic stress conditions such as HS, HSP70 molecular chaperones also function as ATP-driven unfolding/refolding machines that are capable of shifting substrate polypeptides between various folding states together with their co-chaperones such as HSP40 (Lee et al., 2007; Shiber and Ravid, 2014; Palakolanu et al., 2016). The significance of HSP70 regarding functional roles against HS was highlighted by transgenic plants overexpressing *AtHSP70-1* and *NtHSP70-1* (Sung and Guy, 2003; Cazalé et al., 2009; Cho and Choi, 2009). In addition, numerous experimental results have shown that HSP70 is involved in thermotolerance in various crops such as rice (Jung et al., 2013), tomato (Hahn et al., 2011), and pepper (Guo et al., 2014) under HS conditions.

Heat Shock Protein 60 Family

The heat shock protein 60 (HSP60) family (also known as chaperonins, Cpn, and GroEL in *E. coli*) typically functions inside the mitochondria together with the co-chaperone HSP10 to maintain protein homeostasis (Caruso Bavisotto et al., 2020). However, they have also been found in other subcellular compartments including the ER, cytosol, chloroplasts and nucleus, and participate in folding and aggregation of many proteins (Meng et al., 2018). Chaperonins are generally composed of two rings, stacked back to back, consisting of subunits of ∼60 kDa molecular weight (Nguyen et al., 2021). Each oligomer has three domains (1) the equatorial domain (ED), which has the ATP-biding site, (2) the apical domain (AD), which hosts client proteins and (3) the intermediate domain (ID), which

transduces signals from the equatorial domain (Pipaón et al., 2021). When signals are transmitted to the ID from ATP binding and hydrolysis, conformational changes occur in the AD corresponding to the open and closed forms (Xu et al., 1997). Heat shock protein 60 proteins bind several types of proteins before folding to block their aggregation (Parsell and Lindquist, 1993) and stromal chaperones (Hsp70 and Hsp60) are involved in functional conformation of newly transferred proteins to the chloroplast (Jackson-Constan et al., 2001). Most of the HSP60 family proteins are heat inducible and also required for preventing protein aggregation, and mediating folding and refolding in mitochondria under HS conditions (Martin et al., 1992; Sharma et al., 2006).

Small Heat Shock Protein Family

Small heat shock proteins (sHSPs), which have a low molecular mass of 15-42 kDa, are very diverse in plants (Wang et al., 2004; Basha et al., 2006; Morrow and Tanguay, 2012). Small heat shock proteins have a common alpha-crystallin domain (ACD) containing 80–100 amino acid residues on the C-terminal region, and contribute to degradation of proteins with unsuitable folding (Seo et al., 2006). Small heat shock proteins are ubiquitous ATP-independent molecular chaperones that bind and stabilize misfolded or unfolding intermediates of substrate proteins in an energy-independent manner (Ferguson et al., 1990; Miernyk, 1999; Waters and Vierling, 2020).

TRANSCRIPTIONAL REGULATION OF HEAT SHOCK PROTEINS IN PLANTS UNDER HEAT STRESS

Heat-stress response is known to be controlled by complex, tight networks, including selective enhancement and repression of gene expression in various metabolic processes, production of chaperone proteins for cellular protein homeostasis and other protective molecules that prevent targets from detrimental effectors such as ROS. The regulation of this network is critical for plant cells not only to adapt to various environmental conditions linked to temperature, humidity and light, but also to protect them from proteotoxic stresses. HSFs have a central function as major regulators in HSR by regulating transcription of a wide range of genes in several signaling and metabolic pathways (von Koskull-Döring et al., 2007; Guy et al., 2008). Heat shock factors are responsible for rapid synthesis and accumulation of HSPs, molecular chaperones for preventing protein aggregation and maintaining cellular protein homeostasis (Vierling, 1991; Wang et al., 2004; Gupta et al., 2010; Schleiff and Becker, 2011). Heat shock factor activity in each cell is controlled through sophisticated and complex feedback mechanisms and protein interactions, allowing for rapid adjustment and flexibility by diverse chaperones to changing environmental conditions (Akerfelt et al., 2010).

The expression of *HSPs* is induced by HSFs that bind the HSEs in the promoters of heat shock responsive genes (Nover et al., 2001). Under normal conditions, monomeric HSFs are bound to HSP70 in the cytoplasm. When plants are exposed to HS, HSFs are released from HSP70-HSF complexes, and phosphorylated in the cytoplasm, and form a trimer for biding to HSEs in the nucleus (Liu et al., 2006). Overexpression of HSF genes in turn turns on almost all heat shock genes containing the HSE consensus sequence, conferring tolerance to HS. HSP70/90 plays an important role in the regulation of HSFA1 activity. HSP70/90 complex keeps HSFA1 inactive under normal conditions by repressing transactivation activity and nuclear localization of HSFA1 (Yamada et al., 2007; Hahn et al., 2011). Recently, the temperature-dependent repression (TDR) domain has been identified in the central region of HSFA1d, one of the *Arabidopsis* HSFA1s responsible for HS-dependent transactivation activity (Ohama et al., 2017). Overexpression of constitutively active HSFA1d, which lacks the TDR domain, induced the expression of heat shock proteins in the absence of HS, thereby conferring strong thermal stability in the overexpressing plants. Under HS conditions, HSFA1a is released from the HSFA1-HSP70/90 complex and activated. Of note, no TDR domain has been observed in mammalian HSFA1 proteins although the repression of the activities of HSFs by the HSP70/90 complex is generally conserved in both plants and animals. Activated *HSFA1* directly and rapidly regulates expression levels of genes encoding important HS-responsive transcription factors (TFs) such as DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2A (DREB2A), HSFA2, HSFA7a, HSFBs, and MULTIPROTEIN BRIDGING FACTOR 1C (MBF1C) (Yoshida et al., 2011). Subsequently, DREB2A directly regulates the gene expression level of *HSFA3* by creating a coactivator complex with NUCLEAR FACTOR Y, SUBUNIT A2 (NF-YA2), NF-YB3, and DNA POLYMERASE II SUBUNIT B3-1 (DPB3-1)/NF-YC10 (Chen et al., 2010; Sato et al., 2014). *HSFA3* knockout or knockdown transgenic lines caused reduced expression of putative target *HSP* genes under HS, thus HSFA3 is regarded as an important HS-responsive TF (Schramm et al., 2008; Yoshida et al., 2008). Furthermore, HSFA2 contributes to high levels of modifications at specific histone tail residues (H3K4me2 and H3K4me3) of *ascorbate peroxidase 2* (*APX2*), *HSP22*, and *HSP18.2* (Sung et al., 2003; Charng et al., 2007; Lämke et al., 2016). Heat stress memory is maintained for several days, allowing plants to survive when they are exposed to the next HS conditions (Yamaguchi, 2021). Strong/rapid expression of *sHSP* genes including *HSP21*, *HSP22*, and *HSP17.6C* is observed in primed plants compared to non-primed plants (Yamaguchi et al., 2021). *FORGETTER3* (*FGT3*)/*HSFA3* is needed to retain HS memory for several days following HS exposure (Friedrich et al., 2021). A recent discovery showed that genes encoding stem cell regulators such as *CLAVATA1* (*CLV1*), *CLV3*, and *HSP17.6A*, and the primary carbohydrate metabolism gene *FRUCTOSE-BISPHOSPHATE ALDOLASE 6* (*FBA6*) are involved in the HS transcriptional memory in the shoot apical meristem (Olas et al., 2021). *JUMONJI-C DOMAIN CONTAINING PROTEINs* (*JMJs*) that code for H3K27me3 demethylases are regulators of heat acclimation through controlling the methylation status of *HSP* loci (Pan et al., 2007; Xiao et al., 2016; Yamaguchi et al., 2021; Yamaguchi and Ito, 2021).

TABLE 2 | Gene expression pattern response to heat or cold stress in vegetables.

Vegetables	Gene/ protein	Expression pattern		Tissue	Description	References
		Heat (H)	Cold (C)			
Tomato (*Solanum lycopersicum)*	*SlHSP100*	Up		H: leaves	Upregulation detected in both thermotolerant and thermosensitive lines under HS.	Gul et al., 2021
	HSP70 sHSP	Up	*Up (H → C)	H/C: fruits	Protein levels of HSPs were increased under HS. *Increased protein levels at HT remained high for several weeks even when transferred to low temperatures.	Sabehat et al., 1996
	SlHSP20	Up/ Down			Expression of 13 of all tested *SlHsp20* genes was drastically increased in both thermotolerant and thermosensitive lines under HS, except for *SlHsp15.7*.	Yu et al., 2016
	HSFA2 Hsp17-CII	Up		H: flowers	The highest induction of two genes was identified in the anther tissues under HS.	Giorno et al., 2010
	tom111 (homolog from pea *HSP21*), *tom66,* (homolog from pea *HSP18.1*)	Up	**Up (H → C)	H: fruits, flowers, leaves, stems C: Mature-green fruits	The expression of *tom 111* and *tom66* was induced by HT. **The expression was first decreased and re-induced after the heated organs were transferred to low temperature.	Sabehat et al., 1998
	LeHSP17.6	Up	***Up (H → C)	H/C: fruits	Finally, Fruits with heating-and-chilling treatment showed a high level of expression of *LeHSP17.6*. ***Increased expression of *LeHSP17.6* at HT remained during subsequent exposure to low temperatures for at least one week.	Kadyrzhanova et al., 1998
Pepper (*Capsicum annuum*)	*CaHSP70*	Up/ Down		H: leaves	Expression of *HSP70* gene was highly upregulated in the thermotolerant line compared to the thermosensitive line under HS.	Usman et al., 2015
	CaHSP60	Up/ Down	Up	H/C: leaves, stems, roots	Fifteen (93% of total *CaHSP60* genes) *CaHSP60* genes were upregulated under HS and cold stress, and only *CaHSP60-3* was downregulated in both thermosensitive B6 and thermotolerant R9 lines.	Haq et al., 2019
	CaHSP20	Up/ Down		H: leaves, stems, roots, flowers	Generally, the peaks of expression levels of *CaHsp20* genes in the thermosensitive line B6 were higher than the thermotolerant line R9.	Guo et al., 2015
	CaHSP16.4	Up		H: leaves, roots	The expression level of *CaHsp25.9* was higher in leaves than that in roots, and was highest at 2 h after HS in both thermosensitive B6 and thermotolerant R9 lines.	Feng et al., 2019
Soybean (*Glycine max)*	*GmHSP90*	Up		H: leaves	A significant upregulation was observed in 12. *GmHsp90* genes within 30 min at 42°C	Xu et al., 2013
	GmHSP70	Up/ Down		H: leaves	29 genes out of 61 detectable *GmHSP70s* showed upregulation under drought and HS conditions.	Zhang et al., 2015
	GmHSP20	Up	Up	C: leaves	47 soybean *Hsp20* genes were responsive to heat shock stress, and 5 were also induced by cold stress.	Lopes-Caitar et al., 2013
Pea (*Pisum sativum*)	*HSP70 PsHSFA*	Up		H: leaves, cotyledons	The expression of *PsHSFA* and *HSP70* was induced in both leaves and cotyledons under HS.	Aranda et al., 1999
	HSP17.9 HSP18.1	Up		H: leaves	The expression of *HSP17.9* and *HSP18.1* was highly upregulated at the beginning of HS, and declined rapidly after the stress.	DeRocher et al., 1991
Potato (*Solanum tuberosum)*	18 kDa sHSP	Up		H: leaves	The 18 kDa sHSP proteins were induced longer in the heat tolerant cultivars than the heat sensitive cultivars.	Ahn et al., 2004
	HSP100 HSP90 HSP80 HSP70 sHSP		Up (during chilling storage)	C: tuber	Fifteen *HSPs* genes, including *HSP100*, *HSP90*, *HSP80*, *HSP70* and *sHSP* family were consistently upregulated by low temperatures in both RNA and protein levels, which may act to prevent cellular damage from cold stress in potato tubers during postharvest storage.	Lin et al., 2019
Lettuce (*Lactuca sativa*)	*HSP70*	Up		H: leaves, stems	HT induced the expression of a gene encoding HSP70 that interacts with a calmodulin for heat induced bolting tolerance.	Liu R. et al., 2020
	HSP70 sHSP	Up		H: leaves	The *sHSP* and *HSP70* genes were quickly and sharply induced within 1 h treatment of HS.	Kang et al., 2021

Extreme HT causes protein misfolding and denaturation. Unfolded proteins can be degraded by the ubiquitin proteasome system or autophagy (Buchberger et al., 2010; Amm et al., 2014; Xu and Xue, 2019). It has been demonstrated that some ubiquitin E3 ligases and autophagy-related genes play a critical role in plant heat tolerance (Zhou et al., 2014; Li et al., 2015; Liu J. et al., 2016; Gil et al., 2017). Transgenic plants overexpressing ubiquitin or ubiquitin E3 ligases displayed enhanced BTT and/or ATT (Tian et al., 2014; Liu J. et al., 2016), and Zhang Y. et al. (2021) reported that silencing CARBOXYL TERMINUS OF THE HSC70-INTERACTING PROTEINS (CHIP), a chaperone-dependent ubiquitin E3 ligase caused reduced heat tolerance in tomato. CHIP plays a critical role in HSR through the misfolded proteins degradation induced by HS. Transgenic *Arabidopsis* seedlings overexpressing PROTEIN WITH THE RING DOMAIN AND TMEMB (PPRT1) encoding a C3HC4 zinc-finger ubiquitin E3 ligase showed enhanced BTT and ATT (Liu Y. et al., 2020). Moreover, virus-induced gene silencing (VIGS) of tomato *AUTOPHAGY RELATED5* (*ATG5*) and *ATG7* genes resulted in increased sensitivity of tomato plants to HS (Zhou et al., 2014).

Understanding the dynamic behavior involving expression levels of TFs and HSPs under HS will help understand the whole regulatory network to adapt to HT.

Expression Patterns of *HSP* and *HSF* Genes in Vegetables Under Heat Stress

Exposure to extreme temperature stresses such as heat and cold induces cellular changes in plant cells (Guy, 1999; Bita and Gerats, 2013). Plants have evolved various physiological and molecular adaptations to stresses in order to minimize damage and provide cellular homeostasis (Theocharis et al., 2012; Awasthi et al., 2015). In response to the extreme temperature stresses, plants synthesize many stress-responsive proteins including HSP and HSF by regulating gene expression (Guo et al., 2016a; Ul Haq et al., 2019). So far, many studies on gene expression patterns under heat and/or cold stresses in vegetable crops have been reported and the collected information can be seen in **Table 2**.

Tomato (*Solanum lycopersicum* L.)

Tomato is one of the most economically important vegetable crops worldwide (Campos et al., 2021). As global warming leads to extreme weather events, a number of researchers have examined the effects of heat and/or cold stresses on the expression pattern of genes such as *HSPs* and *HSFs*, which play crucial roles in thermotolerance in tomatoes (Tubiello et al., 2007).

Heat treatment has been found to induce chloroplastic *SlHSP100* genes in both thermotolerant and thermosensitive tomato seedlings. The highest upregulation was observed in the genotype 17903, which showed the highest ratio of cell viability and cell membrane stability under HS, implying a crucial role for the gene in ATT (Gul et al., 2021). Besides the role of HSP100 as a chaperone, Sabehat et al. (1996) found that tomato fruits heated and then chilled showed a high level expression of both *HSP70* and *sHSP* family genes (14–25kDa) and enhanced chilling tolerance compared to unheated fruits (Sabehat et al., 1996). Similar results were also reported by Kadyrzhanova et al. (1998) and Sabehat et al. (1998) where the expression of chloroplastic *HSP21* and *HSP17.6* was first decreased and re-induced when the heated fruits were transferred to low temperature. The members of *SlHSP20s* in tomato were also upregulated in both thermotolerant and thermosensitive lines under HS, except for *SlHsp15.7* (Yu et al., 2016). Moreover, it has been reported that the expression of *HSFA2*, transcriptional activator of *HSP* expression, and *HSP17-CII* was highly activated in the tomato anther during its development under HS (Giorno et al., 2010).

Pepper (*Capsicum annuum*)

The production and consumption of pepper has steadily increased worldwide due to its nutritional benefits and spice, but it is thermosensitive (Crosby, 2008; Guo et al., 2014). As with tomato, there has been a growing body of research that explores the expression of *HSP* genes in pepper under temperature stress conditions. Many *HSPs* including *CaHSP70*, *CaHSP60*, *CaHSP20,* and *CaHSP16.4* are upregulated in pepper under HS (Guo et al., 2015; Usman et al., 2015; Feng et al., 2019; Haq et al., 2019). *HSP70* gene was significantly upregulated in the thermotolerant line compared to the thermosensitive line after 2 h of HS treatment at 42°C, indicating that the gene is quickly and sharply induced by heat shock and plays a major role in thermotolerance (Usman et al., 2015). Haq et al. (2019) observed that fifteen *CaHSP60* genes were upregulated under HS and cold stress, and only *CaHSP60-3* was downregulated in both thermosensitive B6 and thermotolerant R9 lines (Haq et al., 2019).

Soybean (*Glycine max*)

Soybeans are members of the legume family of vegetables and have been a staple of Asian cuisines for a long time. Soybean yield is severely affected by temperature stresses. Under low or high temperature stress conditions, HSPs are induced in soybean to prevent cell damage caused by the temperature stresses. Xu et al. (2013) studied the expression of *GmHSP90* in relation to HS, and observed a significant upregulation of this gene in early response to HS (Xu et al., 2013). Expression patterns of soybean 61 *GmHSP70* genes under HS and drought were analyzed. Among those genes, 55 *GmHSP70* genes were highly upregulated during HS, and 29 *GmHSP70* genes showed increased expression under both heat and drought stress conditions, indicating that most of the *GmHSP70* genes play an important role in heat and drought tolerance (Zhang et al., 2015). Similarly, 47 *GmHSP20* genes among 51 *GmHSP20* candidates were found to be highly induced under HS and 5 genes were induced under both heat and cold conditions (Lopes-Caitar et al., 2013).

Pea (*Pisum sativum*)

Pea has long been important in the human diet due to its starch, protein, and fiber content and the many phytochemical substances it contains, but it is a cool season crop which is heat-sensitive (Dahl et al., 2012). Therefore, some researchers have investigated the expression of *HSPs* in pea during HS. DeRocher et al. (1991) observed that the *HSP18.1* mRNA peaked at the beginning of the maximum temperature during 4 h gradual HS (30–42°C) period, and began to decline 6 to 8 h before the amount of HSP18.1 protein reached maximum levels, implying

TABLE 3 | Engineering temperature stress tolerance in plants.

Transgenic plant	Stress	Gene targeted/ transferred	Gene expression/ manipulation	Result	References
Arabidopsis	Heat	*AtHSP101*	Down regulation/Antisense inhibition or co-suppression	Decreased heat tolerance.	Queitsch et al., 2000
		AtHSF1	Overexpression of *AtHSF1*-GUS and GUS-*AtHSF1*	Increased *HSP18* expression level at normal temperatures and enhanced basic thermotolerance.	Lee et al., 1995
		CaHSP25.9 From pepper	Overexpression	Increased heat tolerance. Reduced accumulation of reactive oxygen species (ROS).	Feng et al., 2019
		CaHSP70 from pepper	Overexpression	Increased heat tolerance including basal thermotolerance and acquired thermotolerance.	Guo et al., 2016b
		PfHSP21.4 from Primula	Overexpression	Increased thermotolerance activity. Increased antioxidant enzymes such as ascorbate peroxidase (APX).	Zhang et al., 2014
		TaHSP26 from wheat	Overexpression	Increased thermotolerance. Increased photosynthetic pigments, higher biomass, and seed yield.	Chauhan et al., 2012
			Down-regulation/Antisense inhibition	Showed negligible thermotolerance.	
		LimHSP16.45 from David Lily	Overexpression of *LimHSP16.45*-GFP	Enhanced viability of *Arabidopsis* cells under HS. Induced more superoxide dismutase (SOD) and catalase (CAT) activity.	Mu et al., 2013
	Cold	*CsHSP17.7* *CsHSP18.1* *CsHSP21.8* from *Camellia sinensis*	Overexpression	Increased root length in *Arabidopsis* under low temperature.	Wang et al., 2017
		PfHSP17.2 from Forrest primrose	Overexpression	Enhanced freezing tolerance.	Zhang L. et al., 2018
Tobacco	Heat	*OsHSP101* (*ClpB-C*) from rice	Overexpression	Increased heat tolerance.	Chang et al., 2007
		ZmHSP16.9 from maize	Overexpression	Increased tolerance to heat and oxidative stress.	Sun et al., 2012
		LeHSP21 from tomato	Overexpression	Increased tolerance to heat and oxidative stress.	Zhang et al., 2016
		BcHSP70 from *Brassica campestris*	Overexpression	Increased heat tolerance. Increased the chlorophyll content, SOD and peroxidase (POD) activities.	Wang X. et al., 2016
	Cold	*CaHSP26* from sweet pepper	Overexpression	Protected PSII and PSI from chilling stress.	Guo et al., 2007
		CaHSP22.5 from pepper	Overexpression	Improved the tolerance of chilling stress. Increased the activity of reactive oxygen species-scavenging enzymes.	Li et al., 2018
Rice	Heat	*AtHSP101* (*ClpB-C*)	Overexpression	Increased heat tolerance.	Katiyar-Agarwal et al., 2003
		OsHSP18.6	Overexpression	Increased heat tolerance. Exhibited the lower levels of malondialdehyde (MDA) and greater CAT and SOD activities.	Wang et al., 2015
Tomato	Heat	*HSFA1b* (*AtHSF A1b* and β-*glucuronidase* (*gusA*) fusion gene)	Overexpression	Increased heat tolerance. Increased the activity of soluble isoforms of APX.	Li et al., 2003
		HSP24.4	Overexpression	Increased heat tolerance. Showed tissue specific expression in root, shoot, and stem tissue under HS.	Mahesh et al., 2013
		Unknown (*HT7* mutant)	EMS Micro-Tom mutant	Heat tolerant tomato lines. Highly expressed *SlHSFA1b* and *SlHsp101* than WT respond to HS.	Pham et al., 2020
	Cold	*HSP*	Overexpression	Increased chilling tolerance.	Wang et al., 2005
		HSFA1b (*AtHSF A1b* and *gusA* fusion gene)	Overexpression	Increased chilling tolerance. Increased the activity of soluble isoforms of APX.	Li et al., 2003
		sHSP23.8-M	Overexpression	Protected fruit from chilling injury.	Escobar et al., 2021

(Continued)

TABLE 3 | (Continued)

Transgenic plant	Stress	Gene targeted/ transferred	Gene expression/ manipulation	Result	References
			Knock-down	Decreased chilling tolerance. Showed wilting and skin wrinkles, partial discoloration.	
		SlHSP17.7	Overexpression	Increased tolerance response to cold stress.	Zhang et al., 2020
Potato	Heat	*DcHSP17.7* from carrot	Overexpression	Increased cellular membrane stability and tuberization.	Ahn and Zimmerman, 2006
Pepper	Heat	*CaHSP60-6*	Down regulation/ virus-induced gene silencing (VIGS)	Reduced heat tolerance.	Haq et al., 2019
Carrot	Heat	*HSP17.7*	Overexpression	Increased heat tolerance (with an increase of 68-90% growth).	Malik et al., 1999
			Down-regulation/Antisense inhibition	Decreased heat tolerance (with a decrease of 12-26% growth).	
Soybean	Heat	*GmHsp90A2*	Overexpression	Increased heat tolerance. Reduced chlorophyll loss and stabilized membrane systems.	Huang et al., 2019
			Knockout/ CRISPR/Cas9	Reduced heat tolerance.	

that sHSP levels in plants may also be self-regulated or regulated by some other heat-inducible protein.

Potato (*Solanum tuberosum*)

Potato is a vegetable crop that mainly grows in a temperate climate, so HS can have a negative effect on the yield by inducing physiological defects in tubers (Rykaczewska, 2017). Hence, it is important to examine the accumulation of HSPs in response to HS. Ahn et al. (2004) reported that the 18 kDa sHSP proteins were synthesized for a longer time in the heat tolerant cultivars compared to the heat sensitive cultivars under strong heat shock temperature, suggesting that sHSP plays an important role in the heat tolerance enhancement (Ahn et al., 2004). Fifteen HSPs, including three HSP70s, two HSP80s, one HSP90, one HSP100 and eight sHSPs were consistently upregulated by low temperatures at both the RNA and protein levels to reduce cellular damage and re-build cellular homeostasis in potato tubers under cold stress during postharvest storage (Lin et al., 2019).

Lettuce (*Lactuca sativa*)

Lettuce is an important cool season leafy vegetable with an optimal growing temperature ranging from 17 to 28°C (Holmes et al., 2019). HT can facilitate the accumulation of gibberellin (GA) which promotes lettuce bolding (Fukuda et al., 2012). Under HT, it is suggested that induced expression of genes encoding LsHSPs that interact with a calmodulin confers enhanced tolerance to heat with bolting resistance in lettuce (Liu R. et al., 2020). Recently, putative early heat responsive *HSP* genes were identified by transcriptome profiling in lettuce (Kang et al., 2021). Among them, *sHSP* and *HSP70* genes were quickly and sharply induced within 1 h in response to HS, indicating that these genes could be potential candidates as the breeding targets for the development of heat-tolerant lettuce cultivars.

BREEDING FOR ELEVATED RESISTANCE TO HEAT STRESS

Currently, the greatest risk to crop productivity and yields associated with global climate change is being caused by extreme weather events such as extreme hot and cold weather (Reddy and Hodges, 2000). Therefore, improved tolerance to heat and cold stress might be crucial in increasing yields for most crops. Application of transgenic and genome editing technologies could help to introduce desirable abiotic stress tolerance traits into crop varieties (Sanghera et al., 2011; Lamaoui et al., 2018). In recent years, there has been an increasing effort to reveal functional roles of *HSPs* and *HSFs* using mutagenic and transgenic plants for production of crops with enhanced heat and/or cold tolerance (**Table 3**).

Model Plants

A number of researchers have used model plants such as *Arabidopsis*, tobacco and rice for functional studies (proof of concept) on genes involved in heat and cold stresses because of the ease of genetic experiments (Rensink and Buell, 2004; Koornneef and Meinke, 2010). Queitsch et al. (2000) examined transgenic *Arabidopsis* plants containing *HSP101* antisense and/or co-suppression constructs, and found that they showed normal growth but impaired ATT and BTT, indicating *HSP101* plays a pivotal role in heat tolerance in *Arabidopsis*. In contrast, transgenic *Arabidopsis* plants containing constitutively active HSF-GUS fusion proteins caused increased *HSP18* expression at normal temperature by forming HSF trimers and their binding to DNA, resulting in enhanced BTT (Lee et al., 1995).

In addition, transgenic approaches with other crop genes have also been made with a fair degree of success. Genetically engineered *Arabidopsis* plants overexpressing *HSP* genes from pepper (Guo et al., 2016b; Feng et al., 2019), primula (Zhang et al., 2014), wheat (Feng et al., 2019) and David Lily (Mu et al., 2013) exhibited increased thermotolerance activity. Similar events were

also observed under cold stress conditions by Wang et al. (2017) and Zhang L. et al. (2018). They introduced *CsHSP17.7*, *CsHSP18.1*, *CsHSP21.8,* and *PfHSP17.2* from *Camellia sinensis* and Forrest primrose into *Arabidopsis* for overexpression. Transgenic plants showed increased root length and tolerance to cold stress. Furthermore, overexpression of *OsHSP101* (Chang et al., 2007), *ZmHSP16.9* (Sun et al., 2012), *LeHSP21* (Zhang et al., 2016), *BcHSP70* (Wang X. et al., 2016), *AtHSP101* (Katiyar-Agarwal et al., 2003) and *OsHSP18.6* (Wang et al., 2015) conferred improved HS tolerance in tobacco and rice. These results indicate that *HSP* genes from various crops play a key role in developing thermotolerance.

Vegetables

Vegetable crops are very susceptible to abiotic stresses such as high and low temperatures. Therefore, the development of varieties that are tolerant to heat and cold stresses is an important goal for improvement in crop productivity. Recently investigators have examined the protective roles of HSP and HSF against heat and cold stresses in transgenic vegetables. Li et al. (2003) reported that increased activity of soluble isoforms of ascorbate peroxidase (APX) and tolerance were observed in the transgenic tomato plants overexpressing *AtHSFA1b-gusA* fusion gene under heat and cold stress conditions. In addition, 15 heat tolerant tomato lines were isolated through screening of over 4000 ethyl methanesulfonate (EMS) Micro-Tom mutants. Among the selected heat tolerant mutants, the HT7 line displayed much higher fruit number and total pollen number with enhanced viability under HS conditions. Higher expression levels of *SlHSFA1b3*, which is known as a master regulator that activates HSR (Mishra et al., 2002), and *HSP101* were detected in the leaves of HT7 compared to those of WT after long-term exposure to HS, suggesting that HT7 could be used as a breeding material for production of tomato with improved heat tolerance (Pham et al., 2020). Also, up and downregulated expression of *HSP23.8* made it possible for each transgenic plant to display the opposite phenotype under low temperature conditions: Transgenic plants overexpressing *HSP23.8* gene showed increased cold tolerance whereas decreased chilling tolerance, wilting, skin wrinkles and partial discoloration were observed in the transgenic plant with reduced expression of *HSP23.8* gene (Escobar et al., 2021). Similar studies have reported that the *HSP17.7* gene plays a role in the HS tolerance in potato (Ahn and Zimmerman, 2006) and carrot (Malik et al., 1999). Recently, it has been reported that HS tolerance decreases in pepper when the *CaHSP60-6* gene is down-regulated by virus-induced gene silencing (VIGS) (Haq et al., 2019). In particular, CRISPR-Cas9 based gene knockout was applied to *GmHSP90A2* in soybean, and the *GmHSP90A2* mutant exhibited reduced heat tolerance (Huang et al., 2019). In conclusion, major *HSP* and *HSF* genes are tightly related to thermotolerance of vegetables. Thus, continuous efforts to identify detailed functions and working mechanisms of *HSP* and *HSF* genes are needed for the generation of vegetables with enhanced heat/cold tolerance traits through precise manipulation of genetic elements.

CONCLUSION AND FUTURE PROSPECTS

Climate change including global warming is causing abrupt changes in weather patterns, and extreme weather events that threaten crop yields. Elevated temperatures, in particular, will have a severe influence on the productivity and yields of vegetables in agricultural fields. It is, therefore, indispensible to understand the sophisticated mechanisms vegetable crops use to adapt to changing temperature environments, from the signal perception to gene expression in reponse to HS.

As mentioned above, recent research has elucidated that an interplay of cooperative HSP, HSF, and HSR mechanisms orchestrate the expression of heat-responsive genes as the plant response to HS. Furthermore, research identifying TFs related to abiotic stresses and their molecular functions has contributed to the expansion of knowledge for the production of crops with desired traits through genetic manipulation and/or molecular breeding. Functional and cellular roles of some key TFs such as HSFA1s and DREB2A have been determined in transcriptional networks of HSR at the post-translational levels during HS. Nevertheless, the current information on the functional roles of *HSP* and *HSF* genes in vegetable crops is still insufficient for their practical application to breeding. Transcriptional regulation between HSPs and HSFs, and in-depth working mechanisms and pathways of heat-related proteins during HSR remain to be explored.

Chromatin immunoprecipitation sequencing (ChIP-seq) for protein-protein complexes and reverse ChIP for mining the upstream-gene regulatory sequences have been shown to be effective tools to investigate potential interaction networks between regulatory regions in HSE and proteins, respectively (Machanick and Bailey, 2011; Shim et al., 2021). It will be necessary to utilize these techniques to clarify the in-depth mechanism underlying the gene regulatory relationships in the HSPs and HSFs of vegetable crops during HSR. It is becoming evident that microRNAs, small RNAs, and epigenetic modulations in DNA, RNA, and protein species play a pivotal role in HS memory (Guan et al., 2013; Stief et al., 2014a,b; Lämke et al., 2016). Advances in high-throughput small RNA sequences (RNA-seq) together with methylated DNA and RNA-sequencing combined with IP will be of help in determining the functions of TFs and epigenetic regulators (Pall and Hamilton, 2008; Zhang H. et al., 2018; Shen et al., 2019; Lee et al., 2021). In addition, state-of-art next-generation sequencing (NGS) including quantitative trait loci (QTL)-sequencing, genotyping-by-sequencing (GBS), and genome-wide association studies (GWAS) have been successfully developed and adopted for deciphering comprehensive genome sequences, thus facilitating the identification of a wide variety of molecular markers corresponding to target traits in crops (Han et al., 2016; Jo et al., 2017; Lee et al., 2020; Jha et al., 2021). Candidate and/or identified genes crucial for thermotolerant-traits and HS-related pathways can be used for production of transgenic vegetable crops via genetic engineering. Furthermore, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) and dead Cas9

(dCas9) systems have been extensively introduced into crop biotechnology as powerful tools for gene/genome editing in spite of controversial GMO and non-GMO issues (Liu D. et al., 2016; Pramanik et al., 2020; Gao, 2021; Kim et al., 2021). Indeed, "Sicilian Rouge High GABA tomato" was recently developed by using the CRISPR/Cas9 gene editing technology. It contains high levels of gamma-aminobutyric acid (GABA), an amino acid believed to aid relaxation and help lower blood pressure.[2] All the aforementioned technologies can be utilized for dissecting action modes and intricate networks of HSP, HSF and HSR for thermotolerance in vegetable crops.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

[2] https://the-japan-news.com/news/article/0007780624

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Comparative Transcriptome Analysis of Onion in Response to Infection by *Alternaria porri* (Ellis) Cifferi

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Purple blotch (PB) is one of the most destructive foliar diseases of onion and other alliums, caused by a necrotrophic fungal pathogen *Alternaria porri*. There are no reports on the molecular response of onion to PB infection. To elucidate the response of onion to *A. porri* infection, we consequently carried out an *RNAseq* analysis of the resistant (Arka Kalyan; AK) and susceptible (Agrifound rose; AFR) genotype after an artificial infection. Through differential expression analyses between control and pathogen-treated plants, we identified 8,064 upregulated and 248 downregulated genes in AFR, while 832 upregulated and 564 downregulated genes were identified in AK. A further significant reprogramming in the gene expression profile was also demonstrated by a functional annotation analysis. Gene ontology (GO) terms, which are particularly involved in defense responses and signaling, are overrepresented in current analyses such as "oxidoreductase activity," "chitin catabolic processes," and "defense response." Several key plant defense genes were differentially expressed on *A. porri* infection, which includes pathogenesis-related (PR) proteins, receptor-like kinases, phytohormone signaling, cell-wall integrity, cytochrome P450 monooxygenases, and transcription factors. Some of the genes were exclusively overexpressed in resistant genotype, namely, *GABA transporter1*, *ankyrin repeat domain-containing protein*, *xyloglucan endotransglucosylase/hydrolase*, and *PR-5* (*thaumatin-like*). Antioxidant enzyme activities were observed to be increased after infection in both genotypes but higher activity was found in the resistant genotype, AK. This is the first report of transcriptome profiling in onion in response to PB infection and will serve as a resource for future studies to elucidate the molecular mechanism of onion-*A. porri* interaction and to improve PB resistance in onions.

Keywords: onion, purple blotch, *RNAseq*, *Alternaria porri*, antioxidant, PR proteins

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INTRODUCTION

Purple blotch (PB) is one of the most destructive foliar diseases in onion, caused by *Alternaria porri* (Ellis) Cifferi. Purple blotch is prevalent in all onion-growing countries in the world (Kareem et al., 2012). Symptoms of the disease encompass small chlorotic, water-soaked brown lesions on leaves, and as infection advances lesion enlarges with purple spots, in a humid climate, these lesions are

occasionally seen covered with black-purple spores. In India, its high degree of severity is evidenced by heavy yield losses in both bulb and seed crops, which vary between 2.5 and 85% (Tripathy et al., 2013; Veeraghanti et al., 2017). For the management of PB, mainly chemical fungicides were used, but its excessive and repeated use led to an increase in production cost, deterioration of the environment, and development of resistance in pathogen. Although several bioagents have been reported to antagonize *A. porri* (Tyagi et al., 1990; Prakasam and Sharma, 2012; Abdel-Hafez et al., 2014; Gothandapani et al., 2015), their commercial utility at the field level is limited. Therefore, in such condition, improvement of host resistance by the selection, breeding, and biotechnological tools will be the best sustainable approach (Dar et al., 2020).

To date, limited sources of PB resistance have been reported in onions (Ganesh and Veeregowda, 2007; Behera et al., 2013; Tripathy et al., 2013; Nanda et al., 2016). The PB resistance in onions is controlled by additive and non-additive gene effects (Evoor et al., 2007) and a single dominant qualitative gene (Abubakar and Ado, 2008). Furthermore, the novel PB-resistant gene *ApR1* was mapped using markers (AcSSR7 and ApR-450) linked to PB resistance in the F_2 population developed from Arka Kalyan (AK) and Agrifound rose (AFR) (Chand et al., 2018). Barring these few studies, the mechanism of PB resistance at a molecular level is not fully known.

Plants respond to the invasion of the pathogen through transcriptional regulation, and large-scale approaches such as transcriptional analysis and genome-wide association studies have been widely applied to uncover the molecular mechanism of plant defense mechanisms (Gupta et al., 2016; Bartoli and Roux, 2017; Zhu et al., 2017; Juliana et al., 2018). Significant advances in understanding defense processes have been made in many crops, and the RNAseq for transcriptome analysis has become a powerful tool to investigate plant disease responses in plants where a high-quality genome sequence is not available (Ghodke et al., 2020; Khandagale et al., 2020; Kim et al., 2021).

Onion (*Allium cepa* L.), from the Amaryllidaceae family, is a vegetable of paramount importance in India and other parts of the world (Chinnappareddy et al., 2013). India is the second largest producer of onion among the major onion producer countries of the world with a production of 26.7 million tons from the 1.4-million-hectare area with a yield of 18.6 ton/ha in 2020.[1] Although PB is inflicting significant losses to this economically important crop, very less research is still being performed into the molecular response against in a known PB-resistant cultivar. Therefore, in this study, we examined biochemical and molecular responses in resistant AK and susceptible AFR genotypes when infected by *A. porri*.

MATERIALS AND METHODS

Plant Material and Pathogen

For this study, PB-resistant (AK) and susceptible (AFR) varieties were selected for this study (Chand et al., 2018). Seeds of AK and AFR were procured from the Indian Institute of Horticulture Research, Bangalore and National Horticultural Research and Development Foundation, New Delhi, respectively. Pure culture of *A. porri* was isolated from the experimental field of ICAR-DOGR and is maintained on potato dextrose agar (PDA). The identification of the pathogen was ensured by amplifying the ITS region of the fungal DNA with the primers ITS1 5'TCCGTAGGTGAACCTGCGG3' and ITS4 5'CTGTTGGTTTCTTTTCCTCCGC3' according to White et al. (1990). The amplified fragment was purified and sequenced, and the resulting DNA sequence was aligned with GenBank using BLASTN at the National Center for Biotechnology Information (NCBI) database which showed 100% identity with *A. porri* (LC440611.1) and submitted in NCBI GenBank (OM131604).

Experiment

Seeds of both genotypes were surface sterilized with sodium hypochlorite (4%) for 10 min and 70% alcohol for 30 s followed by a three-time wash with distilled water. The seeds were sown in plastic pots with sterilized soil, and after 45 days, the seedlings were transplanted into fresh pots with similar sterilized soil. Each pot contained 4 plants, 10 pots were used for each replication, and the experiment was performed in triplicate. To prepare a fungal pathogen spore suspension, mycelial plugs from *A. porri* culture plate were transferred to a fresh PDA and incubated at 25 ± 2°C for 10 days. The fungal culture was scraped and mixed with distilled water to obtain a final spore concentration of 10^6 spores/ml. After 30 days of transplantation, the plants were inoculated with a pathogen spore suspension. The leaves were sprayed with pathogen spore suspension and allowed to dry for 2 h, and then the pots were covered with a transparent polythene bag for 24 h to maintain humidity. Control plants were treated similarly, except for pathogen spore suspension; they were sprayed with sterile water. The pots were kept in the greenhouse at 25 ± 2°C, and the leaf tissue was harvested from control and inoculated plants at 5 dpi (day postinoculation), frozen in liquid nitrogen, and stored in –80°C until further studies.

RNA Isolation and Library Preparation and RNA Sequencing

The total RNA of each control (AKC, AFRC) and infected (AKT, AFRT) plant was extracted using the RNeasy Plant Mini Kit (Qiagen). A pool of three plants was used for RNA isolation, and as one replicate, such two replicates were used in this study. Total RNA of each replicate was quantified using NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, United States), and the integrity was assessed on a 1% agarose gel. Further integrity of the isolated RNA was assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States). An equal amount of high-quality RNA having a RIN value above 7 from three plants of each replicate was pooled and used for library preparation. A total of 8 (AKC1, AKC2, AKT1, AKT2, AFRC1, AFRC2, AFRT1, and AFRT2) next-generation sequencing libraries were constructed according to the manufacturer's protocol (NEBNext® UltraTM RNA Library Prep Kit for Illumina®). These libraries

[1] https://www.fao.org/faostat/en/#data/QCL

were sequenced in both directions with a read length of 150 × 2 using the Illumina HiSeq 2500 platform.

De novo Assembly and Sequence Annotation

The quality check for high throughput sequencing reads was performed using FASTQC Toolkit version 0.11.9.[2] The low-quality reads (Q-value $\leq$ 20) were discarded using cutadapt version 2.8,[3] and the remaining reads were considered clean reads. The obtained clean reads of the AK and AFR varieties were assembled using Trinity assembler version 2.12.0[4] to build a mega-assembly. The transcripts were then clustered using the CD-HIT version 4.8.1[5] tool to generate a comprehensive reference. Identity threshold was kept default, i.e., 90%. DESeq2, an R package, was used for differential gene expression analysis between control and treated samples of both resistant and susceptible genotypes. Differentially expressed transcripts were selected based on a cutoff log2 fold change of 2 and a cutoff p-value of 0.05.

The clustered transcriptome was annotated using the DIAMOND BLASTX version 2.0.9.147[6] tool against NCBI's non-redundant protein database (NRDB),[7] UniProt/SwissProt database,[8] and plantTFDB[9] with a cutoff e-value of $\leq 10^{-5}$. UniProt/SwissProt ID mapping functionality was used to get gene ontology (GO) and pathway annotation for transcripts. The Transeq utility from EMBOSS version 6.6.0[10] package was used to convert the transcripts into the longest possible open reading frame. Orthologous groups of protein sequences were identified using a standalone version of emapper version 2.0.1 against eggNOG version 5.0.[11] Protein sequences were classified into families and predicted domains, important sequence signatures using a standalone version of InterProScan 5.39–77.0.[12]To study pathogen receptor genes, we mapped the transcripts on PRGDB[13] manually curated reference protein sequences using the DIAMOND BLASTX utility with an e-value of $\leq 10^{-5}$.

Validation of Differentially Expressed Genes Using Quantitative Realtime-PCR

Total RNA was isolated from leaves of two biological replicates at 5 dpi. The untreated plants were considered as a control in the present experiment. The total RNA was isolated with the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's guidelines. To eliminate potential genomic DNA contamination, RNAs were treated with DNase I (Fermentas, Lithuania). RNAs were quantified using NanoDrop (ND1000), and the integrity of the isolated RNA was assessed on the formaldehyde-agarose gel (1%). First-strand cDNA was synthesized to reverse transcription reaction from 1 μg RNA using the RevertAid First Strand cDNA Synthesis Kit (Fermenats, Lithuania) following the manufacturer's instructions. cDNAs were stored at −80°C until used in quantitative realtime-PCR (qRT-PCR).

Primers for selected PB-induced transcripts were designed using Primer-BLAST[14] to amplify a region of 160–230 bp. Expression analyses of selected differentially expressed genes (DEGs) were performed in LightCycler® 480 II instrument (Roche, Germany). A single 10 μl PCR mixture contained 1 × LightCycler® 480 SYBR Green I master mix (Roche, Germany), 1 μl of cDNA, and 1 μM of each primer (10 μM). The PCR cycling was programmed as follows: initial denaturation at 95°C for 5 min, 45 cycles at 95°C for 10 s, 58°C for 10 s, and 72°C for 15 s. *AcActin* was used as a reference gene. The details of primers used in the present qPCR analyses are depicted in **Supplementary Table 1**. The analyses were performed using two biological replicates along with respective three technical replicates, and the relative fold change in transcript concentration was measured according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Biochemical Analyses

Anti-oxidative and Defense Enzyme Assay

Leaf samples were harvested at 5 dpi from both control and infected plants of resistant and susceptible genotype for estimation of antioxidant enzyme activities of catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), superoxide dismutase (SOD), and phenylalanine lyase (PAL). Enzyme extract was prepared as per Roylawar and Kamble (2017) with modification. The sample was ground in liquid N_2, and 200 mg of this sample was homogenized by adding chilled potassium phosphate buffer of 0.1 M and pH of 7.2 with Na-EDTA (0.1 M) and PVP (0.5%). The supernatant obtained after centrifugation (14,000g) at 4°C for 20 min was used for performing enzyme assays.

Catalase

Catalase was determined by the decomposition of H_2O_2, which was measured by recording the decrease in absorbance at 240 nm (Volk and Feierabend, 1989). For the determination of CAT activity, a 3 ml reaction volume was made up by adding the reaction medium containing 0.1 M potassium phosphate buffer (pH 7.0) and 30 mM H_2O_2 to the enzyme extract. One unit activity of CAT was determined by the amount of enzyme that used 1 μmol H_2O_2 per minute.

Ascorbate Peroxidase

The APX activity was assayed using a modified method of Nakano and Asada (1981). The 1 ml assay mixture contained 0.5 M Tris HCl buffer (pH 7.6), 0.1 mM Na_2EDTA, 0.5 mM ascorbic acid, and 5 mM H_2O_2 along with enzyme extract. To initiate the reaction, H_2O_2 was added at last, and the absorbance at 290 nm was recorded for 3 min. The enzyme activity was calculated by the

[2]https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
[3]https://github.com/marcelm/cutadapt/
[4]https://github.com/trinityrnaseq/trinityrnaseq/wiki
[5]http://weizhong-lab.ucsd.edu/cd-hit/download.php
[6]https://ab.inf.uni-tuebingen.de/software/diamond
[7]http://ftp.ncbi.nlm.nih.gov/blast/db/
[8]https://www.uniprot.org/
[9]http://planttfdb.cbi.pku.edu.cn/
[10]http://emboss.sourceforge.net/download/
[11]http://eggnogdb.embl.de/#/app/downloads
[12]https://www.ebi.ac.uk/interpro/download.html
[13]http://prgdb.org/prgdb4
[14]https://www.ncbi.nlm.nih.gov/tools/primer-blast/

determination of reduction in ascorbate by using an extinction coefficient of 2.8 mM^{-1} cm^{-1} that was expressed in terms of millimole of ascorbate per minute per gram fresh weight.

Guaiacol Peroxidase

Peroxidase activity was assayed by using guaiacol as the substrate by following the method described by Xu et al. (2011). The assay system consisted of 0.1 M phosphate buffer pH 7.0, 30 mM guaiacol, 20 mM H_2O_2, and a suitable aliquot of enzyme in a final volume of 3 ml. The GPX activity was determined spectrophotometrically by measuring the increase in absorbance at 470 nm by the conversion of guaiacol to tetraguaiacol due to its oxidation. The molar extinction coefficient of tetraguaiacol was taken as 26.6 mM^{-1} cm^{-1}. One unit of enzyme activity is defined as the formation of 1 μmol product of tetraguaiacol by the enzyme catalyzing the reaction per minute at 30°C.

Superoxide Dismutase

The SOD activity was determined spectrophotometrically by measuring the ability of the enzyme to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) (Beauchamp and Fridovich, 1971). The assay mixtures contained 50 mM phosphate buffer (pH 7.8), 60 μM riboflavin, 20 mM methionine, 1 mM EDTA, and 1 mM NBT together with enzyme extract. One unit of enzyme activity was taken as the amount of enzyme that caused the 50% inhibition of NBT reduction in the light which recorded a reduction in absorbance reading at 560 nm of up to 50% compared with tubes without enzyme.

Phenylalanine Lyase

The PAL activity was estimated by referring to the method of Khan and Vaidyanathan (1986), with modifications. The estimation of PAL activity was performed based on the rate of conversion of phenylalanine to cinnamate. A reaction mixture was made by adding 1.5 ml of 50 mM Tris-HCL buffer (pH 8.3), 0.3 ml of 1 mM L-phenylalanine, 0.9 ml distilled water, and 0.3 ml of enzyme extract. Furthermore, this reaction mixture was incubated in a water bath at 30°C for 60 min. The reaction was stopped by the addition of 1 ml of 2 N HCl. The absorbance of the solution was recorded at 290 nm using a UV spectrophotometer. A unit of enzyme activity is determined by the conversion of 1 μmol L-phenylalanine to cinnamic acid per minute.

Statistical Analysis

The data of qRT-PCR and enzyme assays were analyzed using one-way ANOVA. Significant differences were analyzed using Duncan's multiple range tests ($p < 0.05$). The analyses were performed using SPSS 16.0 and Microsoft excel. Figures were prepared using OriginPro 8.5.

RESULTS

Symptoms After *Alternaria porri* Infection

Inoculation of *A. porri* spore solution on AK and AFR developed typical PB symptoms. In this study, the expected susceptible variety (AFR) developed numerous larger lesions than AK (**Figure 1**). The percent disease index (PDI) was evaluated at 3, 5, and 7 dpi in the greenhouse which was found higher in AFR compared with AK (**Supplementary Figure 1**). We sequenced the transcriptome at 5 dpi to study the differential molecular response to PB.

RNA Sequencing Data and Gene Expression in Response to *Alternaria porri*

Reads and Assembly Stat

Paired-end RNA sequencing of control and inoculated plants of both varieties was performed in duplicate using the Illumina HiSeq 2500 platform, which yielded raw reads ranging from 46659974 to 119087128 (**Supplementary Table 2**). After data filtering (q30), 97%–99% of reads were survived, and these reads were further used for the construction of *de novo* assembly using Trinity. A final mega assembly comprised a total of 122,660 non-redundant transcripts. The maximum transcript length was 15,682 bases with an average length of 640 bases. N50 value of final assembled transcripts was 1,685 bases. The GC content of the present transcriptome of onion was 42.99% (**Table 1**). RNA sequencing raw reads were submitted to the NCBI SRA database under BioProject: PRJNA796147.

Differential Gene Expression

Differential gene expression was compared between the *A. porri* infected and control leaf samples of both resistant and susceptible onion genotypes at 5 dpi. In susceptible genotype AFR, 8,064 genes were upregulated, and 248 genes were downregulated, whereas in resistant genotype AK, 832 transcripts were upregulated, and 564 were downregulated on infection by *A. porri*. A large portion of transcripts was unknown due to the non-availability of well-annotated genomic resources in onion. The top 100 significant differentially expressed transcripts were schematically represented in the heat map (**Supplementary Figure 2**). Details of differentially expressed genes, such as fold change and functional annotation, are provided in **Supplementary Material 1**.

Functional Annotation

The differentially expressed transcripts were functionally annotated using gene ontology (GO) and orthologous groups (COG) enrichment analyses. Total 188 DEGs of AK were categorized to GO terms belonging to molecular function (MF) of which 103 were upregulated transcripts and 85 were downregulated, while in AFR, 202 were upregulated, and 27 downregulated DEGs were annotated as having MF. In AK, the top three GO terms in the MF category are oxidoreductase activity [GO:0016491], monooxygenase activity [GO:0004497], and metal ion binding [GO:0046872], whereas in AFR, oxidoreductase activity [GO:0016491], metal ion binding [GO:0046872], and chitinase activity [GO:0004568] were dominantly enriched. In the cellular component (CC) category, 49 transcripts were upregulated, and 65 were downregulated in AK, whereas 103 transcripts showed upregulation, and 12 showed downregulation in AFR. GO terms in the CC

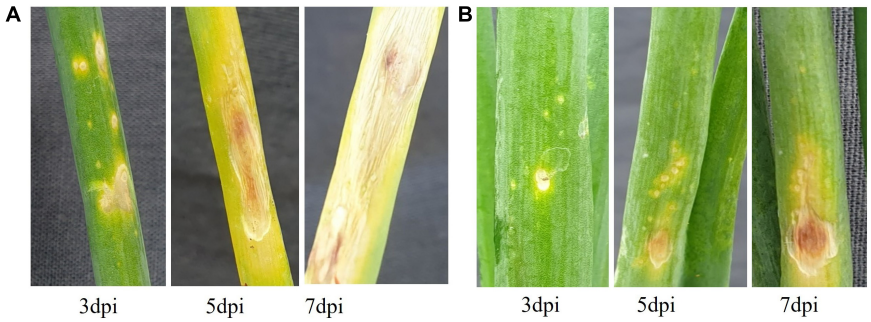

FIGURE 1 | Purple blotch (PB) symptom development in onion after pathogen inoculation; **(A)** Agrifound rose (AFR) and **(B)** Arka Kalyan (AK).

category such as an integral component of the membrane [GO:0016021], chloroplast thylakoid membrane [GO:0009535], and cell wall [GO:0005618], were dominantly enriched in AK. In AFR, an integral component of the membrane [GO:0016021], extracellular region [GO:0005576], and chloroplast thylakoid membrane [GO:0009535] were the top three GO terms upregulated in the CC category. The biological process (BP) category of GO annotation analyses revealed 57 upregulated and 64 downregulated GO terms in AK and 127 upregulated and 16 downregulated GO terms in AFR. GO terms, such as pectin catabolic process [GO:0045490], photorespiration [GO:0009853], and protein-chromophore linkage [GO:0018298], were overrepresented in BP category in AK, whereas GO terms, such as carbohydrate metabolic process [GO:0005975], cell wall macromolecule catabolic process [GO:0016998], and chitin catabolic process [GO:0006032], were highly enriched in AFR.

Gene ontology terms involved in plant defense response were also overrepresented in the MF category such as hydrolase activity, hydrolyzing O-glycosyl compounds [GO:0004553], enzyme inhibitor activity [GO:0004857], and chitin-binding [GO:0008061]. In the BP category, cell wall modification [GO:0042545], glutathione metabolic process [GO:0006749], and defense response [GO:0006952] are also enriched in present data in response to PB disease in onion genotypes. The top ten GO terms in each category are shown in **Figure 2**.

The analyses of the distribution of clusters of COG in DEGs showed that the majority of genes are involved in energy production and conversion, transcription, carbohydrate transport and metabolism, posttranslational modification and protein turnover, signal transduction, cell wall biogenesis, and defense mechanism, which are involved in onion genotypes after *A. porri* infection (**Figure 3**). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway studies demonstrated that most DEGs are involved in glycan metabolism, carbohydrate degradation, lipid metabolism, and alkaloid biosynthesis pathways.

Transcripts of DEGs were blasted against TFDB, and several transcription factors were identified from the present *RNAseq* data. Among them, NAC, ERF, MYB, bHLH, MYB-related, and WRKYs were found to be dominant, and their differential expression in response to PB disease in onion reprogrammed the overall pattern of gene expression in disease state (**Supplementary Figure 4**).

Furthermore, DEGs were examined for species distribution and found that majority of annotated transcripts of AK were matched with *Elaeis guineensis* (23.05%), followed by *Asparagus officinalis* (9.38%) and *Allium cepa* (5.47%). In AFR, the majority of annotated transcripts showed homology with *E. guineensis* (21.32%) followed by *A. officinalis* (12.54%) and *Allium sativum* (5.64%). The top ten species' distribution of DEGs in both genotypes is presented in **Supplementary Figure 3**.

Transcripts of both AK and AFR were blasted against PRGdb, and we found that 73 transcripts showed homology with reference pathogen receptor genes (PRGs) (**Supplementary Figure 3**). Different PRGs were differentially expressed in AK and AFR. Hm2, Serk3A, PBS1, Pid2, and Ve2 were top upregulated PRGs in AFR, while Serk3A, Mlo, PBS1, and Pid2 were top PRGs in AK. Details of PRGs with rgene-id are given in **Supplementary Material 2** and **Supplementary Figure 5**.

TABLE 1 | Assembly statistics of onion transcriptome in response to purple blotch (PB).

Parameters	Mega-assembly
Total No. of transcripts	122660
Length of transcriptome (Mb)	78536108
Max transcript length (bases)	15682
Average transcript length (bases)	640.27
Median transcript length (bases)	286
N50 length (bases)	1685
% GC	42.99

Defense-Related Differentially Expressed Genes in Response to Purple Blotch in Onion

Pathogenesis-Related Proteins in Response to *Alternaria porri*

A total of seven classes of pathogenesis-related (PR) proteins were found to be differentially expressed in onion genotypes with response to PB infection. Expression of *PR-1* (*Antifungal*), *PR-2* (β-*1,3-Glucanase*), *PR-3* (*Chitinases*), *PR-4* (*Chitinases types I, II*), *PR-5* (*Thaumatin-like*), *PR-9* (*Peroxidase*), and *PR-10* (*Ribonuclease-like*) was upregulated by several-fold on *A. porri*

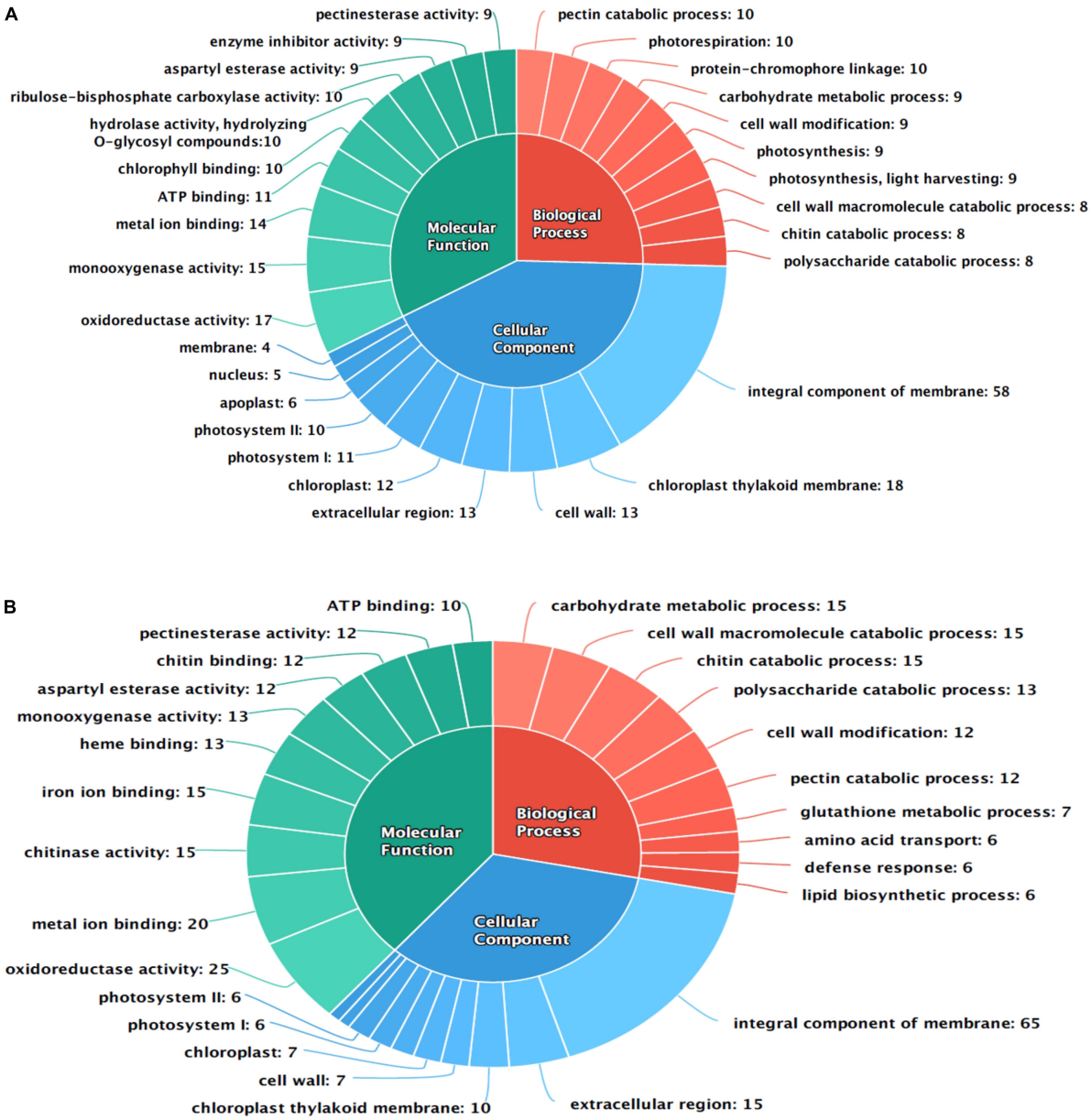

FIGURE 2 | Functional annotation of differentially expressed transcripts in response to PB in onion using gene ontology (GO); **(A)** AK and **(B)** AFR.

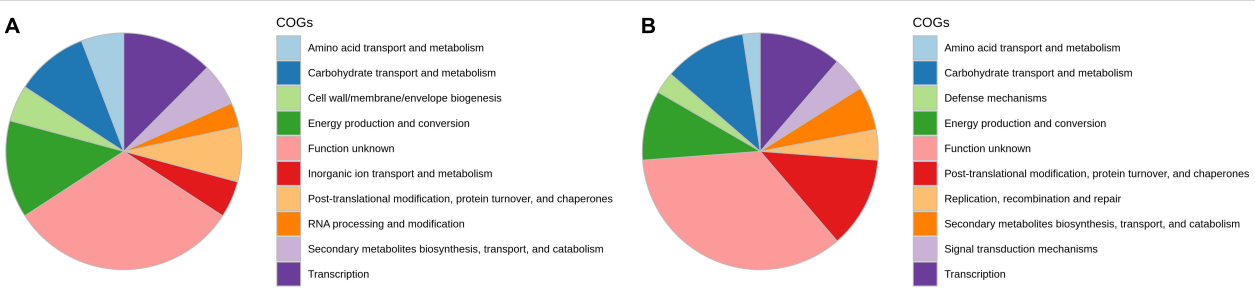

FIGURE 3 | Functional annotation of differentially expressed transcripts in response to PB in onion using orthologous groups (COG) categories; **(A)** AK and **(B)** AFR.

infection in onion. Transcripts for PR-1 were upregulated by 3.6–5.7-fold, PR-2 by 3.7–6.7-fold, PR-3 by 3–8-fold, PR-4 by 5.2–9.2-fold, PR-5 by 3.7-fold, PR-9 by 7.2–13.1-fold, and PR-10 by 4.7–8.1-fold. Among these, PR-5 was only upregulated in resistant genotype AK.

Receptor-Like Kinases in Response to *Alternaria porri*

Receptor-like serine/threonine-protein kinase, *protein kinase domain-containing protein*, *wall-associated receptor kinase-like*, *brassinosteroid LRR receptor kinase*, *CBL-interacting serine/threonine-protein kinase*, and *calcium-dependent protein kinase* were upregulated by 4.8, 3.8, 3.3, 7.8, 5.3, and 2.8 old, respectively, in AK. *Salt tolerance receptor-like cytoplasmic kinase* 1 (6.8-fold), *receptor-like serine/threonine-protein kinase* (6.7-fold), *protein kinase* domain-containing protein (4.1-fold), *brassinosteroid LRR receptor kinase* (6.2-fold), *CBL-interacting serine/threonine-protein kinase* (11.7-fold), and *calcium-dependent protein kinase* (3.4-fold) showed increased expression in AFR.

Genes for Phytohormones in Response to *Alternaria porri*

Genes involved in the phytohormone biosynthesis process were differentially expressed onion genotypes under PB infection. Transcripts for ethylene synthesis, such as *1-aminocyclopropane-1-carboxylic acid synthase* (*ACS*), and *1-aminocyclopropane-1-carboxylic acid oxidase* (*ACO*), were upregulated by 4.7–7.4-fold and 3.6–7.2-fold in present studies. Similarly, transcripts for key ABA biosynthetic gene *9-cis-epoxycarotenoid dioxygenase* (*NCED*) were upregulated by 4.6–6.2-fold in response to *A. porri* infection in onion. *Linoleate 9S-lipoxygenase*, a jasmonic acid marker gene, was upregulated 7.3-fold in AK and 4.5-fold in AFR. Furthermore, *12-oxophytodienoate reductase*, which is involved in jasmonic acid signaling and oxylipin biosynthesis, was overexpressed by 8.6-fold in AK and 3.5-fold in AFR. Transcripts for *Phospholipase A* also showed 5.3-fold increased expression in AK. Transcripts for *auxin-responsive protein* (−5- to −11-fold) and *auxin transporter* (−4.3-fold) were downregulated in AK, whereas *auxin-related protein 2* and *auxin-response protein* were found to be upregulated by 7.4- and 7.6-fold, respectively, in AFR. In addition, several ethylene response factors (ERFs) were also expressed differentially in onion in response to PB infection.

Cell Wall Integrity Genes in Response to *Alternaria porri*

Genes involved in cell wall integrity, such as *pectin methylesterase inhibitors* (*PMEI*) and *polygalacturonase inhibitor proteins* (*PGIPs*), were found to be differentially expressed in onion genotypes under biotic stress. PMEI and PGIP were upregulated by 4.6- and 9.8-fold, respectively, in AK, whereas in AFR, PGIP was upregulated by 10.8-fold. The expression of *xyloglucan endotransglucosylase/hydrolase* with a function in cell wall biogenesis [GO:0042546], cell wall organization [GO:0071555], and the xyloglucan metabolic process [GO:0010411] was only increased in AK. *Pectate lyase* with polygalacturonase activity [GO:0004650] and pectin-catabolic process [GO:0045490] was downregulated by −7.8-fold in AK, while it was upregulated by 4.7-fold in AFR. In addition, transcripts for *pectinesterase* were also showed a 5–12-fold increased expression in both the genotypes.

Cytochrome P450 Monooxygenases in Response to *Alternaria porri*

Several CYPs were differentially expressed in the present *RNAseq* dataset of onion under biotic stress imposed by PB. Transcript levels of *CYP81, CYP81E, CYP86A, CYP89A2, CYP71A1, CYP71A9, CYP736A12, CYP709B2, CYP79A*, and *CYP85A1* were found to be elevated. Among these, *CYP81* and *CYP85A1* were found only in AK, whereas *CYP86A* and *CYP81E* were found to be upregulated only in AFR.

Other Defense-Related Genes

Glutathione S-transferase is an important gene in plant defense in biotic and abiotic stress response, and transcripts for this gene were observed to be increased in AK by 3.2–9.9-fold and in AFR by 3.3–14.5-fold, respectively. Other important defense genes, namely, *E3 ubiquitin-protein ligase*, *hypersensitive-induced response protein 1*, and *BTB/POZ domain-containing protein*, were upregulated by 9. 1-, 3. 8-, 6.5-fold in AK and 7. 5-, 6. 1-, 4.8-fold in AFR, respectively. *GABA transporter1* (*GAT1*) and the *ankyrin repeat domain-containing protein* were highly upregulated by 11.8- and 6-fold only in AK. A few proteases and peptidases were also expressed differentially in onion on PB infection. Thiol protease, aspartic protease, carboxypeptidase, serine carboxypeptidase, and metacaspase were upregulated in both genotypes.

Transcription Factors in Response to *Alternaria porri*

A large number of transcription factors have been expressed differentially in onions in response to biotic stress imposed owing to PB infection. ERF, WRKY, MYB, and NAC are the major TFs that are upregulated in the current *RNAseq* dataset. They are known to play a key role in the plant defense response against abiotic and biotic stress elements by reprogramming the gene expression pattern in the cell. In this study, *ERF1, ERF2, ERF14, ERF96, NAC62, NAC42, NAC47,* and *NAC7* and several other defense-related transcription factors were found to be upregulated.

Metabolism Related Genes in Response to *Alternaria porri*

Transcripts involved in flavonoid biosynthesis were also expressed differentially on PB infection in onion. *Flavonoid glucosyltransferase* and *flavonoid 3′-hydroxylase* were upregulated by 4.1- and 4.5-fold, respectively, in AK, while *flavonoid 3′-hydroxylase* was upregulated 5.8-fold in AFR. *Anthocyanidin synthase* and *Chalcone synthase* were downregulated by −4.4 and −5.6-fold in AK and AFR, respectively.

Several transcripts for genes in carbohydrate metabolism were expressed differentially on *A. porri* infection in onion genotypes. *Glycosyltransferase* (8.2-fold), *sucrose synthase* (4.4-fold), and *xylose isomerase* (3.1-fold) were overexpressed in AK after PB infection. Similarly, in AFR, *glycosyltransferase* (11-fold), *sucrose phosphate synthase* (6.3-fold), *Fructokinase* 1 (4.1-fold), and *Invertase* (3.2-fold) showed upregulation

due to *A. porri* infection. *Glyceraldehyde -3-phosphate dehydrogenase* was downregulated by 3.2–4.3-fold in both onion genotypes in this study. Transcript for *Beta-glucosidase 23* with functional annotation negative regulation of the defense response [GO:0031348] was upregulated in AFR.

Amino acid transporters also showed differential expression in onions in response to PB disease. Transcripts for amino acid transporter protein (6.5-fold) and amino acid transporter domain-containing proteins (4.7 to 8.5-fold) upregulated in AK. Similarly, amino acid transporter protein (6.7-fold) and amino acid transporter domain-containing proteins (6–10-fold) showed upregulation in AFR.

Validation by Quantitative Realtime-PCR

For validation of transcriptome, we selected 15 differentially expressed genes that play an important role in the plant defense response against diseases. These selected genes code for PR proteins and antioxidants, such as *PR1, PR3, PR4*, *PR5, and PR9,* and glutathione-S-transferase. Some of them are also involved in the synthesis of phytohormones, such as *ACS, NCED,* and *LOX*, and few of them code for transcription factors such as *MYB, ERF,* and *NAC*. Furthermore, genes for protein-containing *BTP/POZ domain, ankyrin repeat domain,* and *PGIP* were also validated using real-time qPCR (**Figure 4**). There was a good correlation (R^2 = 0.87) between the levels of expression of genes in *RNAseq* and qPCR assays, which ascertain the reliability and quality of present transcriptome analysis (**Supplementary Figure 6**).

Antioxidant Enzyme Assays

Biochemical changes associated with PB infection were examined for the activity of antioxidant enzymes in the control and infected resistant and susceptible onion genotypes at 5 dpi. It was observed that infection with *A. porri* significantly increased the activity of all antioxidant enzymes examined. The activity of APX, GPX, and PAL was found significantly higher in the resistant genotype AK, whereas CAT and SOD activity was higher in the susceptible counterpart AFR. The catalase activity in infected AFR and AK was 2.8-fold and 2-fold higher than their respective controls, while the activity of ascorbate peroxidase was higher by 1.8 and 3.5-fold in AFR and AK, respectively. The guaiacol peroxidase activity was increased by 2.3-fold in AFR and 2.6-fold in AK after *A. porri* infection. The SOD activity was increased by 3.2-fold in AFR and 2.4-fold in AK after infection. The PAL assay showed a higher increase in activity in AK (2.4-fold) than in AFR (2.1-fold), while there is no significant change in activity that was observed in controls of both the genotypes (**Figure 5**).

DISCUSSION

Onion is an economically important crop in the world, and PB is one of the most devastating diseases of this crop. There is limited information available on the molecular response of onion to PB. *RNAseq* is one of the efficient advanced approaches for studying the molecular mechanism behind plant disease response. The main aim of this study is the transcriptome profiling of PB-resistant and susceptible genotypes.

Pathogenesis-Related Proteins

Pathogenesis-related (PR) proteins were synthesized by a plant in adverse conditions owing to biotic or abiotic stress elements and are a vital component of plant defense response. They are involved in HR or SAR against pathogen infection and are regulated by the complex panoply of signaling pathways. They are classified into 17 families along with a putative new PR-18 group, which comprises fungus and SA-inducible carbohydrate oxidases (Custers et al., 2004; Jain and Khurana, 2018). In this investigation, PR proteins belonging to seven families (PR-1, PR-2, PR-3, PR-4, PR-5, PR-9, and PR-10) were showed upregulation in onion in response to PB disease. PR-4 proteins are comprised of chitinases and also have ribonuclease activity, and these accumulate in response to a pathogen attack or wounding. Bravo et al. (2003) demonstrated that the fungal elicitors, wound, exogenous ABA, and methyl jasmonate treatment resulted in the production of *ZmPR4* in maize. Recently, sugarcane PR-4 was reported to be involved in fungal cell death by virtue of ribonuclease, chitosanase, and chitinase action (Franco et al., 2019). Our previous study reported upregulation of chitinase gene in response to *Stemphylium* blight in onion caused due to a necrotrophic pathogen *Stemphylium vesicarium* (Roylawar et al., 2021). PR-5 (thaumatin-like) imparted fungal disease resistance in transgenic tobacco (Rajam et al., 2007). In addition, it has also been reported that PR1, PR2, PR4, and PR5 are induced in garlic after infection by *Fusarium* (Rout et al., 2016; Chand et al., 2017; Anisimova et al., 2021). PR-9 is comprised of peroxidase and is well known for its role in plant defense as a potent antioxidant in ROS scavenging. These peroxidases were upregulated in apples by the infection of *Alternaria* blotch caused by *Alternaria alternata* (Zhang et al., 2015). Thus, higher expression of PR proteins suggests the development of systemic acquired resistance against *A. porri* in onion.

Transcription Factors

Few ERF transcription factors were upregulated in both genotypes under pathogen attack. ERF1 is known to be induced by phytohormones such as jasmonate and ethylene and imparts resistance against necrotrophic pathogens in *Arabidopsis* (Lorenzo et al., 2003; Berrocal-Lobo and Molina, 2004). Constitutive expression of *ERF1* and *ERF2* activates the pathogen-inducible plant defensin 1.2 (PDF1.2) gene (Maruyama et al., 2013). Similarly, the expression of JA/ET defense genes, such as *PDF1.2a, PR-3,* and *PR-4*, was upregulated by *ERF96* and positively regulates the resistance to necrotrophic fungi, which indicates its importance in ERF regulatory network (Catinot et al., 2015). *ERF14* is also known to play a key role in defense against *Fusarium oxysporum* in *Arabidopsis*, and the expression of other ERFs also depends on the expression of *ERF14.* Further *PDF1.2* and *Chitinase* expression levels were also increased in *ATERF14* overexpression lines, which suggests its prominent role in plant defense (Oñate-Sánchez et al., 2007). Thus, these ERFs might play a role *via* JA/ET signaling in PB disease response in onion. Several WRKY transcription factors

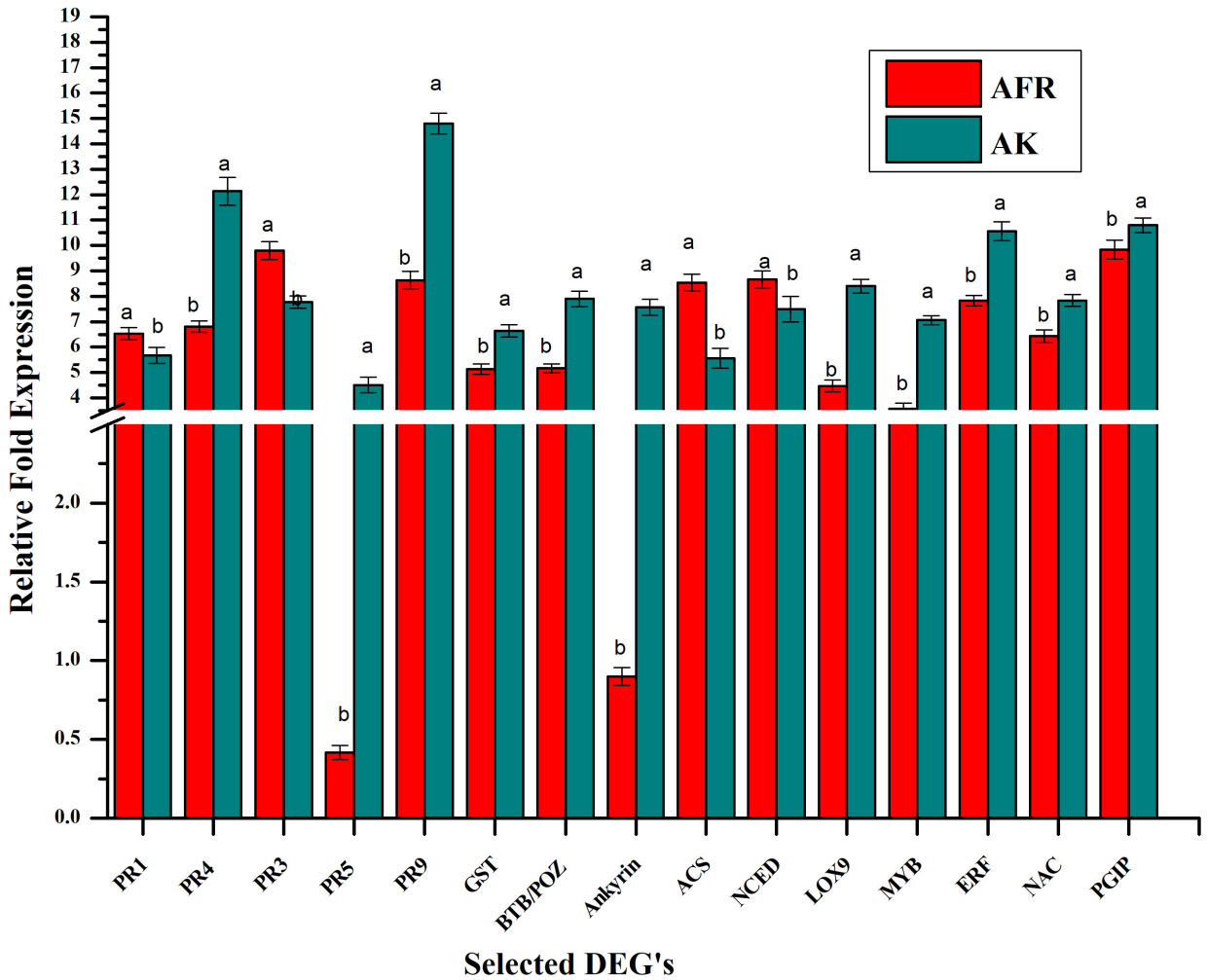

FIGURE 4 | Validation selected differentially expressed genes by relative qPCR analysis in AK and AFR at 5 dpi. Data refer to the most representative of two independent repeated experiments. Each reaction was performed in triplicate, and values represent the average of these three technical replicates. Error bar showed the standard error of the mean, and different letters suggest statistically significant differences as per Duncan's multiple range test ($p < 0.05$).

were differentially expressed in response to *A. porri* infection in onion. *WRKY6* was reported to be a positive regulator for plant resistance to necrotrophic pathogen *via* JA, ET, ABA-induced gene expression, ROS accumulation, and upregulation of PR genes against *Phytophthora infestans* and *Ralstonia solanacearum* (Cai et al., 2015; Hong et al., 2018). Furthermore, *WRKY65* was reported to play a key role in imparting resistance against *Alternaria tenuissima* in herbaceous peony by regulating JA and SA levels (Wang et al., 2020). *MdWRKY75* was reported to increase the resistance to *A. alternata* in apples primarily by jasmonic acid signaling and antioxidant enzymes (Hou et al., 2021). These WRKY TFs are also upregulated in onion in response to PB infection and might be performing a similar function in onion also. Several NAC transcription factors were also upregulated in this study. *NAC* TFs were reported to be involved in the induction of plant defense against *Alternaria brassicicola* in *Arabidopsis* and *Brassica* (Saga et al., 2012; Mondal et al., 2020). NAC7 was reported to be a negative regulator of senescence and stay green trait in maize. RNAi lines of maize exhibited delayed senescence as well as an increase in total biomass (Mondal et al., 2020). In this study, NAC7 was highly upregulated in susceptible genotype (AFR) which might be due to higher senescence of leaves on PB infection. Similarly, several other transcription factors, such as MYB, bZIP, and C2H2, were also differentially expressed under *A. porri* infection in onion genotypes under study. They showed differential expression in other crops also in response to disease and plays important role in plant immunity (Verma et al., 2017; Yuan et al., 2019).

Phytohormones

Phytohormones, such as jasmonic acid, salicylic acid, abscisic acid, and ethylene, play a key role in plant responses to biotic and abiotic stresses. In this study, genes in jasmonic acid and ethylene biosynthesis were highly upregulated in response to *A. porri* infection. JA and ET are mainly involved

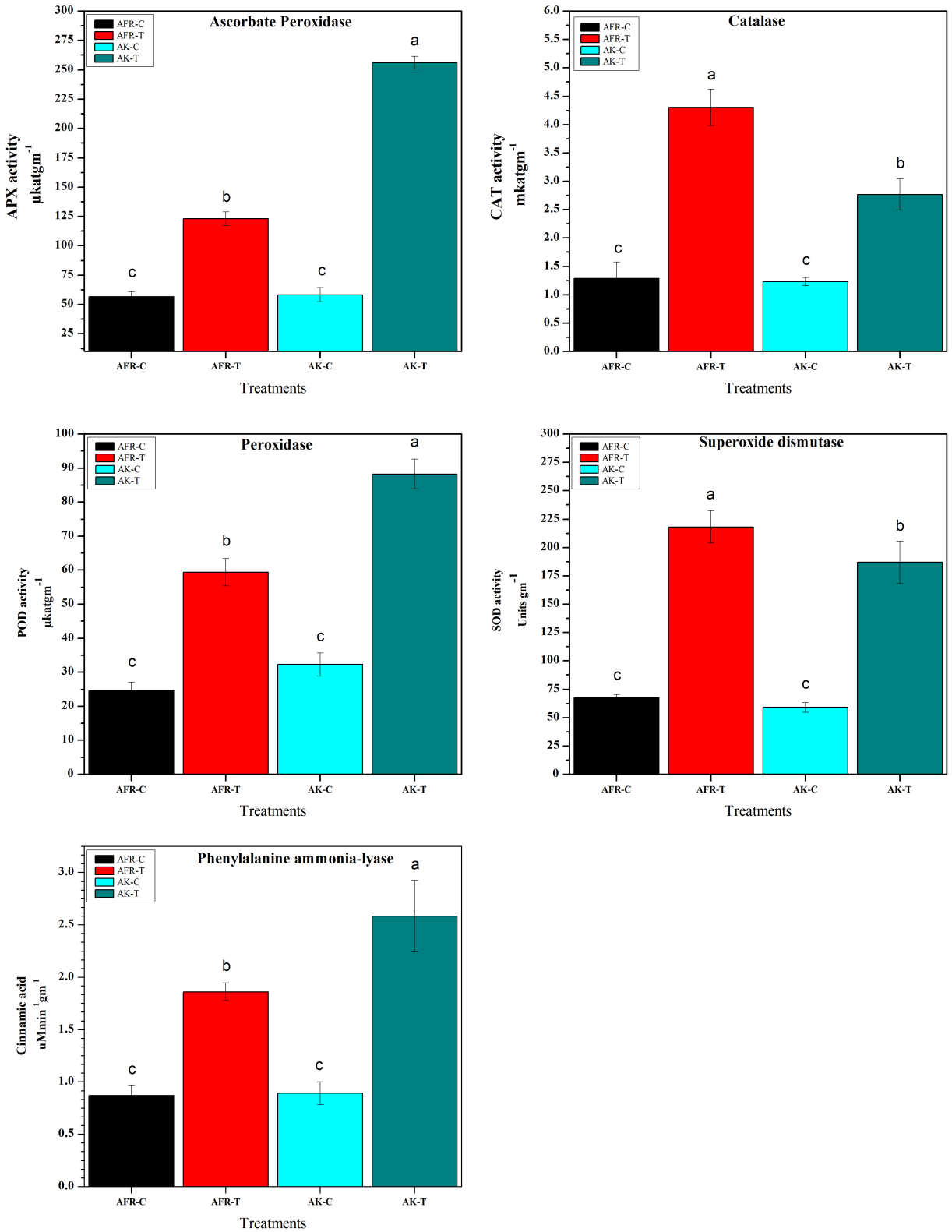

FIGURE 5 | Effects of *Alternaria porri* infection on resistant and susceptible onion antioxidant and defense enzymes at 5 dpi. Data refer to the most representative of two independent repeated experiments. Values are expressed as the average of three biological replicates, each consisting of three plants pooled together. Error bar showed the standard error of the mean, and different letters suggest statistically significant differences as per Duncan's multiple range test ($p < 0.05$).

in defense against necrotrophic pathogens, while SA acts against biotrophic pathogens (Dong, 1998; Glazebrook, 2005; Zhang et al., 2018). *Acyl-CoA oxidase, 12-oxophytodienoate reductase, lipoxygenase,* and *3-ketoacyl-CoA thiolase* transcripts in jasmonic acid biosynthesis were also reported to be upregulated in response to other fungal diseases such as Bakanae disease in rice (Matić et al., 2016), *Fusarium* head blight of wheat (Pan et al., 2018), *Fusarium* wilt of Flax (Galindo-González and Deyholos, 2016), and *Sclerotium* stem rot in peanut (Bosamia et al., 2020). Ethylene is also an important phytohormone that acts hand in hand with JA to fight necrotrophic diseases. Key transcripts in ethylene biosynthesis, namely, ACS and ACO, were highly expressed in onion in response to *A. porri* infection. Transgenic rice with inducible ACS expression exhibited enhanced resistance to *Magnaporthe oryzae* and *Rhizoctonia solani* (Helliwell et al., 2013). Further *RNAseq* studies also found the upregulation of ET synthetic genes in response to the necrotrophic fungal pathogen (De Cremer et al., 2013; Matić et al., 2016; Pan et al., 2018). JA and ET modulate the expression of PR genes through a signaling network comprising various transcription factors and metabolites (Pieterse et al., 2012). *NCED,* a key gene in ABA biosynthesis, was also upregulated in onion genotypes due to infection of *A. porri.* ABA is known to regulate the various processes in plant-pathogen interactions (Fan et al., 2009). It plays an effective role in imparting disease resistance in plants against necrotrophic fungi (Ton and Mauch-Mani, 2004; Boba et al., 2020). PR1 and GST are the marker genes for the salicylic acid and were reported to be induced in the present dataset with response to PB in onion. GST's main role is already known as the removal of toxic compounds and acts as an antioxidant. Upregulation of GST was reported in several plants after infection by necrotrophic pathogens such as *Alternaria brassicicola* (Schenk et al., 2000).

Cell Wall Integrity Genes

The cell wall is the first physical barrier to the pathogen, and numerous changes occur in the cell wall in response to the pathogen (Malinovsky et al., 2014). Necrotrophic fungal pathogens degrade the cell wall matrix with the help of the secretion of different enzymes. The plant maintains cell wall integrity by inhibiting the cell wall degrading enzymes by the expression of different enzymes such as PMEI and PGIPs. The genes for these enzymes were highly upregulated in onion in response to PB pathogen. In *Arabidopsis*, it was found that PMEI helps in maintaining cell wall integrity by inhibiting the pectin methylesterase which imparted resistance against botrytis (Lionetti et al., 2017). PGIPs protect the cell wall from pathogen, and insects attack by inhibiting the depolymerization of pectin in the cell wall by polygalacturonases (Kalunke et al., 2015). PGIPs not only protect pectin from degradation but also lead to the production of longer oligogalacturonides that can be recognized as damage-associated molecular patterns that ultimately activate PTI and slow down the colonization of pathogen (Federici et al., 2006). These genes might also contribute toward defense response against PB in onion.

Defense-Related Genes Exclusively Expressed in Arka Kalyan

A few genes were exclusively showed upregulation in a resistant genotype (AK), PR5, ankyrin repeat domains, GABA transporter, *CYP85A1*, etc. PR5 was reported to induce phytohormones and biotic and abiotic stresses in garlic. Its ectopic expression in *Arabidopsis* increased resistance to *Botrytis* by constitutive expression of defense-related genes, which suggests its broad role in plant defense (Rout et al., 2016). Similarly, ankyrin repeat domains are widely distributed and well-studied protein–protein interaction domains in several processes in plants including response biotic and abiotic stresses. In rice, ankyrin repeat-containing protein involved in providing defense against *Magnaporthe oryzae*, and overexpression lines showed higher expression of SA and JA responsive genes (Mou et al., 2013). Similarly, the expression of ankyrin repeat protein, *GmARP1*, imparted resistance in transgenic soybean against *Fusarium virguliforme* (Ngaki et al., 2016). CYPs are known to play an important role in biotic and abiotic stress response in plants by modulating levels of antioxidant molecules, phytohormones, and other metabolites (Pandian et al., 2020). *CYP85A1* is reported to be involved in brassinosteroid biosynthesis, and its overexpression in tobacco increased tolerance to *Phytophthora nicotianae* in transgenic plants by elevating brassinosteroid level and modulation of phytohormone and defense enzyme activities (Duan and Song, 2019). This suggests that *CYP85A1* might play a role in defense response against pathogen. GABA accumulation after abiotic or biotic stress is reported in several plants (Rashmi et al., 2018), and it might help in enhancing host immunity against fungal pathogens by modulating oxidative enzymes (Shelp et al., 2021). GABA transporter 1 (*GAT1*) involved in GABA influx into the cell (Batushansky et al., 2015). It suggests GABA's important role in plant defense response.

Metabolism-Related Genes in Response to *Alternaria porri* Infection

Secondary metabolites play an important role in plant defense against biotic and abiotic stresses by their antioxidant and antibacterial properties. Onions are a rich source of secondary metabolites such as flavonoids (Khandagale and Gawande, 2019). *Flavonoid glucosyl-transferase* is involved in the glycosylation of these flavonoids. They are known to regulate quercetin and kaempferol levels in plants and govern the plant defense against the pathogen (Campos et al., 2019). *Flavonoid 3′-hydroxylase*, another gene in the flavonoid pathway, was reported to be involved in the fungal pathogen-induced production of phytoalexins (Boddu et al., 2004). Glycosyltransferase is one of the players in JA-SA cross-talk during defense response signaling in plants. The overexpression of *UGT76B1* in *Arabidopsis* increased the resistance against a necrotrophic pathogen; *Alternaria brassicicola* also decreased the senescence with upregulation of JA dependent pathway. On the contrary, silencing of this gene led to reduced tolerance to *A. brassicicola*, induction of SA pathway, and elevated resistance to the biotrophic pathogen (von Saint Paul et al., 2011). Sugars and carbohydrates are the prime sources of energy. Several

carbohydrate metabolisms and energy production genes showed differential expression in this study such as sucrose synthase, sucrose phosphate synthase, and invertase. These are reported to play role in defense response by interaction with phytohormones signaling in plants (Morkunas and Ratajczak, 2014; Tauzin and Giardina, 2014). The upregulation of these genes suggests that they might be playing such a role in defense response against PB in onion as well.

Pathogen Receptor Genes From PRGdb

PRGdb is a database of PRGs that provide constant updates on plant resistance genes (Calle García et al., 2021). In this analysis, we found 73 transcripts having homology with reference PRGs in PRGdb. *Hm1 and Hm2* code for the NADPH-dependent HC-toxin reductases which were reported to protect maize from the toxin produced by a fungal pathogen (Dehury et al., 2014). Serk3a belongs to somatic embryogenesis receptor kinases and is involved in defense response. Loss of function mutants for Serk3a was unable to induce defense response against *P. infestans* in tobacco (Chaparro-Garcia et al., 2011) and also did not exhibit any defense response in potato after oligopeptide Pep-13 treatment (Nietzschmann et al., 2019). PBS1 is a member of receptor-like cytoplasmic kinases and is involved in the pattern triggered defense response in plants (Swiderski and Innes, 2001; Tang et al., 2017). Pid2 is a transmembrane receptor-like kinase that plays role in a rice blast resistance (Sharma et al., 2016). It was reported that E3 ubiquitin ligase and Pid2 interaction involved in the regulation of cell death and immunity against rice blast disease (Wang et al., 2015). Another PRG, Ve2, was found to impart resistance to wilt caused by *Verticillium* (Chen et al., 2017). These pathogen receptor genes might also play a key role in imparting immunity against PB in onion. These PRGs need to be investigated further for a better understanding of plant-pathogen interaction in onion.

Antioxidant and Defense Enzymes

Biotic stress led to the generation of ROS in plants which help to fight pathogen *via* HR, and PCD (Apel and Hirt, 2004; Roylawar et al., 2021) and ROS also act as secondary messengers in signaling defense response (Yan et al., 2007; Hasanuzzaman et al., 2020). In this study, activities of antioxidant and defense enzymes, such as superoxide dismutase, catalase, ascorbate peroxidase, guaiacol peroxidase, and phenyl ammonia lyse, were increased in *A. porri* infected onion genotypes over control plants. GPX protects cells by neutralizing ROS radicals and functions as an antifungal agent in plant disease response (Chavan et al., 2013; Pieczul et al., 2020). GPX is also involved in lignin synthesis which acts as a barrier to pathogen spread in infected tissues (Tayefi-Nasrabadi et al., 2011; Roylawar et al., 2021). APX and CAT both play important roles in scavenging hydrogen peroxide. CAT plays a key role in the detoxification of H_2O_2 generated in peroxisome during pathogen attacks (Meena et al., 2016; Roylawar et al., 2021). Excessive H_2O_2 is scavenged by the ascorbate-glutathione cycle involving APX and was reported that APX activity, as well as gene expression level, gets elevated during pathogen infection (Meena et al., 2016). SOD performs dismutation of highly toxic oxygen radicals to oxygen and comparatively less toxic hydrogen peroxide. SOD activity is often increased in various biotic and abiotic stress situations, and higher activity is correlated with the resistance to the oxidative stress caused by abiotic stress as well as pathogens (Ehsani-Moghaddam et al., 2006; Gill and Tuteja, 2010; Youssef et al., 2020). These enzyme activities were also reported to be modulated by phytohormones (Szöke et al., 2021). Thus, in this study, in onion, reprograming of these genes led to the activation of antioxidant enzymes in response to PB.

CONCLUSION

The present *RNAseq* analysis discovered several DEGs in onion in response to PB disease. Functional annotation analysis by GO and COG revealed that a large number of genes in several BPs including defense-related terms were enriched. This suggests that a large number of genes play role in PB response in onions. Several pathogen recognition genes were also discovered from PRGdb analysis. Defense-related genes, such as PR proteins, antioxidants, phytohormones biosynthesis and signaling, cell wall integrity, and transcription factors, were found to be induced in PB disease in onion. Antioxidant enzymes were found to play a key role in PB resistance in onion. Further investigation is required to identify key candidate genes necessary for imparting PB resistance using genetic engineering. This is the first report of transcriptome analysis of onion in response to PB, and thus, data generated in the present investigation will help researchers for further research in PB-onion interaction.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: National Center for Biotechnology Information (NCBI) BioProject database under accession number PRJNA796147.

AUTHOR CONTRIBUTIONS

KK and SG conceived idea and designed the study. KK and PR performed experiment and statistical analysis. OK, AK, and KK contributed in bioinformatics analysis and visualizations. PK assisted in performing the experiment. AA, MS, and SG provided resources. KK, OK, PR, and PK wrote the manuscript. All authors played role in revising manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.857306/full#supplementary-material

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Multiple Stressors in Vegetable Production: Insights for Trait-Based Crop Improvement in Cucurbits

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Vegetable production is a key determinant of contribution from the agricultural sector toward national Gross Domestic Product in a country like India, the second largest producer of fresh vegetables in the world. This calls for a careful scrutiny of the threats to vegetable farming in the event of climate extremes, environmental degradation and incidence of plant pests/diseases. Cucurbits are a vast group of vegetables grown almost throughout the world, which contribute to the daily diet on a global scale. Increasing food supply to cater to the ever-increasing world population, calls for intensive, off-season and year-round cultivation of cucurbits. Current situation predisposes these crops to a multitude of stressors, often simultaneously, under field conditions. This scenario warrants a systematic understanding of the different stress specific traits/mechanisms/pathways and their crosstalk that have been examined in cucurbits and identification of gaps and formulation of perspectives on prospective research directions. The careful dissection of plant responses under specific production environments will help in trait identification for genotype selection, germplasm screens to identify superior donors or for direct genetic manipulation by modern tools for crop improvement. Cucurbits exhibit a wide range of acclimatory responses to both biotic and abiotic stresses, among which a few like morphological characters like waxiness of cuticle; primary and secondary metabolic adjustments; membrane thermostability, osmoregulation and, protein and reactive oxygen species homeostasis and turnover contributing to cellular tolerance, appear to be common and involved in cross talk under combinatorial stress exposures. This is assumed to have profound influence in triggering system level acclimation responses that safeguard growth and metabolism. The possible strategies attempted such as grafting initiatives, molecular breeding, novel genetic manipulation avenues like gene editing and ameliorative stress mitigation approaches, have paved way to unravel the prospects for combined stress tolerance. The advent of next generation sequencing technologies and big data management of the omics output generated have added to the mettle of such emanated concepts and ideas. In this review, we attempt to compile the progress made in deciphering the biotic and abiotic stress responses of cucurbits and their associated traits, both individually and in combination.

Keywords: cucurbits, stress tolerance, biotic stress, abiotic stress, metabolic pathways breeding, grafting, mitigation

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VEGETABLE PRODUCTION: THE CUCURBIT CONTEXT

The vegetable crops belonging to the family Cucurbitaceae are known as cucurbits or gourds. This important family of vegetables contains 950 species in over 90 genera and is mainly distributed in the tropics and subtropics (Schaefer and Renner, 2011). Cucurbit family includes several genera and represents the largest tropical vegetable group (Roy and Chakrabarti, 2003; Ebert et al., 2021), as summarized in **Table 1**

Wide variability is observed in the genetic makeup of the members with monoploid chromosome number ranging from seven (*Cucumis sativus*) to twenty (*Cucurbita* spp.) (Rai et al., 2007; Choudhary et al., 2016; Samadia and Haldhar, 2017). A great variability also exists in the utilization of these crops, *viz.,* salads (cucumber, gherkins, long melon), sweet dishes (ash gourd, pointed gourd), pickles (gherkins), and desserts (melons). Cucurbit seeds are also high in oil and protein content attesting to their nutritive value (Rahman et al., 2008; Choudhary et al., 2015; Bhargava et al., 2016).

Despite their wide adaptability and varied uses in different parts of the world, their commercial cultivation is increasingly facing the threats of climate change and consequent biotic and abiotic stresses as well as genetic erosion. It is imperative that a holistic approach on the scientific management of various factors affecting the crop performance including development of stress tolerant types, manipulation of metabolic pathways for tolerance/resistance and their crosstalks, as well as propagation strategies for withstanding biotic and abiotic stresses be adopted for successful cucurbit production.

TABLE 1 | Diverse genera of family Cucurbitaceae.

Genera	Common name	Scientific name
Cucumis	Cucumber	*Cucumis sativus L.*
	Muskmelon or Cantaloupe	*Cucumis melo* L.
Citrullus	Watermelon	*Citrullus lanatus* subsp. *vulgaris* (Schrad.) Fursa
Cucurbita	Winter squash	*Cucurbita maxima* Duchesne
	Summer squash	*Cucurbita pepo* L.
	Pumpkin	*Cucurbita moschata* Duchesne
Benincasa	Wax or Ash gourd	*Benincasa hispida* (Thunb.) Cogn.
Lagenaria	Bottle gourd	*Lagenaria siceraria* (Molina) Standl.
Luffa	Ridge gourd	*Luffa acutangula* (L.) Roxb.
	Sponge gourd	*Luffa aegyptiaca* Mill.
Momordica	Bitter gourd	*Momordica charantia* L.
	Spiny gourd	*Momordica dioica* Roxb. ex Willd
Coccinia	Ivy gourd	*Coccinia grandis* (L.) Voigt
Sechium	Cho cho or Chayote	*Sicyos edulis* Jacq.
Trichosanthes	Snake gourd	*Trichosanthes cucumerina* L.
	Pointed gourd	*Trichosanthes dioica* Roxb.

BIOTIC STRESS RESPONSES IN CUCURBITS

Cucurbits are often attacked by a wide range of pests including beetles, fruit flies, aphids, white flies, borers, mites etc. (Sharma et al., 2016; Srivastava and Joshih, 2021; **Table 2**). The pests include those affecting cucurbits worldwide as well as those which are more pronounced in certain regions of the globe, where they attain the status of primary pests. Cucumber moth, *Diaphania indica*, is a potentially damaging pest of cucurbitaceous vegetables worldwide (Chintha et al., 2002; Kinjo and Arakaki, 2002; Hosseinzade et al., 2014; Dai et al., 2018; Jalali et al., 2019; Capinera, 2020; Debnath et al., 2020; Khanzada et al., 2021). The attractiveness of cucurbitaceous host plants for *D. indica* was observed to depend on the species and condition of the plant (uninfested and infested), and sex, mating status and experience of the insect. Females that had experience of cucumber, squash and melon plants were significantly attracted to the same plant, and the larvae were attracted only to volatiles of uninfested cucumber, squash and melon (Jalali et al., 2019). Striped cucumber beetle (StCB) and the western striped cucumber beetle (WStCB) are native to North America and StCB is reported to have attained the status of primary pest in northeastern and midwestern United States and eastern Canada (Haber et al., 2021).

Although pests attack affects all stages of cucurbits, severity and susceptibility depends on the plant type and stage of incidence. Pests like red pumpkin beetle and leaf miner are serious at seedling stage (Bains and Prakash, 1985) while beetles [flea beetles (*Phyllotreta cruciferae*) and spotted cucumber beetles (*Diabrotica undecimpunctata*)] were identified as major pests of cucumber at vegetative stage (Alao et al., 2017). Plant growth promoting rhizobacteria (PGPR) induced resistance was reported to be more effective than insecticides for control of cucumber beetles on cucumber possibly by inducing altered production of allelochemicals acting as beetle attractants, repellents, or feeding stimulants (Zehnder et al., 1997). The fruit fly (*Zeugodacus cucurbitae*), a pest of summer squash, cucumber, pumpkin and bitter gourd (Subedi et al., 2021) attacks only flowers and fruits at crop maturity (Ram et al., 2009). Alao et al. (2017) also demonstrated that at vegetative stage of the plant, insect attack was considerably lower in cucumber compared to watermelon, and was attributed to the presence of antixenosis or antibiosis factors in cucumber. Some of the pests that attack cucurbits like whiteflies, thrips and mites, transmit viruses apart from causing feeding damage (Wisler et al., 1998; Park and Lee, 2005; Messelink et al., 2008; Turechek et al., 2014). Although cultivation under greenhouse conditions is reported to be favorable for cucumber production, it is conducive for the rapid development of insect and mite populations (Messelink et al., 2020).

Climate change has led to resurgence of pests and their spread to new areas and often the resistance of varieties breakdown with the evolution of the pest. The fact that pests often become resistant to commercial pesticide formulations in use necessitates a study on pests and their management as well as identification of resistant genotypes and the traits that confer the resistance response.

Cucurbits are found to be affected severely by several diseases including fungal, bacterial and viral diseases, and nematodes, among which the viruses were reported to cause

TABLE 2 | Major pests of cucurbits.

Pest	Scientific name	References
Fruit fly	*Bactrocera cucurbitae*	Haldhar et al., 2014, 2017, 2018
Leaf eating caterpillar	*Diaphania indica*	
Leaf miner	*Liriomyza trifolii*	
Aphids	*Aphis gossypii*	
Ash weevil	*Myllocerus subfasciatus*	
White flies	*Bemisia tabaci*	Wisler et al., 1998; Wintermantel et al., 2019
Beet armyworm	*Spodoptera exigua*	Haldhar, 2016
Red spotted mite	*Tetranychus urticae* (Koch)	Singh and Raghuraman, 2011
Flower beetles	*Mylabris macilenta, Anthicus crinitus,* and *Anthrenus subclaviger*	Haldhar, 2013
Hadda beetle	*Epilachna vigintioctopunctata*	Haldhar et al., 2014, 2017, 2018
Spotted cucumber beetle	*Diabrotica undecimpunctata*	Sharma et al., 2017
Striped cucumber beetle	*Acalymma vittatum*	Sharma et al., 2017
Melon aphid	*Aphis gossypii*	Ng and Perry, 2004
Red pumpkin beetle	*Aulacophora foveicollis (Lucas)*	Khan et al., 2012

TABLE 3 | Major diseases of cucurbits.

Disease	Causal organism	References
Fungal diseases		
Cucurbit powdery mildew	*Podosphaera xanthii*, *Erysiphe cichoracearum,* and *Sphaerotheca fuliginea*	Lebeda et al., 2016
Downy mildew	*Pseudoperonospora* spp.	Lebeda and Cohen, 2011
Anthracnose	*Colletotrichum orbiculare*	Wehner and Amand, 1995
Fruit rot	*Alternaria alternata*, *Fusarium equiseti*, *Fusarium solani*, *Aspergillus* spp., *Phytophthora capsici*, *Penicillium oxalicum*, *Bipolaris* spp., *Botrytis cinerea*, *Cladosporium tenuissimum*	Al-Sadi et al., 2011.
Damping off	*Pythium aphanidermatum, Phytophthora melonis* (in cucumber)	Al-Sadi et al., 2008
Target leaf spot	*Corynespora cassiicola*	Li et al., 2012
Fusarium wilt	*Fusarium* spp.	Li et al., 2009
Bacterial diseases		
Angular leaf spot	*Pseudomonas syringae*	Bhat et al., 2010
Bacterial wilt	*Erwinia tracheiphila*	Shapiro et al., 2014
Bacterial Fruit Blotch	*Acidovorax citrulli*	Wu et al., 2019
Viral diseases		
	Cucumber mosaic virus (CMV)	
	Cucurbit chlorotic yellows virus (CCYV)	Sydänmetsä and Mbanzibwa, 2016
	Squash vein yellowing virus (SqVYV)	Adkins et al., 2008
	Zucchini yellow mosaic virus (ZYMV)	Tsai et al., 2010; Lecoq and Desbiez, 2012
	Watermelon mosaic virus (WMV)	Ayo-John et al., 2014
	Moroccan watermelon mosaic virus (MWMV)	Arocha et al., 2008
	Papaya ringspot virus (PRSV)	Omar et al., 2011
	Cucumber green mottle mosaic virus (CGMMV)	Molad et al., 2021
Parasites		
Root Knot disease	*Meloidogyne* spp.	Omar and Adam, 2018

the largest number of diseases (McCreight, 2011; **Table 3**). Oomycete pathogens like *Pseudoperonospora cubensis*, causing downy mildew, affects all major cucurbit crops, including cucumber, muskmelon, squashes, and watermelon and can assume epidemic proportions (Holmes et al., 2015). Virus diseases, apart from causing reduction in vegetative growth and crop yield, also results in poor fruit quality and makes the plant susceptible to other pathogens as well (Sastry, 2013). However, some studies have demonstrated that healthy wild gourd plants (*Cucurbita pepo* ssp. *texana*) contract bacterial wilt at significantly higher rates than virus infected plants. Prior infection by Zucchini yellow mosaic virus (ZYMV) was found to delay the subsequent onset and progression of bacterial wilt disease by *Erwinia tracheiphila* (Shapiro et al., 2013). Majority of the fungal diseases in cucurbits caused by *Stagonosporopsis cucurbitacearum* (gummy stem blight and black rot), *Alternaria alternata* (leaf spot), *Fusarium solani* (damping off and wilt), *Alternaria cucumerina* (leaf spot) and *Myrothecium roridum* (foliar and stem lesion) are seed borne (Gannibal, 2011; Farrag and Moharam, 2012; Fish et al., 2012). Bacterial pathogens like *Acidovorax avenae* subsp. *citrulli* which causes fruit blotch of cucurbits is also reported to be seed borne and contaminated seeds is the main source of the bacterial inoculum (Block and Shepherd, 2008). Cucurbits adopt various strategies to resist or tolerate diseases. Despite the fact that several genotypes showing resistance to fungal, bacterial, viral and oomycete diseases are available in the germplasm, long-term planting, variable adaptability of pathogens and suppression of host resistance mechanisms by the pathogens often leads to a gradual decline in plant resistance (Chen et al., 2021; Gao et al., 2021; Zhang S. et al., 2021).

ABIOTIC STRESS RESPONSES IN CUCURBITS

Cucurbits are a vast group of vegetables which contribute to the daily diet of a large portion of the world population and are grown almost throughout the world. These vegetables are a good source of nutrients and hence play a vital role in ensuring nutritional security to mankind. Increasing food demands call for intensive, offseason and year-round cultivation of cucurbits, thereby predisposing these crops to a multitude of stressors like high temperature, drought, salinity, heavy metal toxicity, nutrient deficiency/toxicity, soil pH etc. The climate change scenario has further intensified the predisposition to abiotic stressors- high temperature, drought and salinity being the major players in the global arena.

Plants support their growth and development even under adverse conditions by developing several tolerance and adaptation mechanisms. The biochemical, physiological

and molecular responses elicited in response to abiotic stress are guided by common stress tolerance pathways, shared by most of the cultivated crops.

Heat Stress Response

Global warming is one of the most alarming effects of climate change with a long term impact on agriculture, particularly the vulnerable vegetable crops. Heat stress is a function of temperature, duration/period of stress and rate of increase in temperature. Cucurbits being warm season vegetables, are more likely to be exposed to heat stress particularly the summer crop. High temperatures can influence cell development, synthesis of cell wall, plant hormonal connections, amalgamation of proteins, stomatal regulation (thereby influencing photosynthesis, CO_2 assimilation and respiration) etc. (Hasanuzzaman et al., 2012).

High temperatures adversely affect several physiological, biochemical, morphological and molecular processes and pathways in plants. Seed germination of cucumber and melon is reduced drastically at 45 and 42°C respectively (Kurtar, 2010). The ideal temperatures for crop growth and development are 18.3–23.8°C for squash, pumpkin, muskmelon and cucumber, and 23.8–29.4°C for watermelon. In cucumber (*Cucumis sativus* L.) or watermelon (*Citrullus lanatus* L.), temperatures above 35°C caused a reduction in flowers and sugar content (Lai et al., 2018). Heat stress resulted in reduced biomass, root growth and development, leaf area (Porter and Gawith, 1999; Al-Busaidi et al., 2012; Balal et al., 2016) and decreased fruit length, fruit diameter and reduced fruit weight (Balal et al., 2016). The alterations in cell division, cell elongation, water loss and reduced photosynthetic rate under heat stress resulted in reduced yield, leaf area, biomass etc. (Hasanuzzaman et al., 2013).

The photosynthetic rate is positively correlated with the chlorophyll content in the leaves (Lin et al., 2011). Plant growth and yield are adversely affected due to reduced chlorophyll under high temperature stress and subsequent reduction in photosynthetic rate. In cucumber, heat stress induced reduction in chlorophyll and photosynthetic rate has been observed (Balal et al., 2016; Zhou et al., 2016). Reactive oxygen species (ROS) levels are enhanced in the plant tissues in response to heat stress which results in oxidative stress (Suzuki and Mittler, 2006; Potters et al., 2007; Pucciariello et al., 2012). During the electron transport in photosynthetic process, electron leakage to oxygen molecule results in generation of ROS (Sharma et al., 2012). Plants have different mechanisms (enzymatic and non-enzymatic) to detoxify the ROS. Several antioxidative enzyme activities, i.e., superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), glutathione reductase (GTR), monodehydroascorbate reductase (MDHAR), etc. are generally reduced under heat stress (Balal et al., 2016) and are upregulated in response to stress particularly in tolerant species or in response to ameliorants (Balal et al., 2016).

Another strategy to counteract stress induced osmotic damage is the accumulation of various compatible solutes like proline, glycine betaine (GB), amino acids, sugars, quaternary ammonium and sulphonium compounds etc. (Majumder et al., 2009). In cucumber, increased levels of proline, glycine betaine and total soluble sugars were reported in response to heat stress (Balal et al., 2016).

Screening of cucurbit genotypes based on these traits is an effective strategy to identify stress tolerant lines/varieties. High temperature tolerant varieties have been developed in cucurbits such as AHW-19, AHW-65, Thar Manak (Watermelon), Thar Samridhi (bottle gourd), Thar Karni (ridge gourd), Thar Tapish (sponge gourd) etc. (Saroj and Choudhary, 2020).

Drought Stress Response

Cucurbits are warm season vegetable crops mostly cultivated in the summer season, hence prone to drought stress if not irrigated at critical stages of growth. Drought response is classified into three categories *viz.*, drought escape (shortening the life cycle), drought avoidance (minimizing water loss or maximizing water uptake thereby preventing exposure to stress) and drought tolerance (helps the plant to withstand stress by osmoregulation, osmotic adjustment, stomatal regulation etc.). However, crop adaptation to drought may be achieved through a balance between these three strategies (Saroj and Choudhary, 2020). Hence, a combination of different traits should be used as a screening criterion for drought tolerance, rather than a single trait (Singh and Sarkar, 1991). The important traits to be considered while breeding for drought tolerance are early vigor, root depth and density, low and high temperature tolerance, carbon isotope discrimination, osmoregulation, low stomatal conductance, leaf posture, reflectance and duration, sugar accumulation etc. However, priority should be given to those traits which can maintain stability of yield in addition to overall yield (Parry et al., 2005). Some of the drought tolerant genotypes identified are AHW-65 and Thar Manak in watermelon, VRSM-58, AHS-10, AHS-82 in snapmelon etc. In cucumber, drought stress reduces photosynthetic rate, increases superoxide anion radicals ($O_2^{\cdot -}$), electrolyte leakage and lipid peroxidation products like malondialdehyde (MDA), whereas the activities of key antioxidant enzymes superoxide dismutase (SOD) and peroxidase (POD) as well as soluble sugar and proline contents are decreased (Wang J. et al., 2012; Zhang et al., 2013; Fan et al., 2014; Sun et al., 2016).

Salinity Stress Response

Soil salinity has become a severe problem in agricultural production. It is one of the major factors limiting plant growth and productivity particularly in the arid and semi-arid regions of the world (Parida and Das, 2005). Under salinity conditions, stress is induced due to lower water potential of the root medium, toxic effects of Na^+ and Cl^- and nutrient imbalance by reduction in uptake or shoot transport (Colla et al., 2006). Salinity stress response is multigenic, as a number of processes involved in the tolerance mechanism are affected, such as various compatible solutes/osmolytes, polyamines, ROS and antioxidant defense mechanisms, ion transport and compartmentalization of injurious ions (Sairam and Tyagi, 2004).

In cucumber, the salt tolerance in a genotype was associated with higher relative water content (RWC), total chlorophyll content, and SOD, CAT, and APOX activities, together with the lower MDA and proline contents, and Na^+ and Cl^-

concentrations (Furtana and Tipirdamaz, 2010). Sodium chloride stress induces reduction in biomass, photosynthetic pigments, and proline accumulation, while lipid peroxidation and K^+, Na^+, and Cl^- contents are increased (Hawrylak-Nowak, 2009). The addition of 150 mM of NaCl to the nutrient solution of a floating system where 30 varieties of Cucurbitaceae species were cultivated, affected plant growth parameters (number of leaves, shoot length, diameter and dry weight, root length and dry weight) in a genotype-dependent manner (Modarelli et al., 2020). Salinity reduced chlorophylla content by up to 49% in some genotypes, whereas in others chlorophylla content increased by up to 61%. Similarly, chlorophyllb was reduced by salinity by up to 51% in some genotypes or increased by up to 64% in some others. The increase in photosynthetic pigments was considered as a consequence of the reduction of the leaf area and therefore of the dilution effect. Moreover, salinity increased electrolyte leakage by up to 509%, as compared to the non-salinized control.

Heavy Metal Toxicity

Heavy metal accumulation in soils is of great concern in agricultural production due to the adverse effects on food safety and marketability, crop growth due to phytotoxicity, and environmental health of soil organisms (Gill, 2014). Heavy metals cause irreversible damage to a number of vital metabolic constituents and important biomolecules including injury to plant cell walls and cell membranes. Mercury, lead, cadmium, vanadium, arsenic, chromium etc. are some of the heavy metals which are present as soil pollutants and cause severe damage to the crops raised. A common consequence of heavy metal toxicity is the excessive accumulation of ROS and methylglyoxal (MG), both of which can cause peroxidation of lipids, oxidation of protein, inactivation of enzymes, DNA damage and/or interact with other vital constituents of plant cells.

Mercury (Hg) and lead (Pb) heavy metal stress results in high peroxidase activity in cucumber, bottle gourd, sponge gourd and bitter gourd (Khan and Chaudhry, 2006, 2010). In melon, with increasing cadmium concentration, seedling growth, net photosynthetic rate (P_n), stomatal conductance (g_s), transpiration rate (T_r), and stomatal limitation (L_s) decreased; meanwhile, intercellular carbon dioxide concentration (C_i) increased significantly (Zhang et al., 2016; Khan et al., 2020). Hg induces oxidative stress in cucumber seedlings, resulting in plant injury due to reduced activities of antioxidant enzymes (catalase and ascorbate peroxidase), reduced chlorophyll content, increased lipid peroxidation, protein oxidation etc. (Cargnelutti et al., 2006).

TRAIT GENE DISCOVERY AND FUNCTIONAL ANNOTATION-INDIVIDUAL AND COMBINED STRESS EVENTS

Trait based crop improvement assumes significance in the context of the highly variable environmental conditions, which often results in the co-existence of multiple stresses. Identification of suitable traits/trait-combinations conducive for conferring tolerance in different ecosystems is inevitable. There have been promising reports on the functional characterization of different stress responsive genes in cucurbits or the genes cloned from cucurbits in model crops (Parmar et al., 2017; Nanasato and Tabei, 2020). The High Affinity K^+ Transporter (HKT) genes encode Na^+ and/or K^+ transport systems, active at the plasma membrane and play a crucial role in imparting salt tolerance to different plant species e.g., *HKT 1;5* in barley (Hazzouri et al., 2018) and *CmHKT1;1* in pumpkin (Fu et al., 2018). The YUCCA proteins are critical partners in auxin biosynthesis in plants, which have been reported to regulate response to abiotic stresses and flower development in cucumbers. *CsYUC8* and *CsYUC9* were specifically upregulated to elevate the auxin level under high temperature in *Cucumis sativus*. *CsYUC10b* was dramatically increased but *CsYUC4* was repressed in response to low temperature. *CsYUC10a* and *CsYUC11* act against the upregulation of *CsYUC10b* under salinity stress, suggesting that distinct YUC members participate in different stress responses, and may even antagonize each other to maintain the proper auxin levels in cucumber (Yan et al., 2016). A wholistic genomic and functional analysis of *bHLH* genes was attempted in cucumber to identify 142 bHLH genes, classified into 32 subfamilies, among which five *CsbHLH* genes were found to simultaneously respond to three abiotic stresses (NaCl, ABA and low-temperature treatments). Targeted promoter analysis also revealed many *cis*-elements responsive to multiple stresses and plant hormones (Li et al., 2020). Similar attempts targeting different traits have resulted in the identification of prospective candidate genes, which have been functionally characterized in either cucurbits or in model crops like Arabidopsis. There have been reports on novel candidates such as intrinsically disordered proteins belonging to Plant Group II LEA Proteins with possible roles in multiple stress responses (Abdul Aziz et al., 2021), which could be promising even in cucurbits. The functional annotation of candidate genes in cucurbits has been achieved by traditional over-expression or gene silencing approaches, with recent advancements leading to the adoption of advanced gene interference technologies and CRISPR/CAS mediated gene editing approaches. The successful trait-gene based crop improvement attempts in this direction in different cucurbits have been tabulated in **Table 4**.

POSSIBLE CROSSTALK IN PATHWAYS/MECHANISMS UNDER COMBINED STRESSES

Challenges faced by plants come in multitudes and often a combinatorial response to the simultaneous occurrence of stresses, either abiotic or biotic or cross combinations, is actually displayed by plants. A concerted effort to study cucumber plants exposed to salt stress and thereafter infected with *Pseudomonas syringae* pv *lachrymans* (*Psl*), revealed that there were distinct changes in photochemistry, the antioxidant system, primary carbon metabolism, salicylic acid (SA) and abscisic acid (ABA) contents. The careful examination of hormonal

TABLE 4 | Trait-gene discovery and functional or translational characterization in cucurbits.

Gene	Source	Crop	Target Trait	Remarks
Cbf1	*Arabidopsis thaliana*	Cucumber (OE)	Chilling tolerance	Marker free Gupta et al., 2012
CsWAX2	Cucumber	Cucumber (OE)	Abiotic and biotic stress response	Wang et al., 2015
CsATAF1	Cucumber	Cucumber (RNAi)	Drought stress tolerance	Wang J. et al., 2018
CsCaM3	Cucumber	Cucumber (OE)	High temperature stress tolerance	Yu et al., 2018
CsYUC11	Cucumber	*Arabidopsis thaliana* (OE)	Salinity tolerance	Yan et al., 2016
CsbHLH041	Cucumber	Cucumber *and Arabidopsis thaliana* (OE)	Salinity and ABA tolerance	Li et al., 2020
CMV 2a/2b	Watermelon	Artificial microRNAs	Virus resistance	Liu et al., 2016
CsGPA1	Cucumber	Cucumber (RNAi)	Drought stress tolerance	Liu et al., 2021d
CmRCC1	Pumpkin	Tobacco (OE)	Cold stress tolerance	Wang et al., 2021
Chimeric gene construct containing truncated ZYMVcp and PRSV W cp genes	*Citrullus lanatus* Watermelon	*Citrullus lanatus* Water melon (RNAi)	Virus resistance	Yu et al., 2011
CMV replicase	Defective viral genome mediated resistance against CMV	Lilium	Virus resistance	Azadi et al., 2011
eIF4E	Cucumber	Cas9/subgenomicRNA (sgRNA technology)	Virus resistance	Chandrasekaran et al., 2016
RBOHD	Pumpkin	CRISPR/Cas9-mediated mutagenesis	Salinity tolerance	Huang et al., 2019
RBOHD	Pumpkin	*Arabidopsis thaliana* (OE)	Salinity tolerance	Huang et al., 2019
CsWIP1	Cucumber	CRISPR/Cas9-mediated mutagenesis	Gynoecious phenotype	Hu et al., 2017
ZW-20	Squash	Cucumber	*Zucchini yellow mosaic virus resistance*	Fuchs and Gonsalves, 1995
CZW-3 (CMV, ZYMV, and WMV2)	Squash	Cucumber	Virus resistance	Fuchs and Gonsalves, 1997

OE, overexpression; RNAi, RNA interference.

and redox balance as well as the carboxylate metabolism and activities of some NADPH-generating enzymes indicated that salt-stressed plants were more prone to pathogen infection. There can be critical convergence points and master regulators for the characteristic response to specific abiotic factor-pathogen combination. In case of cucumber, the combinatorial stress response to salt stress and *P. syringae* is dominated by the abiotic factor. Modulation of SA-mediated defense, hormonal, ROS/redox and metabolic signals are responsible for predisposing cucumber plants to *P. syringae* after sequential salt stress episodes making them highly susceptible (Chojak-Koźniewska et al., 2018). Another important stressor is temperature which has profound influence on the occurrence of bacterial diseases caused by *Ralstonia solanacearum* (causal agent of wilt in tomato), *Acidovorax avenae* (causal agent of seedling blight and bacterial fruit blotch of cucurbits) and *Burkholderia glumae* (causal agent of bacterial panicle blight in rice) (Kudela, 2009; Pandey et al., 2017). *Cucumber mosaic virus* (CMV) as an important viral invader of cucurbits has significance in understanding its interactive specificities with other stressors. CMV infection was found to impart improved drought tolerance of *Capsicum annum* (pepper), *S. lycopersicum*, and *Nicotiana tabacum* (tobacco) (Xu et al., 2008; Pandey et al., 2017). The combinatorial effects between abiotic-abiotic and abiotic-biotic pairs can be starkly different. Generally abiotic stress combinations can have "only net effects and no stress interactions," leading to additive deteriorative effects due to co-occurrence of two stresses together. It will be different for a plant-pathogen system wherein, it may lead to enhanced or reduced susceptibility to the pathogen; some pathogens also modulate abiotic stress tolerance. In case of heat–pathogen and drought–pathogen stress combinations, wherein multiple individual stresses or sequential stresses occur one after the other, either prior priming leading to stress memory or predisposition can be the consequence (Pandey et al., 2017). *CsbHLH041* is an important regulator in response to multiple abiotic stresses like salinity and water deficit in cucumber (Li et al., 2020). Phytohormonal variations can result in a common response against both biotic and abiotic stressors such as similar morphological root changes under CMV infection and a heavy metal challenge like cadmium stress (Vitti et al., 2013). It was very recently identified that there are distinct metabolite signatures, with special reference to amino acids, associated with response to salt and drought stresses in *Cucumis melo* L. (Chevilly et al., 2021). High histidine contents and the ability to sequester salts in vacuoles are expected to confer salt stress tolerance capacity. However, varieties or cultivars with enhanced levels of isoleucine, glycine, serine and asparagine exhibited drought stress tolerance. There was a retardation in tolerance to abiotic stresses when the phenylalanine levels were high (Chevilly et al., 2021).

POSSIBLE STRATEGIES TO ACHIEVE COMBINED STRESS TOLERANCE

A. Propagation Methods as a Means for Combining Multiple Stress Tolerance Traits

Vegetable grafting is a unique horticultural technique used in the propagation of fruit vegetables due to the multitude of advantages over the conventional propagation methods. Vegetable grafting was primarily developed and practiced with an objective of avoiding the damage caused by soil borne pathogens and pests (Cohen et al., 2007). The scope for grafting has further widened for combating abiotic stress tolerance, with the advancement in our understanding of the rootstock mediated effect on superior performance of scion, exploiting the physiological stress tolerance reserved in the wild species (Colla et al., 2010, 2012). Grafting has emerged as a viable alternative to relatively slower breeding approaches for enhancing environmental stress tolerance in fruit vegetables (Flores et al., 2010). Grafting is a special method of adapting plants to counteract environmental stresses by grafting superior commercial cultivars onto specific vigorous rootstocks (Lee and Oda, 2003).

Cucurbits are the first group of vegetables where grafting was widely popularized to combat biotic stress particularly Fusarium wilt. Research on cucurbit grafting began in the 1920s with the use of *Cucurbita moschata* as a rootstock for watermelon in Japan. Grafting is a quick, less expensive and viable solution for combating soil borne pathogens and their novel races, in comparison to the tedious breeding approach adopted for developing resistant cultivars (Davis et al., 2008). Watermelon, cucumber and melons are the major cucurbits which are propagated using grafted seedlings in order to overcome biotic and abiotic stresses.

(a) Grafting for Biotic Stress Tolerance in Cucurbits

In cucurbits, grafting has proven to impart resistance/tolerance to several fungal, bacterial and nematode infections, soil borne pathogens and even some viral as well as foliar pathogens. The most devastating soil borne pathogen of cucurbits is *Fusarium oxysporum* causing Fusarium wilt (FW). In watermelon and cucumber, grafting is the most popular alternative for controlling Fusarium wilt. Some of the achievements in combating biotic stress in cucurbits through grafting has been summarized in **Table 5**.

(b) Grafting for Abiotic Stress Tolerance in Cucurbits

Grafting has also emerged as an effective adaptive technique to overcome abiotic stressors including drought, flooding, waterlogging, salinity, heavy metal contamination, suboptimal and supraoptimal temperatures, nutrient deficiencies, toxicities etc. When the plants are exposed to these abiotic factors beyond the threshold level for optimal biochemical/physiological activity or morphological development, it results in reduction of plant performance and subsequent yield reduction. In cucurbits, several studies involving different rootstocks have proven their efficiency in alleviating the adverse effects of a number of abiotic factors; some of these have been summarized in **Table 6**.

Under the present climate change scenario multiple stresses in combination or separately, pose severe threat to vegetable production including cucurbits. Use of rootstocks conferring multiple stress resistance could be a sustainable and eco-friendly alternative to the more complicated traditional/molecular breeding approaches to develop multiple stress resistant varieties (**Table 7**). The melon hybrid (*Cucurbita maxima x Cucurbita moschata*), figleaf gourd (*Cucurbita ficifolia*), pumpkin, bottle gourd and sponge gourd rootstocks have the potential to impart multiple stress tolerance to scions of different cucurbits. Other wild and cultivated species of cucurbits could also be explored for their capabilities to confer multiple stress resistance to susceptible species/varieties.

B. Genetic Manipulation Avenues for Developing Stress Tolerance (Conventional/Molecular Breeding/Biotechnological)

(a) Breeding for Biotic Stress Tolerance

Screening of germplasm for resistance, utilization of the identified resistant lines as donors for recombination breeding or backcross breeding, interspecific crosses, mutation breeding and manipulation using propagation strategies are the widely used conventional strategies for the development of biotic stress tolerant cucurbit genotypes. Cucurbits are widely affected by viral diseases and among them bottle gourd is found to be moderately resistant to viral disease caused by CMV and yellow mosaic virus (ZYMV) (Provvidenti and Gonsalves, 1984; Provvidenti, 1995; Ling and Levi, 2007). It also displays resistance to fungal diseases like Fusarium wilt (Yetişir and Sari, 2003) and powdery mildew (Kousik et al., 2008) and has been exploited in its use as rootstock for watermelon (Yetişir and Sari, 2003; Keinath and Hassell, 2014).

Resistance to viruses as well as other pests and diseases has also been identified in wild or semi-domesticated types of bitter gourd (*M. charantia var. muricata*) (Asna, 2018). Previous studies also demonstrated the utility of mutation breeding in the development of biotic stress tolerant genotypes. Bitter gourd cultivar MDU 1 developed through mutation breeding from the landrace MC 013, displayed tolerance to pumpkin beetle, fruit fly and leaf spot diseases (Rajasekharan and Shanmugavelu, 1984). The reported cucurbit genotypes resistant to specific pathogens/pests are tabulated in **Table 8**.

(b) Breeding for Abiotic Stress Tolerance

Genomic/genetic resources and plant transformation protocols have recently been developed and standardized for cucurbits (Nanasato et al., 2013; Sun et al., 2017; Montero-Pau et al., 2018, 2017). The characterization of the wild relatives of cucurbits have aided in finding their potential use in breeding and other related crop improvement initiatives (e.g., Holdsworth et al.,

TABLE 5 | Rootstocks for combating biotic stress in cucurbits.

Sl No.	Crop/Scion	Rootstock	Stress tolerance imparted	Region	Condition	References
1	Watermelon	*Lagenaria siceraria* (16S-71)	*Fusarium oxysporum* f. sp. *niveum*	China	Open field	Zhang M. et al., 2021
2	Oriental melons (cv. makuwa) and pickling melon	Cucurbita moschata (Shirokikuza) and *C. maxima* × *C. moschata* (Shintosa)	*Fusarium oxysporum*	Japan	Open field	Sakata et al., 2008
3	Watermelon	*Citrullus* sp. (RS-18, RS-10, RS-11)	*Fusarium oxysporum*	Bangalore (India)	Open field	Pal et al., 2020
4	Watermelon	*L. siceraria* (WMXP-3938)	*Phytophthora capsici*	United States	Open field	Kousik et al., 2012
5	Watermelon (cv. Fiesta)	*C. lanatus* var. *citroides* (RKVL 315 and 318)	Nematode *(Meloidogyne incognita)*	–	Open field	Thies et al., 2010
6	Cucumber	*Cucurbita maxima* and *C. moschata*	*Fusarium oxysporum; Pythium aphanidermatum*	Egypt	Open field	Reyad et al., 2021
				Oman	Green house	Deadman et al., 2009
7	Cucumber (cv. Caspian 340)	*C. maxima*	*Pythium aphanidermatum*	-	Open field	Rostami et al., 2015
8	Cucumber (cv. Centenario)	*Lagenaria siceraria* (Lag 53)	Nematode *(Meloidogyne incognita)*	Mexico	Green house	Suárez-Hernández et al., 2021
9	Cucumber	*Benincasa hispid*a	Black root rot (*Phomopsis sclerotioides*)	–	–	Yamaguchi and Iwadate, 2009
10	Cucumber, melon and watermelon	*Cucumis pustulatus*	Nematode (*Meloidogyne incognita*) and Fusarium wilt	China	Green house	Liu et al., 2015
11	Melon	*Cucumis melo, Cucurbita maxima* × *Cucurbita moschata*	*Fusarium oxysporum*	South Korea Italy	Open field	Lee, 1994; Nisini et al., 2002
12	Inodorous melon	*Cucurbita maxima* Duchesne × *Cucurbita moschata* Duchesne (RS841, P 360, ES99-13, Elsi)	*Fusarium oxysporum* f. sp. *melonis* and *Didymella bryoniae*	Italy	Green house	Crino et al., 2007
13	Honey dew melon (cv. Honey yellow) Galia melon (cv. Arava)	*Cucumis metulifer* line (USVL-M0046)	Root knot nematode *Meloidogyne* spp.	Florida (United States)	Green house	Guan et al., 2014
14	Oriental Melon	*C. moschata, C. metuliferus,* and *Sicyos angulatus*	Fusarium wilt	–	–	Davis et al., 2008
15	Bitter gourd	*Luffa aegyptiaca*	*Fusarium oxysporum*	–	Open field	Lin et al., 1998
16	Bitter gourd	*Citrullus colocynthis, Cucumis metuliferus, Cucurbita moschata*	Nematode (*Meloidogyne incognita*)	Tamil Nadu (India)	Glass house	Tamilselvi et al., 2017

2016). It was also interesting to note that many wild relatives have multiple stress tolerance capacities, both biotic and abiotic. Potential and documented use of wild cucurbits in breeding with special emphasis on possession of traits such as abiotic and biotic stress tolerance is tabulated in **Table 9**. It has also been reported that many of the wild cucurbits are under the threat of being endangered, demanding conservation interventions owing to their tolerance potentials (Khoury et al., 2020).

Concerted efforts have been made to identify and characterize the potential species and genotypes, among the different cucurbits, considered tolerant to drought or heat or a combination of both stresses (Saroj and Choudhary, 2020; Mkhize et al., 2021). The potential tolerant sources have been tabulated in **Table 10**.

The successful strategy for identifying the candidate tolerant sources and developing elite donors is inclined towards a physiogenetic approach including careful analysis of the key physiological traits distinctly critical for each stress (**Figure 1**) and subsequent characterization of the genetic basis for the respective trait manifestation. However, it is pertinent that there may be common physiological traits that can be capable enough to confer tolerance to multiple stresses. Abiotic stress tolerance, in particular, is a complex trait; the component primary, secondary (constitutive or induced) and integrative traits will have their distinct individual relevance under different stresses, along with their contributory significance.

(c) Molecular Breeding and Biotechnological Approaches for Crop Improvement in Cucurbits

Strategies adopted for development of stress tolerant genotypes in cucurbits including conventional breeding, propagation techniques, mitigation strategies etc. have contributed immensely to the successful cultivation of members of the cucurbit family. However, traditional approaches are often time

TABLE 6 | Rootstocks for combating abiotic stress in cucurbits.

Sl No.	Crop/Scion	Rootstock	Stress tolerance imparted	Region	Condition	References
1	Cucumber	*Luffa cylindrica*	Drought	China	Growth chamber	Liu, 2016
2	Cucumber	*Cucurbita moschata*	Salinity; low temperature	China	Open field	Niu et al., 2019
				China	Open field	Zhu et al., 2008
				Japan	Green house	Shibuya et al., 2007
3	Cucumber	Figleaf gourd (*Cucurbita ficifolia* Bouché) and bur cucumber (*Sicyos angulatus* L.)	Low temperature	–	–	Schwarz et al., 2010
	Cultivar 'Infinity'	Figleaf gourd (*Cucurbita ficifolia*), bottle gourd (*Lagenaria siceraria* cv. Sharda)	Low temperature	Jodhpur (India)	Unheated green house	Kumar et al., 2019
4	Cucumber (cv. Jinyou No. 35)	*Momordica charantia* L. (Changlv)	Heat Stress tolerance	China	Plastic arched shed	Xu et al., 2018
5	Cucumber (cv. Ekron)	*C. sativus* L., *C. maxima x C. moschata*	Salinity tolerance	Italy	Green house	Colla et al., 2013
	(cv. Jinchum No. 2)	*Cucurbita ficifolia*		China	Green house	Huang et al., 2010
6	Cucumber (cv. Gian Co F1)	VSS-61 F1 *Cucurbita pepo* (squash) and Ferro *Cucurbita maxima* × *C. moschata*	Heat and Salinity stress	Egypt	Net house	Bayoumi et al., 2021
7	Cucumber (cv. Jinyou 35)	*Cucurbita moschata* (Jinmama 519)	Chilling tolerance	China	–	Fu et al., 2021
8	Cucumber (cv. Akito)	*C. maxima* x *C. moschata* Shintoza	Copper toxicity	Italy	Green house	Rouphael et al., 2008b
	(cv. Creta)	*C. maxima* × *C. moschata* Power	Ni and Cd toxicity	–	–	Savvas et al., 2013
	(cv. Ekron)	*C. maxima* × *C. moschata* (P360)	Acidity and Al toxicity	Italy	Green house	Rouphael et al., 2016
9	Cucumber (cv. Sharp 1, cv. Natsubayashi)	*Cucurbita sp.* (Shintosa-1gou, Hikaripower-gold, Yuyuikki-black)	Organic pollutant (dieldrin)	Japan	Open field	Otani and Seike, 2007
10	Watermelon (cv. Crimson tide)	*Lagenaria siceraria*	Flooding tolerance	Turkey	Green house	Yetisir et al., 2006
11	Watermelon (cv. Ingrid)	*Cucurbita maxima* × *Cucurbita moschata* (PS1313)	Drought tolerance	Italy	Green house	Rouphael et al., 2008a
	(cv. Zaojia 8424)	(Qingyan zhenmu No. 1)	Nitrogen use efficiency	–	Open field	Nawaz et al., 2017
	(cv. Crimson Sweet)	*C. maxima* × *C. moschata* (Shintoza) *Citrullus colocynthis* (L.) Schrad (Esfahan)	Drought	Italy	Green house	Bikdeloo et al., 2021
12	Watermelon (cv. Mahbubi)	*Cucurbita pepo* (Tiana F1 hybrid); *Cucurbita maxima*	Cd toxicity	Iran	Green house	Shirani-Bidabadi et al., 2018
13	Watermelon (cv. Zaojia 8424)	*Cucurbita maxima* × *Cucurbita moschata* (Qingyan zhenmu No. 1) and *Lagenaria siceraria* (Jingxinzhen)	Vanadium toxicity	China	–	Nawaz et al., 2018
14	Bitter gourd	*Cucurbita moschata*	Low temperature	United States	Green house	Wang J. et al., 2018
15	Bitter melon (cv. New Known You #3)	*Luffa cylindrica* (cv. cylinder #2) *Momordica charantia*	Flooding tolerance	China China	Pot study Pot study	Liao and Lin, 1996; Peng et al., 2020

consuming and restricted by the available variation in the gene pool. Biotechnological interventions can result in rapid and sustainable development of crop varieties having high quality and stress tolerance.

(i) Genome Sequencing, Mapping and Marker Assisted Selection

Genome sequencing facilitates all subsequent analyses of genome structure, organization and function. Genome sequences have been published for major cucurbit family members like cucumber (Huang et al., 2009; Osipowski et al., 2020), melon (Garcia-Mas et al., 2012; Ruggieri et al., 2018), water melon (Guo et al., 2013), zucchini (Montero-Pau et al., 2018; Xanthopoulou et al., 2019), *C. maxima* (Sun et al., 2017), *C. moschata* (Sun et al., 2017), bottle gourd (Wu et al., 2017), wax gourd (Xie et al., 2019) etc. Genomic information has facilitated the discovery of genes and pathways associated with several stress response pathways leading to the development of stress tolerant varieties or genotypes.

In cucumber, several quantitative trait loci (QTLs) associated with resistance to virus, fungi and bacteria have been mapped. QTLs associated with abiotic stress tolerance like cold, water stress, temperature (Dong et al., 2020; Liu et al., 2021c), drought, salt (Liu et al., 2021a) etc. have also been identified in cucumber which can be utilized in breeding programs. Phytophthora crown rot resistance in *C. moschata*. was detected on chromosome 4 (QtlPC-C04), 11 (QtlPC-C11), and 14 (QtlPC-C14) by bulk segregant analysis and potential linked markers for utilization in marker assisted selection (MAS) (Ramos et al., 2020). A genome-wide association study (GWAS) based on 5,330 single-nucleotide

TABLE 7 | Prospective rootstocks for multiple stresses tolerance interventions.

Sl No.	Rootstock*	Crops	Biotic/abiotic stress tolerance
1	*Cucurbita maxima x Cucurbita moschata*	Cucumber	Fusarium wilt, Pythium, salinity, heat, Ni, Cd, Al toxicity, acidity.
		Watermelon	Fusarium wilt, drought, Nitrogen use efficiency, Vanadium toxicity
		Melon	Fusarium wilt, *Didymella bryoniae*
2	*Cucurbita moschata*	Bitter gourd	Nematode, low temperature
		Cucumber	Salinity, low temperature
		Oriental melon	Fusarium wilt
3	*Lagenaria siceraria*	Watermelon	Fusarium wilt, *Phytophthora capsici*, Flooding, Vanadium toxicity
		Cucumber	Nematode (*Meloidogyne incognita*)
4	*Luffa cylindrica*	Cucumber	Drought
		Bitter gourd	Flooding
5	*Cucurbita ficifolia*	Cucumber	Low temperature, salinity

The information on the rootstock can be derived from **Tables 5, **6**.*

TABLE 8 | Biotic stress resistant genotypes identified cross different cucurbits.

Pathogen/ pest	Crop	Resistant genotype identified	References
Tomato leaf curl New Delhi virus	*Luffa cylindrica Roem.*	DSG-6, DSG-7, DSG-9, and DSG-10	Islam et al., 2010
	L. cylindrica (L.) *Roem*	IIHR-137, IIHR-138, IIHR-Sel-1	Kaur et al., 2021
Potyviruses	*Cucumis melo*	PI 414723 and PI 124112	Martín-Hernández and Picó, 2021
Mosaic diseases	*M. charantia var. muricata*	IC 213312, AC-16/1, AC-16/4, AC-16/9, and AC-16/21	Asna, 2018
Broad spectrum virus diseases	*Lagenaria siceraria*	USVL#1-8 and USVL#5-5	Ling et al., 2013
Fruit rot	*Cucumis sativus* L.	PI109483, PI178884, and PI214049	Colle et al., 2014
Downy mildew	*Cucumis sativus* L.	PI 197088	Berg, 2020
Cucurbit powdery mildew	*Momordica charantia* L.	THMC 153 and THMC 167	Dhillon et al., 2018
Powdery mildew	*Citrullus lanatus*	PI 632755, PI 386015, PI 189225, PI 346082, PI 525082, PI 432337, PI 386024, and PI 269365	Tetteh et al., 2010
Powdery mildew	*Cucumis sativus* L.	PI 418962, 418964, 432860, 432870, 197085, 197088, 605930, 279465, 288238, 390258, 390266, 330628, 426169, 426170, 321006, 321009, and 321011	Block and Reitsma, 2005
Powdery mildew	*M. charantia var. muricata*	IC 213312, AC-16/1, AC-16/4, AC-16/9, and AC-16/21	Asna, 2018
Powdery mildew	*Cucumis sativus* L.	PI 197088	Wang Y. et al., 2018
Anthracnose	*Cucumis sativus* L.	Dual, Regal, Slice, and Gy 3	Wehner and Amand, 1995

polymorphisms (SNPs) in bottle gourd accessions detected *HG_GLEAN_10011803* to be likely the major-effect candidate gene for resistance against FW in bottle gourd (Yanwei et al., 2021). Wang Y. et al. (2018) identified three major-effect contributing QTLs for downy mildew resistance viz., *dm5.1*, *dm5.2,* and *dm5.3* and a major-effect QTL *pm5.1* for powdery mildew resistance in cucumber. CsGy5G015660, encoding a putative leucine-rich repeat receptor-like serine/threonine-protein kinase (RPK2), was identified as a strong powdery mildew resistance candidate gene in a Korean cucumber inbred line, by genome wide SNP profiling and corresponding RT-PCR analyses (Zhang C. et al., 2021).

SSR marker ECM230 linked to the major QTL in melon (*Cucumis melo* L.) was reported to be useful in selection for resistance to CCYV (*Cucurbit chlorotic yellows virus*) (Kawazu et al., 2018). Two additive QTLs affected the whitefly attack and a major QTL that reduces acceptance by *Aphis gossypii* and 10 genome locations on five linkage groups involved in resistance to hemipterans in melon have been identified (Boissot et al., 2010). In cucumber, resistance to Watermelon mosaic virus (WMV) is controlled by a single recessive gene designated as wmv02245 and was mapped to chromosome 6 (Chr.6) (Tian et al., 2016). The bottle gourd genome sequence has facilitated the mapping of Prs, conferring Papaya ring-spot virus (PRSV) resistance, on chromosome 1 and the potential of a CAPS marker tightly linked to the Prs locus in marker-assisted selection of PRSV resistance in bottle gourd has been demonstrated (Wu et al., 2017). Zhang et al. (2014) identified SSR17631 marker, which could be used to screen cucumber resources with Fusarium wilt resistance in molecular marker-assisted selection breeding. The identified QTLs and associated markers can be effectively utilized in screening, selection and gene pyramiding for multiple stress tolerance in cucurbits.

(ii) Transgenic Development for Crop Improvement

Majority of the transgenics developed in cucurbits are for development of virus resistance (Gaba et al., 2004). Transgenic watermelon carrying a single chimeric transgene comprising a silencer DNA from the partial N gene of Watermelon silver mottle virus (WSMoV) fused to the partial coat protein (CP) gene sequences of CMV, Cucumber green mottle mosaic virus (CGMMV) and WMV demonstrated that fusion of different viral CP gene fragments in transgenic watermelon contributed to multiple virus resistance via RNA-mediated post-transcriptional gene silencing (PTGS) (Lin et al., 2012). Transgenic cucumber and melon lines harboring a hairpin construct of the Zucchini yellow mosaic potyvirus (ZYMV) HC-Pro gene displayed resistance to systemic ZYMV infection (Leibman et al., 2011). Transgenic oriental melon carrying untranslatable chimeric DNA with partial CP sequences of ZYMV and PRSV caused RNA-mediated PTGS conferring high degrees of resistance to ZYMV and PRSV W in *C. melo* (Wu et al., 2010). Transgenic watermelon with resistance to CMV infection was developed by expressing artificial microRNAs that target CMV 2a/2b genes (Liu et al., 2016).

TABLE 9 | Wild relatives of genus *Cucurbita* and their documented tolerance/resistance potentials.

Taxon	Tolerance/Resistance potentials
Cucurbita argyrosperma C. Huber subsp. *sororia* (L. H. Bailey) L. Merrick and D. M. Bates	Resistant to BYMV and TmRSV
C. cordata S. Watson	Drought-tolerant; resistant CMV, TRSV, BYMV
C. digitata A. Gray	Drought-tolerant; resistant to CMV, TmRSV
C. ecuadorensis H. C. Cutler and Whitaker	Resistant to papaya ringspot virus, WMV, powdery mildew, downy mildew
C. lundelliana L. H. Bailey	Resistant to SqLCV, CMV, powdery mildew
C. okeechobeensis (Small) L. H. Bailey subsp. *Martinezii* (L. H. Bailey) T. C. Andres and Nabhan ex T. W. Walters and D. S. Decker	Resistant to CMV, BYMV, TRSV, bacterial leaf spot, powdery mildew, downy mildew
C. okeechobeensis (Small) L. H. Bailey subsp. *okeechobeensis*	Resistant to CMV, BYMV, TRSV, bacterial leaf spot, powdery mildew, downy mildew
C. palmata S. Watson	Drought-tolerant; resistant to CMV, TRSV, BYMV, TmRSV
C. pedatifolia L. H. Bailey	Drought-tolerant; disease resistance unstudied; potential as bridge species between xerophytic and mesophytic species
C. radicans Naudin	Drought-tolerant; resistant to CMV, TmRSV; BYMV
C. x scabridifolia L. H. Bailey	Drought-tolerant

TABLE 10 | Abiotic stress tolerant genotypes/species of cucurbits.

Abiotic stress	Crop	Resistant genotype identified	Source
Drought	*Cucumis melo* var. *momordica*	VRSM-58	Bihar, India
	Cucumis melo var. *chate*	Arya	Rajasthan and Haryana, India
	Watermelon	AHW-65; Thar Manak	ICAR-CIAH, Bikaner, India
Heat	*Cucumis melo* var. *callosus*	AHK-119, AHK-200	ICAR-CIAH, Bikaner, India
	Lagenaria siceraria	Thar Samridhi	
	Luffa acutangula	Thar Kami	
	Luffa cylindrica	Thar Tapish	
	Cucumis melo var. *utilissimus*	Thar Sheetal, AHC-2, AHC-13	
	Citrullus lanatus	AHW-19, AHW-66, Thar Manak	
	Cucumis melo	MIn 28, CU 311	Turkey
	Cucumis melo var. *flexuosus*	Armenian Cucumber	Egypt
Drought and Heat	*Cucumis melo* var. *callosus*	AHK-119, AHK-200	ICAR-CIAH, Bikaner, India
	Cucumis melo var. *momordica*	AHS-10, AHS-82	
Drought and Salt	*Cucumis melo* Cv. *reticulatus*	Galia type Cv.1	Pre commercial melons from Enza Zaden, Netherlands
	Cucumis melo Cv. *inodorus*	Piel de Sapo Cv. 3	
Heavy metal tolerance (Pb)	*Citrullus lanatus*	NBT, ZM5	–

(iii) Non-transgenic Biotechnological Approaches

Heavy restrictions placed on genetically modified organisms (GMOs) have resulted in the adoption of non-transgenic approaches in crop plants. CRISPR/Cas9, the novel and efficient tool for genome editing, was used in cucumber for the disruption of the *eIF4E* for the development of virus-resistant plants without otherwise affecting the plant genome (Chandrasekaran et al., 2016). Use of CRISPR/Cas9-mediated gene modification for *Clpsk1* loss-of-function in watermelon seedlings made them more resistant to infection by *Fusarium oxysporum* f. sp. *niveum* indicating its effectiveness for watermelon improvement (Zhang et al., 2020). Strategies using the ability of dsRNAs to activate the plant RNA silencing mechanism has also been exploited in cucurbits. Exogenous application of *in vitro*-produced dsRNA molecules derived from the HC-Pro and CP genes of ZYMV, conferred significant protection ZYMV in watermelon and cucumber (Kaldis et al., 2018).

C. Novel Stress Tolerance Pathways and Mechanisms- the "Omics" Way

The evolution of high throughput next generation sequencing technologies have aided in the generation of immense omics resources for unraveling the more complex stress acclimation responses in cucurbits as has been demonstrated in many other crop species in the past two decades. The Cucurbit Genome Database (CuGenDB) developed by the Fei Lab at Boyce Thompson Institute, United States, serves as the integral portal for functional and comparative genomics (Zheng et al., 2019). The team has added on more tools to their armory with the development of CucCAP (Grumet et al., 2020), which helps in harnessing genomic resources for disease resistance and management in cucurbit crops. The expression repertoire in terms of transcriptomic and proteomic studies have also found place in the cucurbit quest for tolerance to abiotic and biotic stresses. RNA sequencing attempts have been made to prospect genes involved in long-term waterlogging tolerance in cucumber, unraveling transcript abundance specified to "plant hormone signal transduction pathway" in the "environmental information processing" category (Kreska et al., 2021). Salt stress specific transcriptomic analysis revealed the differential regulation of genes associated with carbon metabolism, biosynthesis of amino acids, carbon fixation in photosynthesis, nitrogen metabolism and fatty acid degradation in cucumber (Jiang et al., 2020). This study assumes significance in the context of the role of H_2S in alleviating salinity stress wherein, proteome analysis indicated differential regulation of proteins involved in sulfur metabolism such as Cysteine synthase 1, Glutathione S-transferase U25-like, Protein disulfide-isomerase,

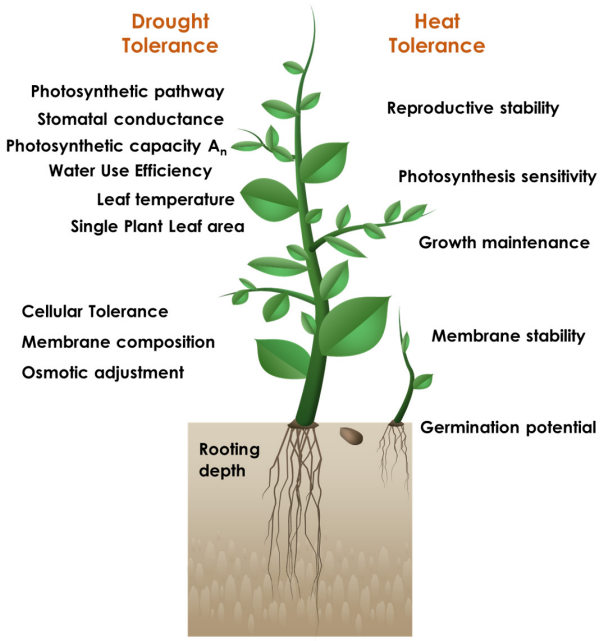

FIGURE 1 | Physiological trait-based screening for identification of drought and heat stress tolerant sources.

and Peroxidase 2 (Jiang et al., 2020). WRKY transcription factors were reported to regulate downy mildew resistance in cucumber as evidenced by the higher expression of pattern recognition receptor (PRR) proteins unravelled by transcriptome analysis (Gao et al., 2021). Phenylpropanoid biosynthesis pathway emerged as a key regulator of resistance to *Corynespora cassiicola* stress in cucumber as revealed by transcriptome and miRNA analysis (Wang X. et al., 2018). Sucrose biosynthesis and ABA signal transduction were reported to be the key molecular regulations under drought stress, specifically induced after 4 days of drought stress in cucumber (Wang M. et al., 2018). Organellar genome influence, with special emphasis to chloroplastic and mitochondrial genomes in regulation of multiple traits have been highlighted by interventions brought about by next generation sequencing and *omics* in cucumber (*Cucumis sativus* L.) (Pawełkowicz et al., 2016).

In *Cucurbita pepo* subsp. *pepo*, down regulation of SA precursor related enzyme, *CpPAL* (Phenyl ammonia lyase) was found to be associated with susceptibility, while defensin overexpression was found to be related to tolerance (Ayala-Doñas et al., 2021). Targeted metabolomics studies have been attempted to understand the response of cucumber to silver and silver nanoparticles (Zhang et al., 2018), sulfur (Liu et al., 2021b) and elevated atmospheric CO_2 (Li et al., 2018). An interdisciplinary approach involving different fields of plant sciences to culminate in adopting ionomics as an integrated assessment of elemental accumulation, will hold potential because molybdenum and iron are reported to mutually govern their homeostasis in cucumber (*Cucumis sativus*) plants (Vigani et al., 2017; Pita-Barbosa et al., 2019).

D. Stress Mitigation Strategies for Cucurbits by Exogenous Amelioration

Cucurbits are a class of vegetables often grown in hot and dry tropics, making them vulnerable to the exposure of multiple stresses. The knowledge and information on the different stress adaptive traits and mechanisms have paved the way for employment of different biostimulants and chemical ameliorants for sustainable management of different stressors. Mycorrhizal associations in cucurbits have been proven to be beneficial both under optimal and stressful conditions. The symbiotic interaction with arbuscular mycorrhizal fungi (AMF) has profound influence when multiple stresses occur at the same time. There are commercial examples of mycorrhizal consortia such as MycoApply®, a four-species consortium, which facilitates nutrient and water uptake[1]. A consortium of three plant growth-promoting rhizobacterium (PGPR) strains (*Bacillus cereus* AR156, *Bacillus subtilis* SM21, and *Serratia* sp. XY21), has been reported to confer systemic tolerance to drought stress in cucumber, by maintaining assimilation and growth vigor and offering protection against oxidative stress damage (Wang C. J. et al., 2012). Humic acid is a highly beneficial biostimulant used in different crops to stimulate shoot and root growth and enhance tolerance to stresses, which has also been demonstrated in cucumber to influence yield and mineral nutrient uptake under salinity stress exposure (Demir et al., 1999). Foliar spray of Moringa leaf extract was found to be beneficial in enhancing growth, harvest index, WUE, photosynthetic stability, osmoregulation and membrane stability in *Cucurbita pepo* under drought stress (Abd El-Mageed et al., 2017). Similarly, under salinity stress, seed treatment/irrigation with a bacterial consortia of *Bacillus species, Bacillus pumilis, Trichoderma harzianum, Paenibacillus azotoformans,* and *Polymyxa* plays a role in maintaining growth by regulating ion homeostasis in *Cucurbita pepo* (Yildirim et al., 2006). Soil amelioration with *Ascophyllum nodosum* was beneficial in *Cucumis sativus* against salinity stress, which helped in maintaining fruit yield (Demir et al., 1999). Resistance against *Fusarium oxysporum* induced wilt in cucumber was effectively enhanced by a combination treatment with GAWDA® (an antioxidant formulation designed and patented in Egypt) and an AMF consortia (Elwakil et al., 2013), and exogenous nitrate nutrition operating through modulation of photorespiration (Sun et al., 2021).

Brassinosteroids (BR) are naturally occurring plant steroids with growth regulatory potential, which has been reported to impart chilling stress tolerance in cucumber (*Cucumis sativus*) by a chemico-genetic regulation of oxidative stress management. BR-induced activation of plasma membrane-bound NADPH oxidase (RBOH) results in the upregulation of signaling molecules in the form of H_2O_2, which has a role in activating subsequent stress response pathways (Xia et al., 2009). Exogenous application of 24-Epibrassinolide was found to alleviate the detrimental effects of root zone temperature fluctuations in cucumber seedlings by regulating hormonal and ion homeostasis (Anwar et al., 2018, 2019a,b;

[1]https://www.valentbiosciences.com/soilhealth/solutions/abiotic-stress-mitigation-for-cucurbit-vegetables/

Anwar and Kim, 2020). Amelioration of chilling stress in cucumber seedlings by triadimefon (Feng et al., 2003), selenium (Hawrylak-Nowak et al., 2010), and SA (Kang and Saltveit, 2002) have also been reported. Drought tolerance was found to be enhanced in cucumber plants treated with natural carbon materials like shungite, which led to an increase in antioxidant potential thereby reducing cellular damage (Kim et al., 2019). Although there are many strategies employed to combat stress incidence in cucurbits, development of novel technologies in the form of ameliorative treatments can be effective, provided the right traits and target mechanisms come under the purview of the mitigation strategy.

FUTURE PERSPECTIVES IN CROP IMPROVEMENT FOR MULTIPLE STRESS TOLERANCE IN CUCURBITS

Cucurbits are vulnerable to simultaneous exposure of multiple stressors and hence interventions at various levels are imperative

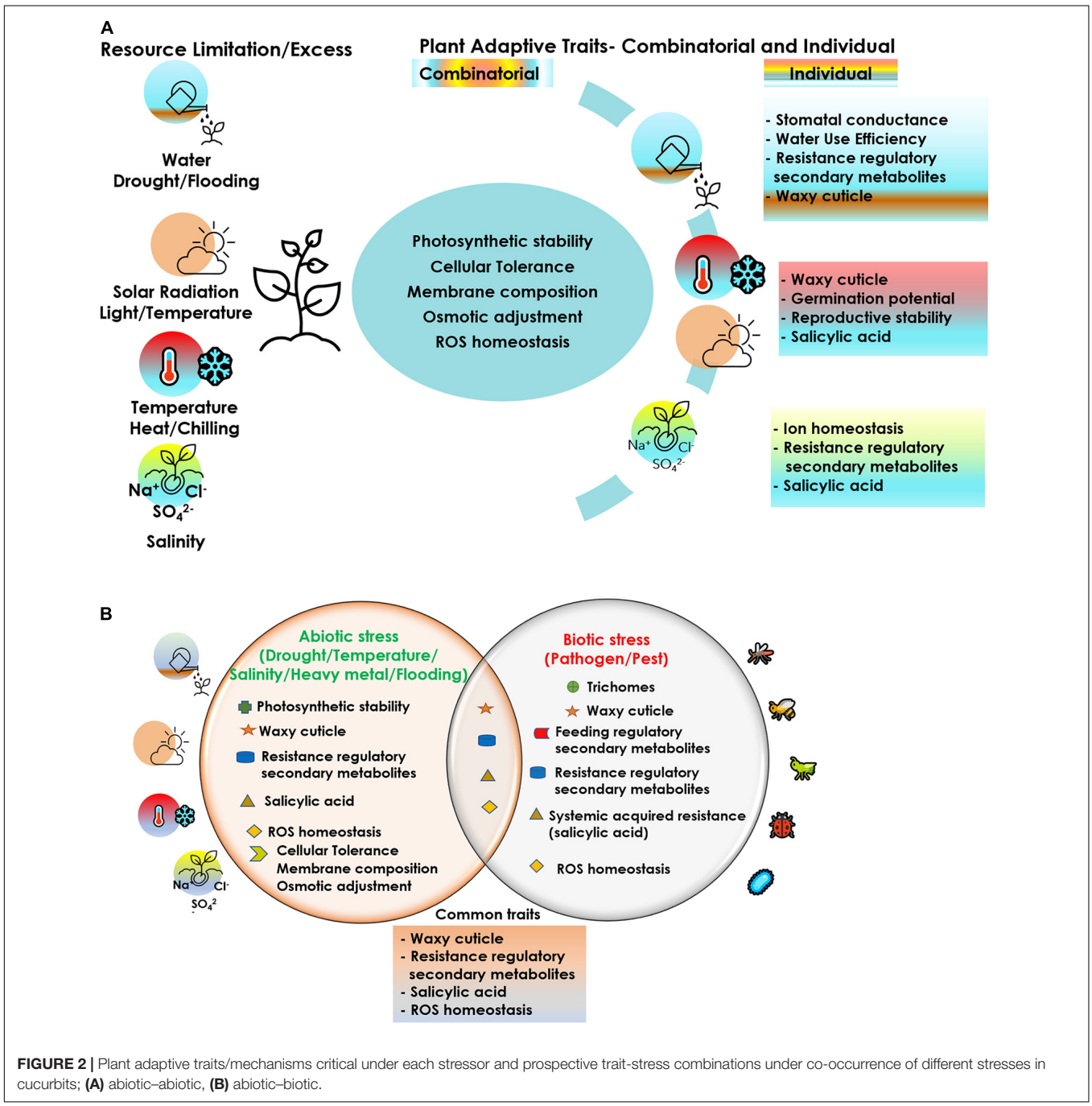

FIGURE 2 | Plant adaptive traits/mechanisms critical under each stressor and prospective trait-stress combinations under co-occurrence of different stresses in cucurbits; **(A)** abiotic–abiotic, **(B)** abiotic–biotic.

to achieve sustained crop production, even under adverse climatic conditions. The choice of the apt component trait for achieving tolerance to a stress episode is the key towards effective crop improvement. Crop improvement relies on the available diversity or creation of diversity as the source of desirable traits, including abiotic and biotic stress resistance. Genetic diversity is facing serious threat due to habitat loss owing to human intervention and climate change. Efforts for collection and conservation of cucurbit germplasm including related species, distant species, wild relatives and landraces needs to be expedited for their utilization in breeding programs, biotechnological approaches and propagation methods. Grafting has evolved as a relatively cheap and quick option for managing the biotic and abiotic stressors. Although a good number of commercial rootstocks have been identified, particularly in the temperate regions, these attempts are very rare in the tropical regions. Hence, systematic testing of genotypes and wild relatives of cucurbits for their potential use as rootstocks against multiple stresses should be a research priority. Rootstock breeding in cucurbits leading to development of vigorous intra and interspecific hybrid rootstocks conferring tolerance to multiple stresses needs urgent attention. Hence, it is very crucial to screen for the right plant traits/characters/mechanisms for employing any of the prospective strategies discussed in this review. A comprehensive account of the different plant adaptive traits/mechanisms critical under each stressor (**Figure 2**) can aid in the adoption of the correct trait(s) combination and the approach in the event of a multiple stress exposure. With climate change posing novel, varied and often multiple stresses to the crops, strategies for screening for multiple stress resistance needs to be evolved and employed. Even a moderate level of resistance for multiple stress factors can confer tolerance to stresses through cross-talk between various pathways and mitigate their damaging effect. Targeted manipulation of tolerance traits will be possible if a better understanding of the combined stress effects is realized. Breeding approaches should focus on gene pyramiding for multiple stress tolerance in cucurbits through a combination of conventional and biotechnological approaches. Robust markers, genomic prediction tools and phenotyping facilities need to be developed for rapid screening and identification of stress tolerant genotypes in cucurbits. Previous attempts in this direction in vegetable crops like onion, including gene prospecting for drought tolerance (Kutty et al., 2012) and molecular marker identification by bulk segregant analysis of drought tolerance in F2 population (Kutty et al., 2013), concerted with physiological, morphological and biochemical characterization of drought stress response in different onion cultivars (Kutty et al., 2014) have been promising. Genes/gene products regulating multiple molecular mechanisms thus identified can be combined together by novel gene stacking approaches to alter multiple traits required for combined stress tolerance, as has been demonstrated in model crop plants (Vemanna et al., 2013; Pruthvi et al., 2014; Parvathi et al., 2015). Multiple trait manipulation employing a 'super regulatory gene/QTL' capable of coordinating numerous trait-related genes can help in developing genotypes tolerant to multiple stresses (Parvathi and Nataraja, 2017; Parvathi et al., 2021). Such initiatives would help in congregating the indispensable traits in an ideal genotype/accession, that would be critical for crop acclimation under a combination of stress challenges. Advanced gene editing technologies can be of great value in the view of multigenic control over tolerance in a multiple stress scenario. A careful and concerted trait-specific approach for each stress combination is imperative to achieve optimal realizable crop performance.

AUTHOR CONTRIBUTIONS

All authors listed have made an equal, direct, and intellectual contribution to the work, and approved it for publication.

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De novo Transcriptome Analysis of Drought-Adapted Cluster Bean (Cultivar RGC-1025) Reveals the Wax Regulatory Genes Involved in Drought Resistance

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Cluster bean (*Cyamopsis tetragonoloba* L.) is one of the multipurpose underexplored crops grown as green vegetable and for gum production in dryland areas. Cluster bean is known as relatively tolerant to drought and salinity stress. To elucidate the molecular mechanisms involved in the drought tolerance of cluster bean cultivar RGC-1025, RNA sequencing (RNA-seq) of the drought-stressed and control samples was performed. *De novo* assembly of the reads resulted in 66,838 transcripts involving 203 pathways. Among these transcripts, differentially expressed gene (DEG) analysis resulted in some of the drought-responsive genes expressing *alpha dioxygenase* 2, low temperature-induced 65 kDa protein (LDI65), putative vacuolar amino acid transporter, and late embryogenesis abundant protein (LEA 3). The analysis also reported drought-responsive transcription factors (TFs), such as NAC, WRKY, GRAS, and MYB families. The relative expression of genes by qRT-PCR revealed consistency with the DEG analysis. Key genes involved in the wax biosynthesis pathway were mapped using the DEG data analysis. These results were positively correlated with epicuticular wax content and the wax depositions on the leaf surfaces, as evidenced by scanning electron microscope (SEM) image analysis. Further, these findings support the fact that enhanced wax deposits on the leaf surface had played a crucial role in combating the drought stress in cluster beans under drought stress conditions. In addition, this study provided a set of unknown genes and TFs that could be a source of engineering tolerance against drought stress in cluster beans.

Keywords: drought stress, transcriptome, wax genes, cluster bean (*Cyamopsis tetragonoloba* L.), differentailly expressed genes

INTRODUCTION

Cyamopsis tetragonoloba (L.) Taub. (Cluster bean) is a drought-adapted annual legume crop with lower water requirements than many other dryland legume crops. Cluster beans can grow in marginal soils because of their high water use efficiency, deep tap rooting system, etc. In India, cluster bean is cultivated for its green vegetables, foraging cattle, green manure, and dry pods for

guar gum production (Rao and Shahid, 2011; Global Agricultural Information Network [GAIN], 2014). Globally, India ranks first and produces about 80% of the world's cluster beans, and Rajasthan is the top state, making 75% of the total production in India. Due to high prices and export demand for guar gum, the cultivation of cluster bean is gradually increased in India from the year 2010 onward, and the total area is about 5,345 ha with 615 kg per ha yield during the agricultural year 2018–2019 (DoA, Government of India, Annual Report). The guar gum produced from cluster beans is rich in galactomannan, which is 78–82% of the seed's endosperm. Guar gum is an essential non-toxic agrochemical, mostly an export product, and a source of polysaccharide emulsifier used primarily in the food, cosmetic, and pharmaceutical sectors (Mudgil et al., 2014). In addition, it is also used in the oil and gas industry as a gelling agent and as an additive in the milling industry (Coveney et al., 2000). The co-products for guar are guar meal and guar bagasse used to produce biofuels and other value co-products (Gresta et al., 2017).

Although cluster bean is considered a highly valued crop, its productivity is lesser than other legume crops; consequently, a significant gap exists between demand and export of guar. Biotic and abiotic factors are major limiting factors for guar yield enhancement. Thus, there is a need to increase the productivity of cluster beans to meet the demand-supply gap of cluster beans and their derivatives through genetic enhancement (Kumar et al., 2020). To increase guar output, it is necessary to produce cluster bean cultivars with improved abiotic stress tolerance, particularly drought resistance, for growing in the semi-arid tropics. The genetic improvement of cluster beans for enhanced drought resistance is not achieved due to insufficient genomic resources and inadequate germplasm availability. *C. tetragonoloba* genome size has been estimated to be approximately 580 Mbp using flow cytometry (Tyagi et al., 2019). The present study adopted next-generation sequencing (NGS) technologies to understand the detailed information of the drought-stressed transcriptome of cluster beans. This technology enables the identification of differentially expressed genes (DEGs), the deciphering of metabolic pathways involved in drought resistance, gum biosynthesis, and the identification of DNA-based markers, all of which may open up new avenues for molecular breeding to improve cluster bean production, gum quality, and stress resistance.

For the past few decades, extensive research has been carried out on applying omics technologies to identify many candidate genes, proteins, and metabolic pathways of various crop species under different stress conditions (Panda et al., 2021; Raza et al., 2021). In recent years, transcriptome technology has become an essential tool for analyzing the molecular mechanisms of abiotic stresses in plants (Cai et al., 2019; Hasan et al., 2019). Global transcriptome profiling of drought-stressed grain legumes, such as chickpea (Garg et al., 2011; Hiremath et al., 2011; Kumar et al., 2019), groundnut (Brasileiro et al., 2015; Zhao et al., 2018), and lentil (Singh et al., 2017; Morgil et al., 2019), revealed a set of DEGs involved in various metabolic pathways under stress conditions. Following transcriptome analysis, Wu et al. (2016) have identified 22 NAC TFs from drought-tolerant and drought-sensitive genotypes of common bean. Transcriptome analysis of drought-tolerant and drought-sensitive genotypes of wheat showed significant induction or repression of genes involved in secondary metabolism, nucleic acid synthesis, protein synthesis, and transport in the tolerant genotype when compared with the sensitive genotype (Kumar et al., 2018). RNA sequencing (RNA-Seq) analysis has been employed to elucidate drought-tolerance molecular mechanisms in other crops, such as cotton (Hasan et al., 2019), buckwheat (Hou et al., 2019), and Proso millet (Zhang et al., 2019). To date, very few studies on the transcriptome analysis of *C. tetragonoloba* have been published. For instance, Rawal et al. (2017) reported an RNA-Seq-based transcriptome from the leaf, shoot, and flower tissues of Guar; Tanwar et al. (2017) detailed the transcriptome of leaf tissues from two leaf tissue guar varieties M-83 and RGC-1066. Al-Qurainy et al. (2019) published a transcriptome of guar, accession BWP 5595 under various treatments, such as drought, salinity, and heat stress. The present study was focused on targeted transcriptome deep sequencing of a drought-adapted cultivar RGC-1025 to characterize the genes responsible for the drought resistance. The gene information thus obtained would pave the way for using DEGs in developing strategies for drought resistance through various approaches. Moreover, transcriptome data sets could be valuable for novel gene discovery and the marker-assisted selective breeding of cluster bean species.

MATERIALS AND METHODS

Screening Cluster Bean Cultivars for Drought Tolerance

Initially, four cluster bean cultivars, namely, RGC-1025, RGC-1038, RGC-1055, and RGC-1066, were screened for their drought tolerance based on various parameters, such as germination, seedling growth, biomass, relative water content (Barrs and Weatherley, 1962), cell membrane injury (Leopold et al., 1981), malondialdehyde (MDA) (Hodges et al., 1999), total chlorophylls (Arnon, 1949), and total proline content (Bates et al., 1973).

Plant Samples, Processing, and Sequencing

Seeds of cluster bean (*C. tetragonoloba* L.) cultivar RGC-1025 were sterilized in 0.5% (W/V) sodium hypochlorite solution for 5 min, then rinsed thoroughly, and soaked in distilled water for 30 min. Seeds were sown in earthen pots containing soil and farmyard manure in a 3:1 proportion maintained in the departmental botanical garden. After 20 days post-sowing, drought stress was induced by withholding water to one set of pots, and respective fully watered controls were maintained in another set of pots. Ten days after stress imposition, fresh leaf samples from five plants were collected, pooled, flash-frozen in liquid nitrogen, and transported immediately to the sequencing facility.

For total RNA-seq, total RNA was extracted using the Qiagen RNeasy Plant Mini Kit with DNAse treatment (Thermo Fisher Scientific, United States) as per the manufacturer's instructions. The quality and quantity of the RNA were estimated using

a NanoDrop Spectrophotometer (Thermo Fisher Scientific, United States) and Qubit Fluorometer (Thermo Fisher Scientific, United States). The integrity of the RNA samples was analyzed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, United States). RNA-seq libraries were prepared with Illumina-compatible NEB Next® Ultra™ II Directional RNA Library Prep Kit (New England BioLabs, MA, United States). In total, 500 ng of total RNA was taken for mRNA isolation, fragmentation, and priming. Fragmented and primed mRNA was subjected to first-strand synthesis followed by second-strand synthesis. The double-stranded cDNA was purified using JetSeq Clean Beads (Bioline Meridian Bioscience, Australia). Purified cDNA was end-repaired, adenylated, and Illumina multiplex barcode adapters were ligated as per NEBNext® Ultra™ II Directional RNA Library Prep protocol, followed by second-strand excision using USER enzyme at 37°C for 15 min. Adapter-ligated cDNA was purified using JetSeq Beads and was subjected to 10 cycles for indexing (98°C for 30 s, cycling (98°C for 10 s, 65°C for 75 s) and 65°C for 5 min) to enrich the adapter-ligated fragments. The final PCR product (sequencing library) was purified with JetSeq Beads, followed by a library-quality control check using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, United States). A total of 7,629,816 short reads were obtained, 150 bp length paired-end (P.E.) reads and average fragment size of ∼400 bp were used, and three biological replicates were processed for sequencing analysis with Illumina HiSeq™ 4000, which was out-sourced at Genotypic Technologies, Bengaluru, India.

Read Quality Control, Adapter Removal, *de novo* Assembly, and Clustering

The reads were processed for quality assessment and low-quality filtering before the FastQC tool assembly. The reads were then processed by removing the adapter sequences and low-quality bases (<q30) using the Cutadapt tool. Processed reads were assembled using a graph-based approach by the rnaSPAdes program. The characteristic properties, such as N50 length, average length, maximum length, and a minimum length of the assembled contigs, were calculated. *De novo* transcriptome assembly of the processed reads from all the libraries was done using Bowtie2 with end-to-end parameters. In the second step of the assembly procedure, clustering of the assembled transcripts based on sequence similarity is performed using the Cluster Database at High Identity with Tolerance (CD-HIT)-EST program[1] with 95% similarity between the sequences. This reduces the redundancy without excluding sequence diversity used for further transcript annotation and the DEG analysis.

Functional Annotation of Transcripts

All unigenes were annotated using the BLASTX search tool on *Viridiplantae* transcripts from the UniProt database containing 8,058,045 protein sequences and the NCBI non-redundant database (N.R.). The cutoff e-value was $10^{-5,}$ and the minimum similarity was more significant than 40%. Gene ontology annotation was carried out using the Blast2go program and visualized using Web Gene Ontology Annotation Plot (WEGO).[2]

Differentially Expressed Gene Analysis and Pathway Analysis

DESeq, an R package, was used for differential expression analysis. Sequencing (variable library size/depth) bias among the samples was removed by library normalization using size factor calculation in DESeq. DESeq normalized expression values were used to calculate fold change for a given transcript. The regulation for each transcript was assigned based on log2-fold change. The transcripts that show a log2-fold change less than −1 are represented as downregulated. The values greater than one are upregulated and between −1 and 1 are termed neutrally regulated. Gene Ontology (GO) enrichment analysis and pathway analysis for DEG were done against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. KAAS server was used to analyze and characterize associated pathways. To obtain the highly significant differential expression genes, the criterion of the absolute value of reads per kilobase of transcript per million reads (RPKM) ratio > 1,000 was used.

Mining of Simple Sequence Repeats

Simple sequence repeats (SSRs) were identified using the MISA Perl script in each transcript (MIcroSAtellite identification tool).[3] A simple repetition of motif length ranging from 1 to 6 bp was identified with recommended default parameters of MISA.

qRT-PCR Analysis of Gene Expression

To evaluate the gene expression pattern from the DEG analysis, the total RNA extracted, as mentioned earlier, from control and drought-stressed cluster bean variety RGC-1025 was used for the SYBR Green qRT-PCR assay. The qRT-PCR assay was performed for 16 different stress-responsive genes selected from DEG analysis. These genes include *aldo-keto reductase 1* (*AKR1*), *late embryogenesis Abundant14* (*LEA14*), *non-specific lipid transfer protein*, TFs *MYB30*, *NAC4*, *scare crow-like protein1* (GRAS TF's), *BHLH*, *GATA*, *malate dehydrogenase* (*MDH*), *aquaporin*, *DNA helicase*, *nitrate reductase*, *proline dehydrogenase* (*PRODH*), *serine hydroxy methyltransferase* (*SHMT*), and *thaumatin, trehalose 6-phosphate phosphatase* (*TRE6PH*) with actin and tubulin genes as an internal control. For cDNA synthesis, 1 μg of total RNA from control and drought-stressed cluster bean RGC-1025 samples was treated with a Turbo DNASE treatment kit (Thermo Fisher Scientific, United States) as per the manufacturer's protocol to remove any DNA traces. cDNA was synthesized using Revert Aid M-MuLV Reverse Transcriptase (Thermo Fisher Scientific) as per the manufacturer's instructions. qRT-PCR mix was comprised of 1× using Power SYBR Green Master Mix (Ambion, United States), 20 ng of cDNA, and 0.2 μM of forward and reverse primers. **Supplementary Table 1** shows the primers used for the investigated genes. The RT-PCR analysis was done on Applied Biosystems Step One Real-Time PCR machine with standard cycling comprising 95°C for 30 s, 40

[1]http:www.bioinformatics.org/cd-hit/

[2]http://wego.genomics.org.cn/cgi-bin/wego/index.pl

[3]http://pgrx.ipk-gatersleben.de/misa/

cycles of 95°C for 1 s, 60°C for 20 s, and a melt curve analysis. Relative quantification was studied using $2^{-\Delta\ \Delta}$ CT method (Livak and Schmittgen, 2001). Each gene was analyzed in three biological samples, and three reaction replicates were performed for each biological sample.

Estimation of Epicuticular Wax and Scanning Electron Microscope Imaging of Leaf Surfaces for Wax Deposits

Leaf surface waxes exteriorly deposited were extracted and quantified by a colorimetric assay reported by Mamrutha et al. (2010). Carnauba wax was used as the standard for the wax quantification assay. The wax content is represented as μg/gm fresh weight.

An scanning electron microscope (SEM) examined the epicuticular wax crystals. The third and fourth leaves of drought-stressed and control plants were cut to 0.5 cm, mounted onto standard stubs, and coated with gold particles using a fully automated vacuum spotter smart coater (DII-29030SCTR, JOEL, United States). The surfaces of the coated samples were observed through an SEM (JOEL-JSM-IT500, Japan).

RESULTS

Screening Cluster Bean Cultivars for Drought Tolerance

Cluster bean cultivars, namely, RGC-1025, RGC-1038, RGC-1055, and RGC-1066, were screened for drought tolerance and we found significant differences at the cultivar level in morphological and biochemical traits under stress treatments. Results revealed that among four cultivars evaluated; cultivar RGC-1025 showed a lesser decrease in seedling growth, better biomass, and relative water content; lesser extent of cell membrane injury, lesser MDA, total chlorophylls content, and significantly higher levels of osmoprotectant, proline when compared to other cultivars (**Supplementary Tables**), suggesting the relative tolerance of cultivar RGC-1025 over other cultivars to drought stress. Therefore, we further extended transcriptome studies to understand the molecular mechanisms conferring the drought tolerance of cultivar RGC-1025.

Reads and *de novo* Assembly

To construct the transcriptome of cluster bean cultivar RGC-1025, high-quality RNAs from three replicates of drought-stressed and unstressed conditions (control) were sequenced. An average of 18 million paired-end reads were used for the downstream analysis after pre-processing. In total, 76,129,816 short reads were obtained using Illumina HiSeq 4000 Technology (**Table 1**). Most of the reads had > 99.9% score. Around 97.5% of the reads were retained in both the samples post-filtering. The cleaned-up reads were assembled using the Bowtie2 tool. CD-HIT was used to cluster redundant and similar isoforms. Finally, 66,838 transcripts were clustered with an average length of 955 bp. The non-redundant transcripts were considered as unigenes and were further analyzed.

Characterization of Unigenes

All the obtained unigenes were annotated against the Uniprot Viridiplantae sequence database, NCBI non-redundant database, with a cutoff E-value of 10^{-5}. Around 55.98% of the unigenes were found to have hits in the public databases. The transcripts with more than 30% identity were considered during the analysis. These unigenes were classified as 31 functional categories. The significant fall under the category of DNA templated transcriptional regulation (2.34%) among the biological processes, the category of integral component of the membrane (25.4%) under cellular components, and the category of adenosine 5′-triphosphate (ATP) binding (13.14%) under molecular function. The highest transcript matches during the functional annotation with members of the family Fabaceae, such as *Glycine* (10,131), *Mucuna* (5,826), *Cajanus* (4,533), *Cicer* (2,632), *Vigna* (2,183), *Phaseolus* (2,111), *Arachis* (1,806), *Trifolium* (1,597), and *Medicago* (1,524) (**Figure 1**).

Functional Classification

Differentially expressed genes were subjected to GO analysis to achieve functional classification. As a result, 37,418 DEGs fall into (i) molecular function, (ii) biological process, and iii) cellular components. In total, 30,900 (50.2%) DEGs were associated with molecular function terms, such as ATP binding encoding transcripts, followed by metal ion binding transcripts, DNA binding transcripts, zinc ion binding transcripts, nucleic acid binding transcripts, protein kinase activity transcripts, etc., and 16,410 (26.6%) DEGs were annotated with cellular component terms, represented by the integral component of the membrane encoding transcripts, followed by nucleus components transcripts, cytoplasm components transcripts, ribosome transcripts, plasma membrane transcripts, retrotransposon nucleocapsid transcripts, etc., and 14,191 (23%)

TABLE 1 | Sample wise assembly statistics of cluster bean cultivar RGC-1025 samples.

S. no	Samples	Control	Stressed
1	Raw reads	43,110,222	33,019,594
2	Processed reads	42,051,780	32,208,984
3	Percentage of reads retained	97.5%	97.5%
4	Alignment to clustered transcripts (%)	89.00%	90.65%
5	Number of transcripts identified	123,594	106,025
6	Maximum contig length	21,802	15,999
7	Minimum contig length	31	31
8	Average contig length	489.6	521.9
9	Median contig length	261	266
10	Total contigs length	6,050,9220	55,334,262
11	Total number of non-ATGC characters	5,596	4,419
12	Contigs ≥ 100 bp	123,411	105,933
13	Contigs ≥ 200 bp	97,982	84762
14	Contigs ≥ 500 bp	26,684	25,767
15	Contigs ≥ 1 Kbp	15,221	14,891
16	Contigs ≥ 10 Kbp	5	7
17	Contigs ≥ 1 Mbp	0	0
18	N50 value	875	1003

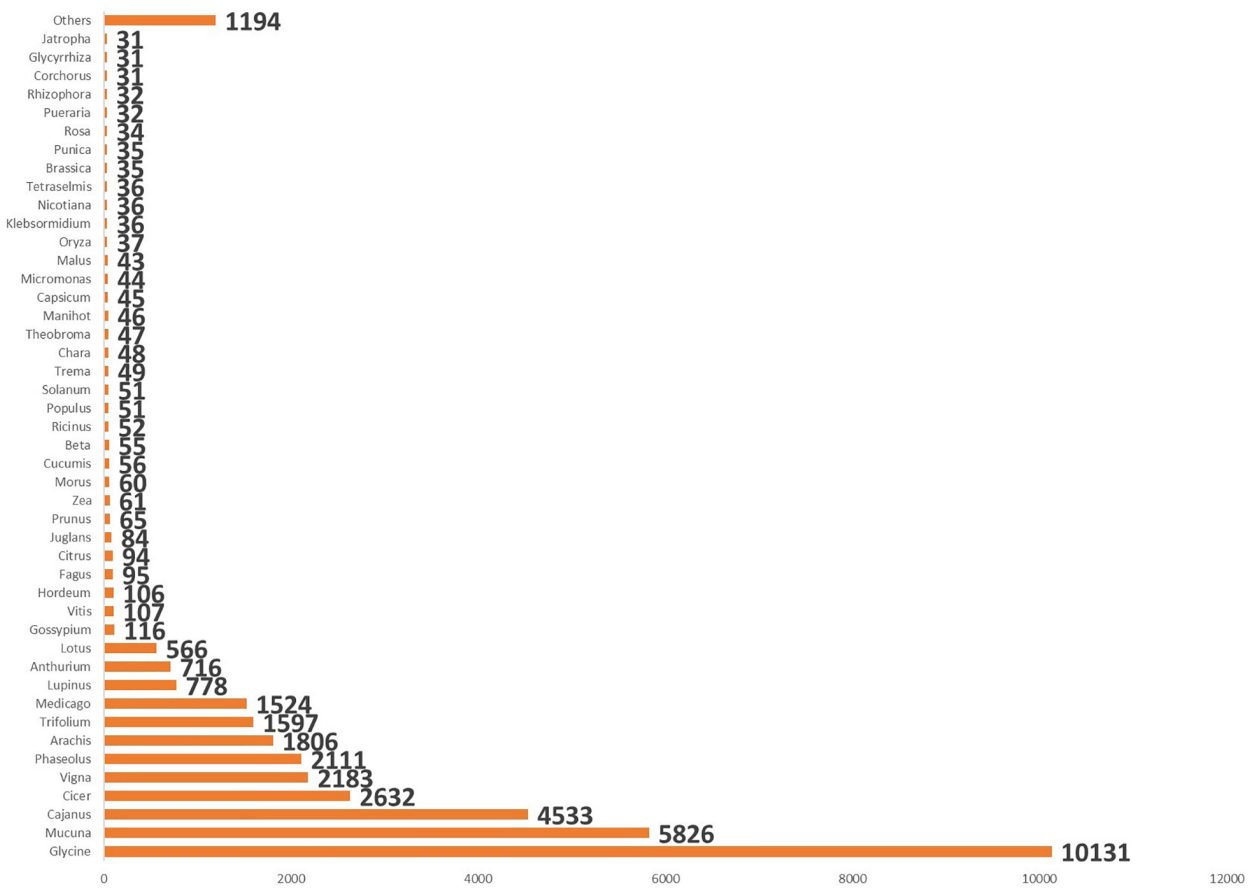

FIGURE 1 | Bar chart shows the number of BlastX hits of cluster bean cultivar RGC-1025 with maximum hits from members of the family Fabaceae.

DEGs were associated with biological process terms. This study also observed that identical DEG sequences could exist in more than one category. The most represented "biological process" subcategories identified were DNA-templated transcriptional regulation encoding transcripts followed by translation components, carbohydrate metabolic process transcripts, DNA integration transcripts, signal transduction transcripts, intracellular protein transcripts, etc. (**Figure 2** and **Supplementary Tables 2–4**). In general, 12,866 genes were found to be upregulated, 16,177 genes were downregulated, and 27,782 genes had shown no change in their expression levels in cluster bean cultivar RGC-1025 due to drought stress. More interestingly, 3,745 transcripts were expressed only in the stressed sample.

Differentially Expressed Genes in Kyoto Encyclopedia of Genes and Genomes Pathways

Kyoto Encyclopedia of Genes and Genomes is an online database that deals with genomes and enzymatic pathways, and its identifiers were looked to predict biochemical pathways related to DEGs. Among 66,838 transcripts in unigene pathways, 17,211 transcripts against the KEGG pathways were identified. Of the 203 pathways identified, the top forty pathways are shown in **Supplementary Table 5**. DESeq analysis of the transcripts revealed that the enzymes with the most frequency of expression in the category of upregulated genes were alpha dioxygenase 2 (9.4-fold), low temperature-induced 65 kDa protein (LTI65; 9.2-fold), putative vacuolar amino acid transporter (9.05-fold), hexosyl transferase (EC 2.4.1.-; 7.95 fold), late embryogenesis abundant protein3 (LEA 3; 7.79-fold), Putative anthocyanidin 3-O-glucoside 2″-O-glucosyltransferase (EC 2.4.1.297; 7.44-fold), Glucosyltransferases, Rab-like GTPase Activators and Myotubularins (GRAM) domain protein/abscisic acid (ABA)-responsive-like protein (putative GRAM domain, P.H. domain-containing protein) (7.30-fold), and cytochrome P450 monooxygenase (EC:1.14.14.80; 7.14-fold). In the category of downregulated genes, the genes encoding the following proteins were found to be downregulated, such as putative CDP-alcohol phosphatidyl transferase class-I family protein 3 (EC 2.7.8.1; 0.5-fold), NEDD4-binding protein 2 (0.49-fold), dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (EC 2.3.1.-; 0.49-fold),

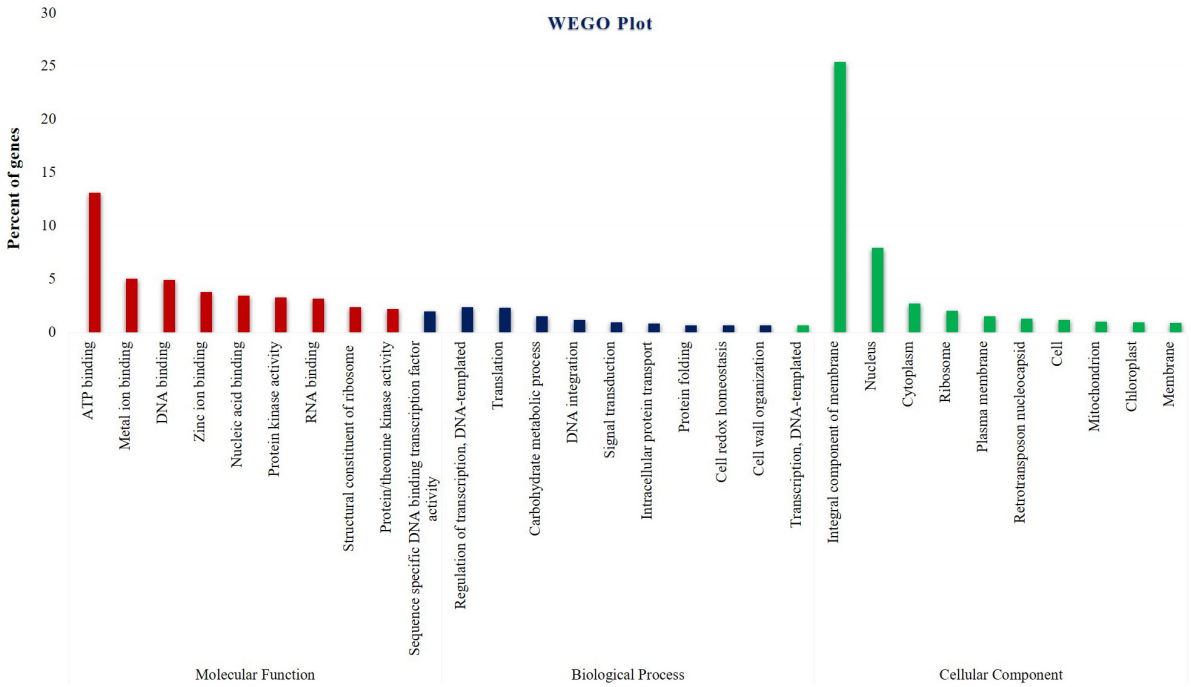

FIGURE 2 | Pie chart shows drought-stressed cluster bean cultivar RGC-1025 Gene Ontology classification of transcripts into biological process, molecular functions, and cellular components.

putative cyclic nucleotide-gated ion channel 14 (0.498), ATP binding cassette (ABC) transporter G family member 28 (0.49-fold), N-(5-phosphoribosyl) anthranilate isomerase (putative phosphoribosyl anthranilate isomerase) (EC 5.3.1.24; 0.49-fold), F-box/FBD/LRR-repeat protein (0.49-fold), and adenylate kinase (EC:2.7.4.3; 0.49-fold). The heat map of DEGs showed the genes expressed (**Figure 3**).

Differentially Expressed Transcription Factors

Among the drought stress-responsive upregulated TF families, NAC family TF was the most abundant (26%), followed by MYB TFs (12%) and WRKY TFs (9%) (**Figure 4A**). **Figure 4B** depicts the top 40 upregulated TFs under drought treatments in cluster bean cultivar RGC-1025. Most renowned drought stress-responsive TFs upregulated include NAC4, NAC3, NAC29, NAC 104, and NAC18 from the NAC family, followed by WRKY12, WRKY50, WRKY6, WRKY33, WRKY30, WRKY24, WRKY42, WRKY53, WRKY70, and WRKY7 from WRKY family, further followed by other TFs, such as homeobox domain TFs, scarecrow/GRAS TFs, and ethylene-responsive TFs. Among the downregulated TF families, the hemophagocytic lymphohistiocytosis (HLH) TF family was the most abundant (20%), followed by the cellular TF family (10%) and homeobox domains (∼8%) (**Figures 4C,D**).

Simple Sequence Repeat Mining

A total of 21,494 SSRs were identified in the cluster bean data, 14,434 (67.15%) are mono-nucleotide repeats, 3,072 (14.29%) are di-nucleotide repeats, 3,516 (16.35%) are tri-nucleotide repeats, 341 (1.58%) tetra-nucleotide repeats, 69 (0.32%) pentanucleotide repeats, and 63 (0.25%) hexanucleotide repeats (**Figure 5**). The 21,494 potential SSRs identified from *de novo* transcriptome sequencing data represent a significant addition to the limited set of genic-SSR markers available in cluster bean cultivar RGC-1025.

Validation of Differentially Expressed Genes by Quantitative Real-Time RT-PCR

To verify the reliability of the expression profiles from RNAseq data, qRT-PCR analysis was performed for DEG (**Figure 6**). Results of the qRT-PCR assay for 16 stress-responsive genes revealed consistency in gene expression patterns as compared to that of the DEG analysis of the transcriptome of RGC-1025. The relative fold change of both the qRT-PCR and NGS-DEG is represented (**Figure 6**). Upregulation of stress-responsive TFs, such as NAC4, MYB30, scarecrow-like protein (SCL-1), primary helix-loop-helix TF (SlbHLH22), and TF bHLH 22, and the overexpression of candidate stress-responsive functional genes, such as DNA helicase, MDH, AKR1, LEA14, PDH, and SHMT, during drought stress by improving the ROS scavenging system, increasing osmotic potential, stomatal regulation, pH stability, respiration, and β-oxidation of fatty acids could support further the drought tolerance of cluster bean cultivar RGC-1025 (Ahmad et al., 2017; Waseem et al., 2019). The correlation among the expression patterns of the genes in NGS and qRT-PCR represents the consistency of the data in the current study.

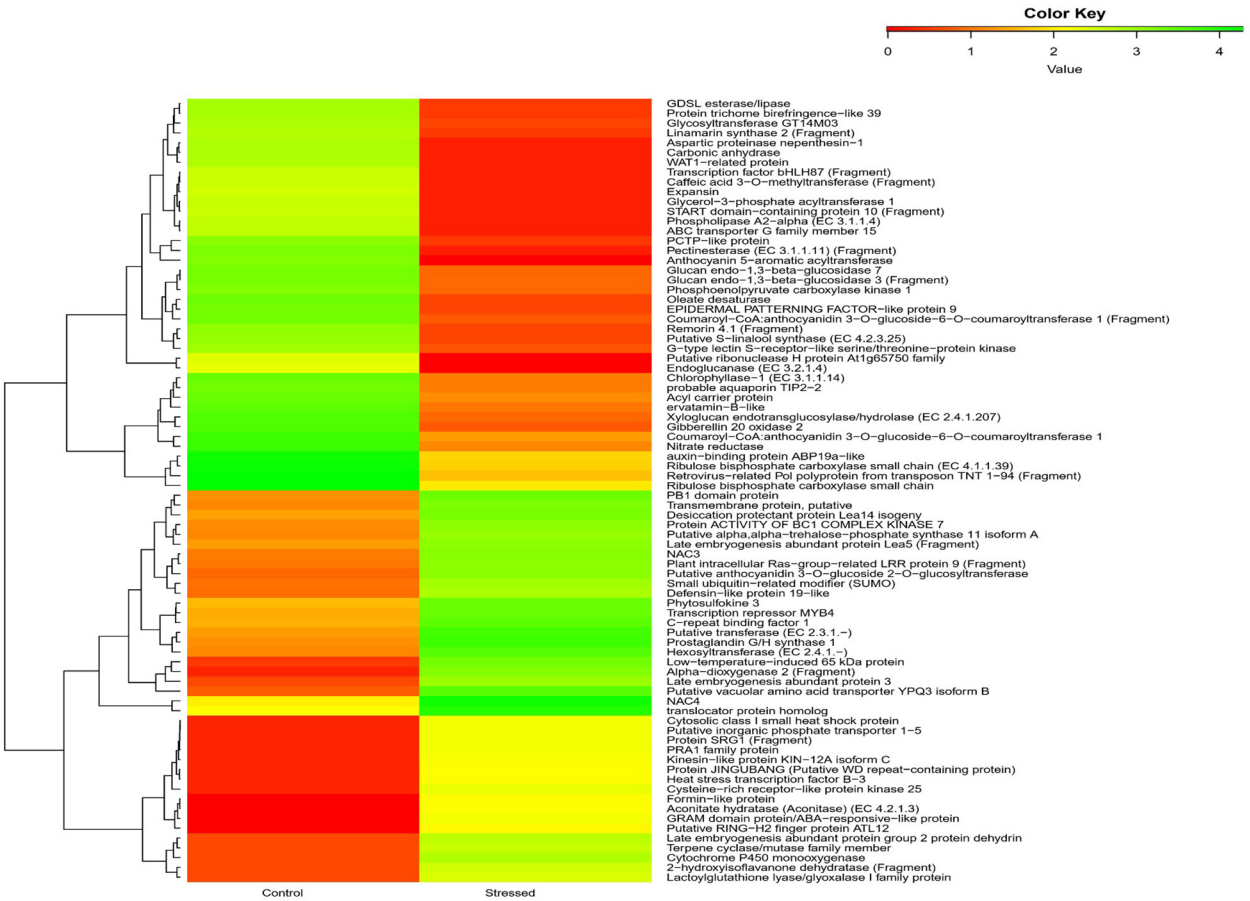

FIGURE 3 | Heatmap shows the top 40 upregulated and downregulated genes of cluster bean cultivar RGC-1025 under control and drought stress.

Epicuticular Wax Content and Scanning Electron Microscope Imaging of Leaf Surfaces

The epicuticular wax content of the cluster bean cultivar RGC-1025 during the drought stress was 610.36 ± 0.53 μg/dm^2 as compared to the control 432.41 ± 0.4 μg/dm^2, which is 41.15% higher as compared to its control. These epicuticular wax data were supported by the SEM imaging of the leaf surfaces of control and drought-stressed plants. The SEM image of the leaf surface of RGC-1025 under drought stress showed enhanced wax crystal deposits (**Figure 7**).

Mapping of the Wax Biosynthesis Pathway in Cluster Bean Using Differentially Expressed Gene Data

Among the genes differentially expressed in wax biosynthesis pathway, there were upregulated genes that include *KCS1* that encodes β-ketoacyl-CoA synthase 1 that is involved in elongation of 24C fatty acids, *WSD1* that encodes wax ester synthase/diacylglycerol acyl transferase, which involves in wax ester biosynthesis, *KCR1* that encodes β-Ketoacyl-CoA reductase, which is involved in very long-chain fatty acid elongation (VLFCA elongation), FATB that encodes acyl-acyl carrier protein thioesterase, which is engaged in supply of saturated fatty acids for wax biosynthesis, *CER4/FAR3* that encodes alcohol forming fatty acyl CoA reductase, which is involved in formation of C24:0 and C26:0 primary alcohols, protein WAX2 encoding gene, CER17 also called Eceriferum1, which encodes for acyl-CoA desaturase-like 4 protein that is involved in n-6 desaturation of very long-chain acyl-CoAs, ABC transporter G family member 11, which encodes ABC transporter proteins that is involved in secretion of surface waxes in interaction with CER5, which is an another ABC transporter protein, and lipid transfer protein gene that encodes a lipid transport protein, which has role in cuticular wax export or accumulation. Finally, the upregulation of these wax genes in the present study through transcriptome DEG data reveals that these gene products are responsible for accumulating or producing epicuticular wax in cluster bean cultivar RGC-1025 (**Figure 8**). qRT-PCR analysis of selective wax genes showed significant changes in the expression patterns and an increase in the expression of the KCS1 gene was 1.85-fold, WSD1 gene was 1.81-fold, KCR1gene was 3.73-fold, FATB gene was 1.8-fold, CER4/FAR3 gene was 0.4-fold, protein WAX2 gene was 2.36-fold, CER17 gene was 2.89-fold, ABC transporter G family member11 gene was 1.95-fold, and lipid transfer protein gene was 1.49-fold.

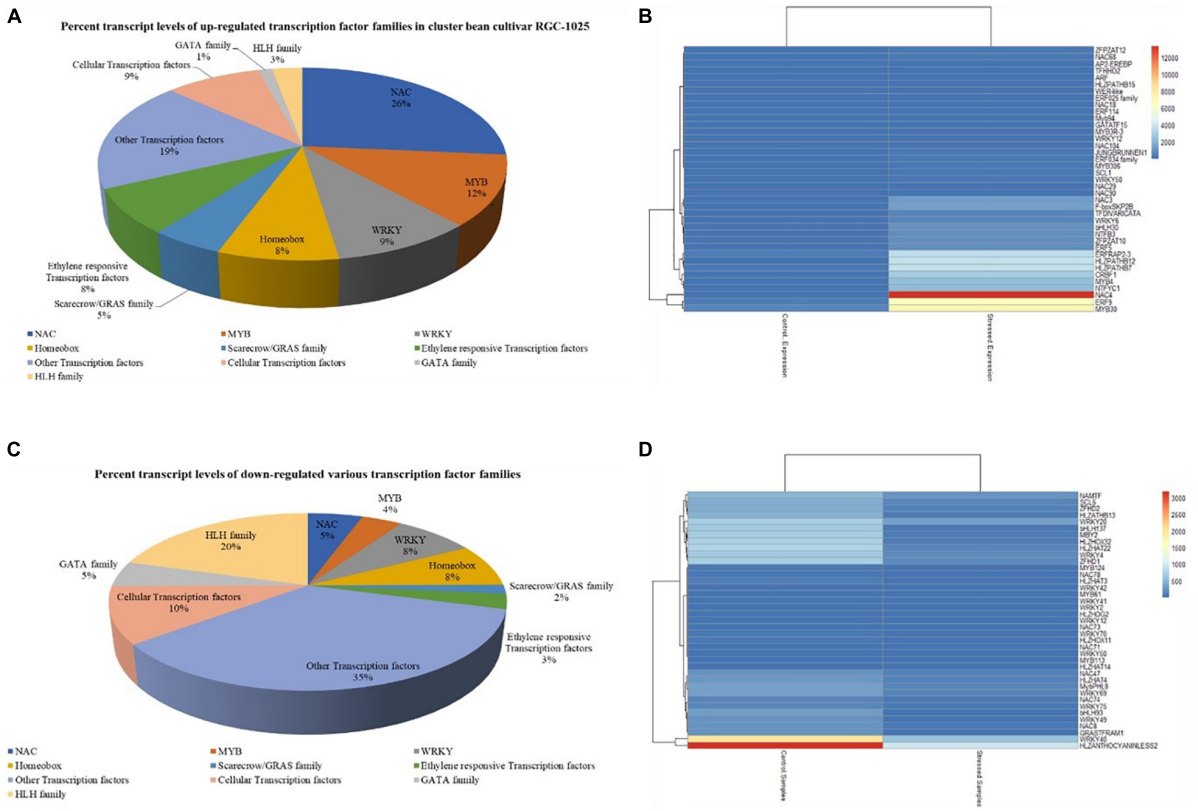

FIGURE 4 | (A) Upregulated transcription factor families of drought-stressed cluster bean cultivar RGC-1025. **(B)** Heatmap of the top 40 upregulated transcription factors of cluster bean cultivar RGC-1025 in drought stress. **(C)** Downregulated transcription factor families of drought-stressed cluster bean cultivar RGC-1025. **(D)** Heatmap of the top 40 downregulated transcription factors of cluster bean RGC-1025 in drought stress.

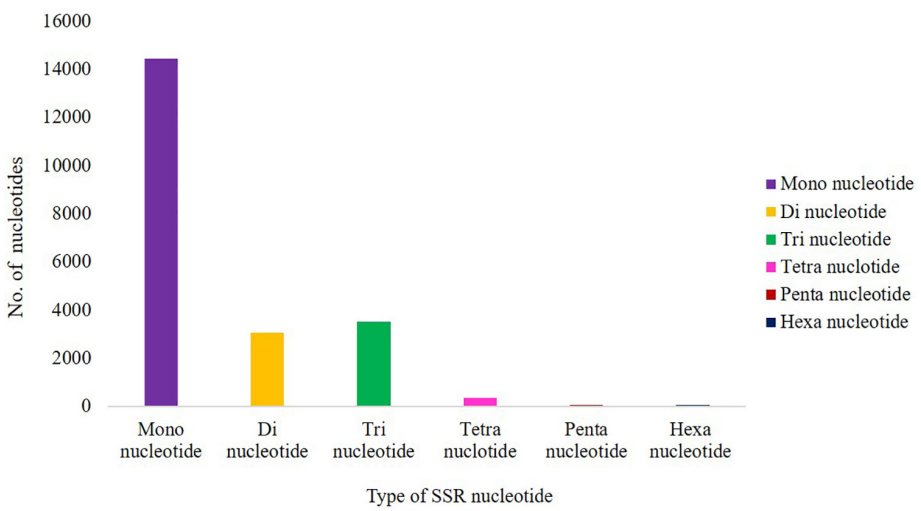

FIGURE 5 | Distribution of different classes of SSRs in cluster bean cultivar RGC-1025.

DISCUSSION

Cluster bean (*C. tetragonoloba* L.) is an annual legume crop grown in arid and semiarid regions. Due to the lack of genomic resources, presently, conventional breeding is the only means of cluster bean improvement. In this regard, the availability of genomic resources can serve as a good platform for cluster bean improvement (Naoumkina et al., 2007; Tanwar et al., 2017).

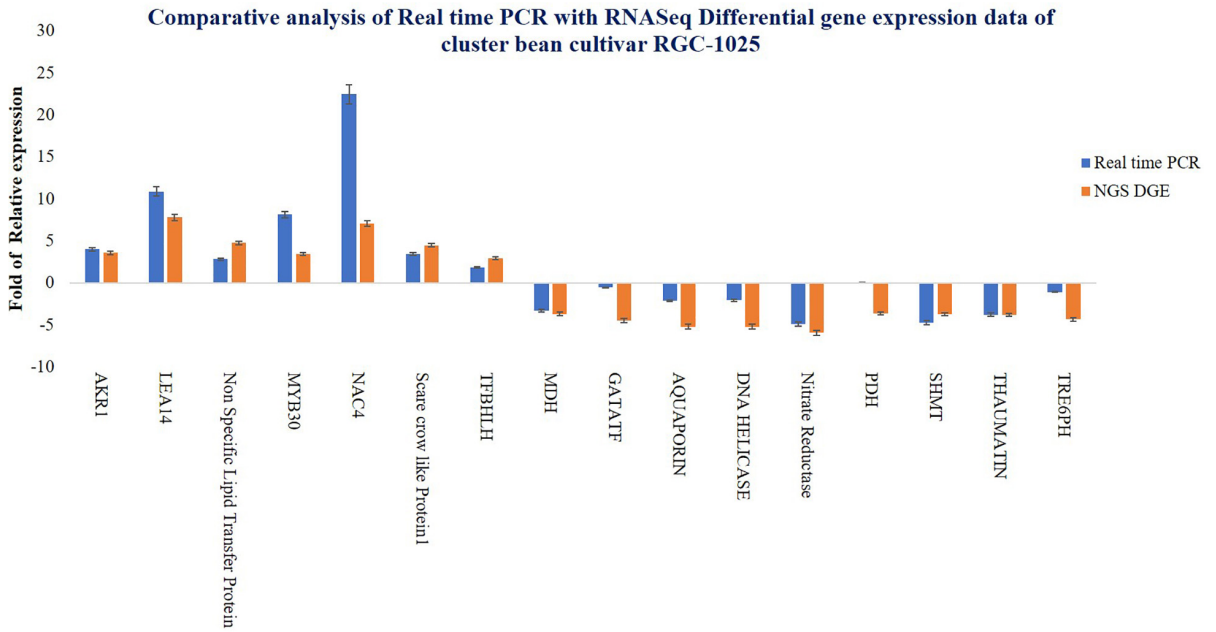

FIGURE 6 | Comparison of real-time PCR data and RNA-Seq differential gene expression data of cluster bean cultivar RGC-1025.

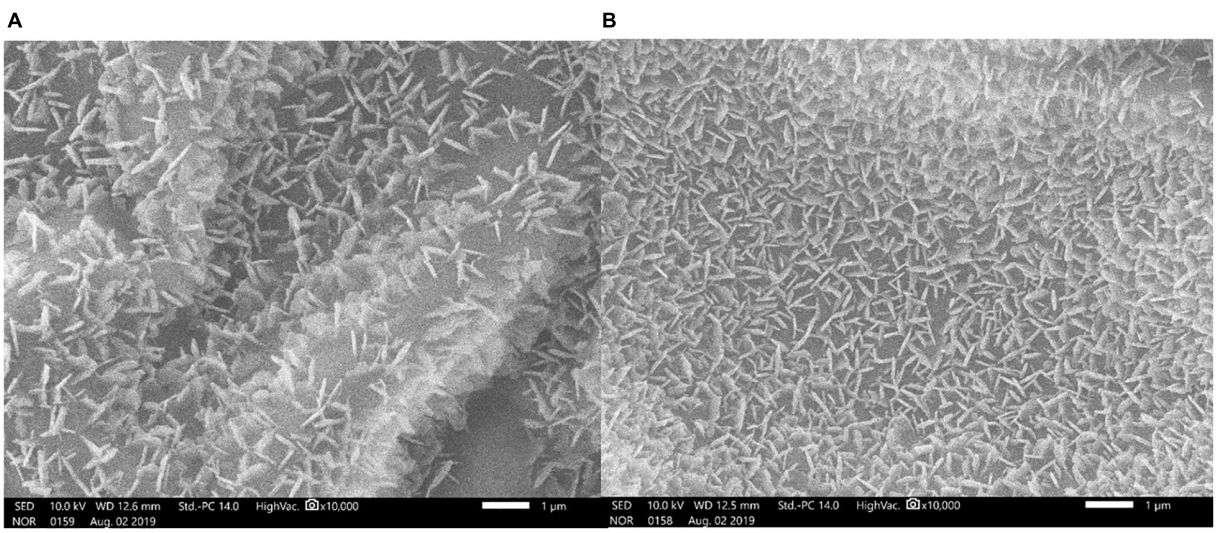

FIGURE 7 | Scanning electron microscopy (SEM) analysis (2,000 × with 20 µm bar scale) of epicuticular wax depositions on the leaf surfaces of cluster bean cultivar RGC-1025 **(A)** control leaf and **(B)** drought-stressed leaf.

Cluster bean is known as relatively tolerant to abiotic stresses. Genotypic variation in stress tolerance exists in cluster bean cultivars (Alshameri et al., 2017), implying that it is a valuable repository for genes that are resistant to these abiotic stresses, and to use this genetic tank, the present study implemented a *de novo* transcriptome analysis of a drought-tolerant cluster bean cultivar RGC-1025.

The RNA-Seq (NGS) method offers a holistic view of the transcriptome, revealing many novel transcribed regions, splice isoforms, genic microsatellites, and the precise location of transcription boundaries (Cloonan et al., 2008; Wang et al., 2009; Li et al., 2010; Wilhelm et al., 2010). These technologies have been widely exploited in numerous plant species to produce molecular markers using transcriptome analysis (Dutta et al., 2011; Wang et al., 2014). In the present study, Illumina HiSeq 4000 Technology generated 76,129,816 short reads from the control and drought-stressed samples of cluster bean cultivar RGC-1025.

A cluster bean is a non-model plant without prior genome knowledge; BLASTX was used to search for sequence similarity and compare the assembled unigenes of the cluster bean

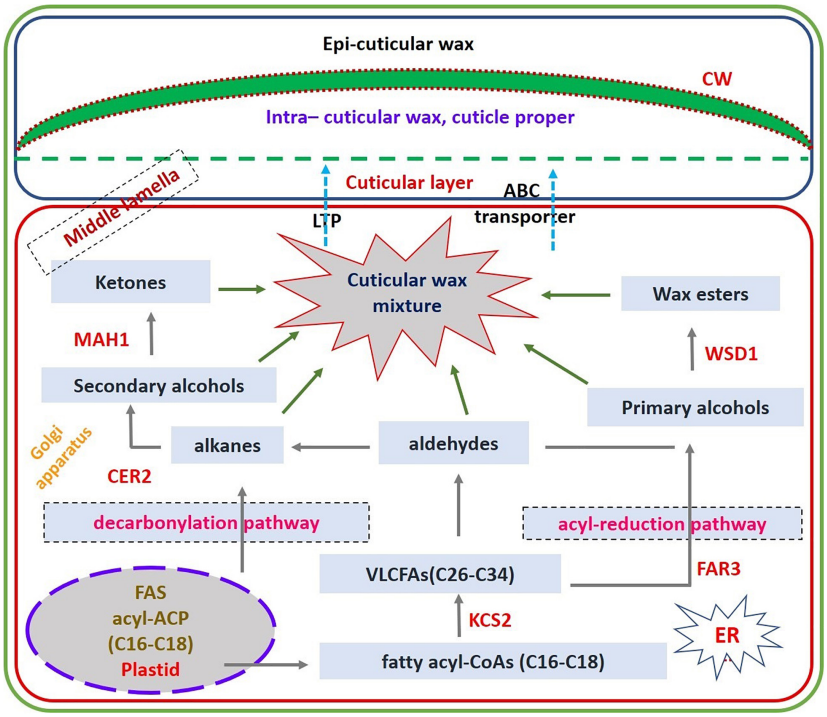

FIGURE 8 | Upregulated genes involved in Wax biosynthesis pathway.

transcriptome against multiple databases. Around 55.98% of the unigenes were obtained and annotated against the Uniprot Viridiplantae sequence database and NCBI non-redundant database, with a cutoff E-value of 10^{-5}. According to species distribution analyses, many plant species have sequences that are homologous to cluster bean sequences. The highest transcript matches during the functional annotation with members of the family Fabaceae, such as *Glycine* (10131), *Mucuna* (5826), and *Cajanus* (4533).

Gene Ontology analysis provides a set of dynamically controlled and structured vocabularies for describing the roles of genes in any organism (Ashburner et al., 2000). Based on the sequence homology, 37,418 DEGs were assigned GO terms and classified into three categories, namely, molecular function, biological process, and cellular components. The results of this study agree with those of other plant leaf transcriptome investigations (Wu et al., 2015; Bose Mazumdar and Chattopadhyay, 2016). Alpha-dioxygenase (-DOX) is engaged in the catalysis of fatty acid oxygenation, resulting in the production of a recently found category of oxylipins, which plays a crucial role in shielding tissues from oxidative damage and cell death under drought stress (Tirajoh et al., 2005). Shi et al. (2015) reported that LTI30 protein positively regulates drought stress resistance in Arabidopsis through the modulation of ABA sensitivity, hydrogen peroxide levels, and proline accumulation. Yang et al. (2015) reported from their study that putative cationic amino acid transporter 9 (CAT9) mutation resulted in chlorotic leaves and overexpression resulted in the formation of stems and inflorescence transgenic *Arabidopsis* plants. Magwanga et al. (2018) also established the role of LEA proteins in cotton drought stress tolerance. Zheng et al. (2020) reported that the overexpressing phenotype of *Oryza sativa* ABA responsive protein 1 (OsABAR1), a GRAM protein-containing protein, showed resistance to drought and salinity. Identifying many DEGs in this study could help to gain in-depth knowledge of the diverse metabolic activities involved in the stress-resistant mechanisms of cluster beans. According to the gene function analysis, the KEGG database revealed that among 66,838 transcripts, 17,211 transcripts were allocated to 203 unigene pathways. A similar pattern was discovered in the transcriptome of *Phyllanthus amarus* leaves (Bose Mazumdar and Chattopadhyay, 2016).

Transcription factors are regulatory proteins involved in various regulatory processes, such as biotic and abiotic stress adaptation (Nakashima et al., 2014; Joshi et al., 2016). TF genes, such as NAC, WRKY, MYB, and bZIP, have been linked to drought stress responses (Gahlaut et al., 2016). NAC genes are TFs specific to plants and are involved in growth, development, and stress responses. Shi et al. (2018) reported that *GmWRKY12* confers drought and salt tolerance in soybean. Auxins usually induce scarecrow-like genes and interact with histone deacetylase, resulting in chromatin modeling in drought stress (Gao et al., 2004; Sánchez et al., 2007). Similarly, Scarecrow-like protein 1, one of the GRAS proteins, was upregulated in this study during drought stress. Zhu et al. (2014) studied the role of the *SlNAC4* TF in combating drought and salinity stress through RNAi-silenced transgenic tomato plants. Yu et al. (2016) also proved that *Cicer arietinum NAC4 (CarNAC4)* TF overexpression

in *Arabidopsis* conferred resistance to drought and salinity stresses. Liu et al. (2013) reported enhanced dehydration and drought tolerance through overexpression of *AhNAC3* in tobacco through enhanced superoxide scavenging. Tang et al. (2017) reported that the overexpression of the peanut *NAC4* gene conferred drought tolerance in tobacco. These differentially expressed TFs propose their significant role in combating drought stress in cluster bean cultivar RGC-1025. SSRs are the most useful molecular markers for genetics and plant breeding applications (Hiremath et al., 2012). In the present study, 21,494 SSRs were identified in the cluster bean data, and the frequency distribution of SSR markers agrees with previous reports in guar (Kuravadi et al., 2014; Kumar et al., 2016).

Cuticular wax prevents non-stomatal water loss, allowing plants to adapt to water-limited conditions (Kerstiens, 1996; Buda et al., 2013). Cuticular waxes deposited on the plant's organs play a critical role in sustaining harsh environmental conditions, such as drought (Jenks and Ashworth, 1999; Goodwin and Jenks, 2005). Drought stress enhances the increased deposition of waxes in many plants (Bondada et al., 1996; Samdur et al., 2003; Cameron et al., 2006). Lee and Suh (2013) and Mamrutha et al. (2017) reported that wax biosynthesis and its pathway genes are regulated at transcriptional, post-transcriptional, and translational levels. Guo et al. (2016) showed that drought-induced accumulation of wax biosynthesis positively correlated with drought-tolerant crops, such as wheat. In the present study, the ECERIFERUM1 was upregulated by 7.82-fold during the drought stress, revealing the upregulation of the wax biosynthesis pathway. Bourdenx et al. (2011) reported that overexpression of ECERIFERUM1 promotes wax's very long-chain alkane biosynthesis and influences plant response to biotic and abiotic stresses. Xu et al. (2003) showed that an ABC transporter family gene, *AtTGD1,* is involved in the inter-organelle lipid transfer in Arabidopsis. Mizuno et al. (2013) reported that an ABC transporter gene, *Sb06g023280*, is responsible for epi-cuticular wax biosynthesis in Sorghum. Elango et al. (2020) assessed the epicuticular wax variability in the extensive genetic pool of Sorghum, and a genome-wide association mapping study showed genic regions associated with epicuticular wax production. Hence, the enhanced epicuticular wax content and the deposition of wax crystals on the leaf surfaces are essential components of plants for enhanced drought tolerance to overcome non-stomatal water loss.

CONCLUSION

In summary, the Cluster bean cultivar RGC-1025 is proved to have enhanced drought tolerance that was evident from DEGs and analyzed from the transcriptome sequencing. The transcriptome sequencing and analysis revealed that the differential expression of the different stress responsible and constitutive cellular TFs, the enhanced traits, such as enhanced wax biosynthesis, and the upregulation of various genes involved in wax biosynthesis played a key role in RGC-1025 to combat drought stress efficiently.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, #PRJNA669348.

AUTHOR CONTRIBUTIONS

CS conceptualized and supervised this study and wrote the manuscript. BR performed the experiments. BR, AA, and MP analyzed the transcriptome data. NJ, BV, and NJ performed RT PCR analysis. All authors equally contributed to manuscript revision, read, and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.868142/full#supplementary-material

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Physiological and Molecular Approaches for Developing Thermotolerance in Vegetable Crops: A Growth, Yield and Sustenance Perspective

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Vegetables are a distinct collection of plant-based foods that vary in nutritional diversity and form an important part of the healthy diet of the human being. Besides providing basic nutrition, they have great potential for boosting human health. The balanced consumption of vegetables is highly recommended for supplementing the human body with better nutrition density, dietary fiber, minerals, vitamins, and bioactive compounds. However, the production and quality of fresh vegetables are influenced directly or indirectly by exposure to high temperatures or heat stress (HS). A decline in quality traits and harvestable yield are the most common effects of HS among vegetable crops. Heat-induced morphological damage, such as poor vegetative growth, leaf tip burning, and rib discoloration in leafy vegetables and sunburn, decreased fruit size, fruit/pod abortion, and unfilled fruit/pods in beans, are common, often rendering vegetable cultivation unprofitable. Further studies to trace down the possible physiological and biochemical effects associated with crop failure reveal that the key factors include membrane damage, photosynthetic inhibition, oxidative stress, and damage to reproductive tissues, which may be the key factors governing heat-induced crop failure. The reproductive stage of plants has extensively been studied for HS-induced abnormalities. Plant reproduction is more sensitive to HS than the vegetative stages, and affects various reproductive processes like pollen germination, pollen load, pollen tube growth, stigma receptivity, ovule fertility and, seed filling, resulting in poorer yields. Hence, sound and robust adaptation and mitigation strategies are needed to overcome the adverse impacts of HS at the morphological, physiological, and biochemical levels to ensure the productivity and quality of vegetable crops. Physiological traits such as the stay-green trait, canopy temperature depression, cell membrane thermostability, chlorophyll fluorescence, relative water content, increased reproductive fertility, fruit numbers, and

fruit size are important for developing better yielding heat-tolerant varieties/cultivars. Moreover, various molecular approaches such as omics, molecular breeding, and transgenics, have been proved to be useful in enhancing/incorporating tolerance and can be potential tools for developing heat-tolerant varieties/cultivars. Further, these approaches will provide insights into the physiological and molecular mechanisms that govern thermotolerance and pave the way for engineering "designer" vegetable crops for better health and nutritional security. Besides these approaches, agronomic methods are also important for adaptation, escape and mitigation of HS protect and improve yields.

Keywords: high temperature, vegetables, heat, environment, climate change

INTRODUCTION

Vegetables are parts of plants cultivated worldwide for consumption as flowers (e.g., cauliflower, broccoli), fruits (e.g., okra, tomato, cucumber, capsicum), leaves (e.g., spinach, lettuce, brassica, cabbage), tubers (e.g., potato, sweet potato), pods and seeds (e.g., common bean, chickpea, broad bean, mungbean, peas) (Peet and Wolfe, 2000). Vegetables contain secondary metabolites with bioactive properties, including carotenoids (e.g., carrots, pepper, tomato, spinach), polyphenols (e.g., tomato, cabbage), glucosinolates (e.g., brassica), saponins (e.g., beans, pea), and terpenes (e.g., carrots, tomato) (Crozier et al., 2006). These bioactive compounds are metabolic intermediates of primary metabolic processes, which are not essential for plant growth but are used in plant defense responses and plant-insect interactions and can stimulate human health. Clearly, vegetables are an important part of the human diet as they replenish our body with various nutrients, including vitamins, dietary minerals, fibers, proteins, antioxidants, carbohydrates, small amounts of fat, and phytochemicals with anticarcinogenic, antiviral, antifungal, and antibacterial properties (Osagie and Eka, 1998; Teng et al., 2021). While not a major energy source, vegetables nourish our bodies with much-needed minerals and vitamins. According to Food and Agriculture Organization (FAO) statistics, vegetables are the source of dietary requirements about 60% of vitamin A and 90% of vitamin C (Gruda, 2005). Vegetables can earn extra income for farmers as they are seasonal plants with higher yields per hectare than staple crops (Abewoy, 2018). The market value of vegetables is assessed by their quality; FAO and WHO provide many quality attributes for grading vegetables, e.g., color, size, shape, texture, aroma, shelf life, and storability (Gruda, 2005). Vegetables are categorized into two groups according to their growing season; warm-season vegetables include capsicum, common bean, cucumber, cowpea, okra, tomato, and mungbean (Peet and Wolfe, 2000), while cool-season vegetables include brassica, broad bean, broccoli, cabbage, cauliflower, lettuce, radish, spinach, soybean, pea, and potato (Peet and Wolfe, 2000) (**Table 1**).

Like other crops, vegetables are also affected by environmental changes that can render vegetable cultivation unprofitable. Abiotic stresses, mainly the high temperature (heat stress. HS), severely limit crop quantity, quality, nutritional status, and production (Boote et al., 2005; Aleem et al., 2021). High temperatures affect the overall growth and development of vegetable crops by altering morphology, physiology, and enzymatic activities. Heat stress (HS) accelerates phenology, shortening the vegetative and reproductive stages. HS reduces vegetable quality, such as changing the color and texture of fruits (e.g., cucumber, pepper, and tomato) (Zipelevish et al., 2000). In general, HS affects morphological, physiological, and biochemical processes of the plant by hampering photosynthetic activity, source-sink relationship, and altered enzymatic activities (Bita and Gerats, 2013; Janni et al., 2020). The quality of vegetables is also impacted by HS, through a change in color and texture of fruit (e.g., cucumber, pepper, and tomato) (Zipelevish et al., 2000). HS also affects the nutritional status of vegetables; for instance, reducing lycopene in tomato (Gross, 1991) and β-carotene in spinach and lettuce (Oyama et al., 1999) and increasing nitrate levels to harmful levels for human consumption.

Due to climate change, in most regions of the world, rising temperatures will decrease quantity and quality of vegetables crops. Studies of Waithaka et al. (2013) suggested that changes in the climate (increased temperatures) will also provide avenues to grow crops in areas where they could not be grown previously. Climate change scenarios further suggest that development of crop and cultivar choice—especially for water-limited or high-temperature areas—will be an important strategy to have adequate yields under changing climate (Thomas et al., 2007). Hence, targeted studies are needed to assess the impact of high-temperature stress on the growth, yield, and quality (taste, flavor, color, nutritional content) of vegetable crops, with suitable agronomic strategies, developed to create heat-tolerant cultivars or mitigate HS.

HEAT STRESS AND VEGETABLES

High temperatures adversely impact plant growth and development (Hasanuzzaman et al., 2013). The constantly rising average surface temperature due to global warming is stressful for all plant growth and development phases, limiting metabolism and productivity, particularly in tropical and subtropical countries (Li et al., 2018). According to the newly released sixth assessment report of IPCC (2021), temperature during the twenty-first century is likely to increase by 1.5°C of warming within just the next two decades, and by 4.5°C, depending on the rate of greenhouse gas emissions. As plants are sedentary

TABLE 1 | Threshold temperature for some vegetable crops at different stages of plant development.

Crop	Family	Threshold temperature (°C)	Response	References
Cool season vegetables				
		Vegetative stage		
Broccoli (*Brassica oleracea* var. *italica*)	Brassicaceae	30°C	Reduced growth and development	Hatfield and Prueger, 2015
Cabbage (*Brassica oleracea* var. *capitata*)	Brassicaceae	30°C	Reduced growth and development	Warland et al., 2006
Cauliflower (*Brassica oleracea* var. *botrytis*)	Brassicaceae	25°C	Reduced leaf growth	Lin et al., 2015
		Reproductive stage		
Brassica (*Brassica napus*)	Brassicaceae	29°C	Reduction in flower number	Morrison and Stewart, 2002
Broad bean (*Viciafaba*)	Fabaceae	30/22°C	Accelerate Floral development	Bishop et al., 2016
Broccoli (*Brassica oleracea* var. *italica*)	Brassicaceae	35°C	Arrest of inflorescence development	Björkman and Pearson, 1998
		Seed filling/maturity stage		
Chickpea (*Cicer arietinum* L.)	Fabaceae	30°C	Reduced yield	Summerfield and Wien, 1980
Lettuce (*Lactuca sativa*)	Asteraceae	24°C	Reduced yield	Jenni, 2005
Pea (*Pisum sativum*)	Fabaceae	25.6°C	Reduced yield	Pumphrey and Ramig, 1990
Potato (*Solanum tuberosum*)	Solanaceae	30/20°C	Reduced yield	Hancock et al., 2014
Warm season vegetables				
		Vegetative stage		
Cucumber (*Cucumis sativus*)	Cucurbitaceae	38°C	Impede growth and development	Yu et al., 2022
Okra (*Abelmoschus esculentus*)	Malvaceae	35°C	Decreased leaf size	Hayamanesh, 2018
		Reproductive stage		
Capsicum (*Capsicum annuum* L.)	Solanaceae	33°C	Inhibition of fertilization or early fruit development	Erickson and Markhart, 2002
Common bean (*Phaseolus vulgaris*)	Fabaceae	34/24°C	Reduced pollen viability	Boote et al., 2005
Soybean (*Glycine max*)	Fabaceae	26/20°C	Delay flowering and distort pod development	Nahar et al., 2016
Tomato (*Lycopersicon esculentum*)	Solanaceae	32/26°C	Abnormalities in male and female reproductive tissues	Peet et al., 1998
		Seed filling/maturity stage		
Cowpea (*Vigna unguiculata*)	Fabaceae	36/27°C	Reduced yield	Craufurd et al., 1998
Okra (*Abelmoschusesculentus*)	Malvaceae	35°C	Reduced yield	Hayamanesh, 2018

organisms, they acclimate to HS by using avoidance mechanisms or programmed cell death (Mittler et al., 2012; Singh, 2013; Zhang T. et al., 2020). Each vegetable crop has temperature threshold for its growth and development; HS will occur beyond the upper threshold for temperature (Wahid et al., 2007; Prasad et al., 2008, 2017). HS impedes photosynthesis through reduced carbon assimilation, ATP reduction, and oxidative damage to chloroplasts, with simultaneous reductions in dry matter accumulation and yield (Sharkey, 2005; Farooq et al., 2011). HS adversely affects vegetative and reproductive plant parts (Bita and Gerats, 2013); thus, the impact of HS varies depending on the developmental stage and crop species (Prasad et al., 2017; Li et al., 2018) (**Table 2**).

IMPACT ON VEGETATIVE GROWTH

Moderate high temperatures stimulate early vegetative growth and accelerate physiological maturity (Nahar et al., 2015).

TABLE 2 | Noticeable symptoms of heat stress in some vegetable crops.

Crop species	Symptoms	References
Cabbage (*Brassica oleracea* var. *capitata*)	Loosening or bolting of heads, smaller and tighter heads, rough leaf texture	Chang et al., 2016
Capsicum (*Capsicum annuum*)	Sun scald, yellowing and wilting	Moretti et al., 2010
Cauliflower (*Brassica oleracea* var. *botrytis*)	Leafy and uneven heads, puffy buds, yellow eyes and leaves, narrow leaves and hollow stems	Lin et al., 2015
Common bean (*Phaseolus vulgaris*)	High fiber in pods, brown and reddish spots in pods	Moretti et al., 2010
Lettuce (*Lactuca sativa*)	Tip burn, bolting, loose puffy heads, decreases β-carotene content	Han et al., 2013
Potato (*Solanum tuberosum*)	Secondary growth and heat sprouting	Hancock et al., 2014
Spinach (*Spinacia oleracea*)	Reduced leaf area and shoots dry weight, reduces β-carotene content	Chitwood et al., 2016
Tomato (*Lycopersicon esculentum*)	Fruit cracking, sunscald, hampered lycopene synthesis, blossom end rot, internal white tissue, blotchy ripening,	Moretti et al., 2010

During seed germination, HS reduces germination percentage and seedling emergence, reduces radical and plumule growth in germinated seedlings, and causes abnormal seedlings and poor seedling vigor (Hasanuzzaman et al., 2013). At later stages of vegetative growth, HS reduces plant height, leaf area, and leaf, stem, pod, root, and total biomass (Kumar et al., 2013). Leafy vegetables require proper growth and development of vegetative parts for realizing only the yield but also the quality. In 45-day-old cabbage plants exposed to 40°C for 6, 12, 24, 48, or 72 h, HS caused loosening or bolting of heads, smaller and tighter heads, and rougher leaf texture (Chang et al., 2016). Likewise, in 30-day-old cauliflower plants exposed to 40°C for 6, 12, 24, 48, 72, or 96 h, HS caused uneven heads, puffy buds, yellow eyes, narrow leaves, reduced leaf growth, and reduced petiole-to-blade ratio (Lin et al., 2015). HS (34.5°C) further delayed the curd induction stage and decreased the chlorophyll content in cauliflower plants; effects were more distinct in heat susceptible genotypes where they were unable to develop curd at high temperature and continued their vegetative growth until temperature fall below 30°C (Aleem et al., 2021). Exposing 4- to 5-leaved lettuce seedlings to 42/37°C for 3 days reduced seedling germination and caused tip burn, rib discoloration, and bolting (Jenni and Yan, 2009; Han et al., 2013). In spinach exposed to 35°C for 21 days, HS decreased seed germination (Chitwood et al., 2016). In potato, high temperature (30–40°C) inhibited tuber development and blocked the tuberization signal (Reynolds and Ewing, 1989). Potato plants exposed to 30/20°C (day/night) for 1 week had reduced yields by 16% compared to plants grown at 22/16°C due to decreased carbon transport to the sink organ (Hancock et al., 2014). Further, reduced yield has been reported in 50 potato cultivars when exposed to heat stressed conditions (35/28°C) than control conditions (22/18°C) (Zhang G. et al., 2020). Likewise, in 6–7-leaved radish seedlings exposed to 40°C for 12 and 24 h, HS affected fleshy taproot growth and development, reducing quality and yield (Zhang et al., 2013) (**Figure 1**).

IMPACT ON REPRODUCTIVE GROWTH

Reproductive stage is highly sensitive to HS; even a single degree increase for a few hours can be fatal for proper reproductive growth, contributing to poor yields (Prasad et al., 2017). However, studies on reproductive tissues are difficult to assess because gamete development and fertilization are major events that occur over short periods. Here, we categorize the effects of HS in vegetables during three stages of reproduction: pre-fertilization (flower bud initiation, flowering, male and female gametophyte development), fertilization (pollen dehiscence, pollination, pollen reception by stigma, pollen tube growth and fertilization), and post-fertilization events (fruit/pod set, seed development, seed filling) (**Figure 2**; **Table 3**).

Pre-fertilization Events

Flower Bud Initiation

High-temperature stress causes flower bud abortion and abscission of reproductive organs in many crop species, including tomato (Levy et al., 1978; Pressman et al., 2002; Sato et al., 2002), common bean (Konsens et al., 1991), pea (Guilioni et al., 1997), brassica (Angadi et al., 2000), capsicum (Aloni et al., 2001; Erickson and Markhart, 2002), resulting in severe yield losses. Common bean grown at 32/27°C (from flowering to pod maturity) experienced greater abscission and drop of flower primordia (2–5 mm) and flower buds (>5 mm) than at 27/17°C (Konsens et al., 1991). In capsicum, high-temperature stress (33°C for 120 h) affected flower buds (<2.5 mm) and early pistil development less than stamen development, whereas buds (3–4 mm) during tetrad formation and dissolution were highly sensitive to elevated temperature, leading to pollen sterility (Erickson and Markhart, 2002). Flower and flower bud abscission also occurred in heat-stressed (35/15°C for 7 days at early stage) brassica species (Angadi et al., 2000). HS (32/28°C) severely affected flower initiation and development in tomato (Levy et al., 1978; Sato et al., 2002). HS (32/26°C for 8 days before anthesis) in capsicum reduced and altered sucrose mobilization and utilization by flower buds and flowers, resulting in fruit drop and abscission and thus reducing yield by 17% compared to normal sown (28/22°C) (Aloni et al., 2001).

Flowering

HS during flowering reduces flower numbers by damaging flower organs, reducing yield (Morrison and Stewart, 2002). HS also decreases the number of flowering branches and thus flower numbers per plant (Harsant et al., 2013). Damage to flower organs has been reported in many crops, including chickpea (Tickoo et al., 1996), common bean (Suzuki et al., 2001; Omae et al., 2012), and mungbean (Kaur et al., 2015). Early flowering and flower abortion are other impacts of HS, as reported in pea

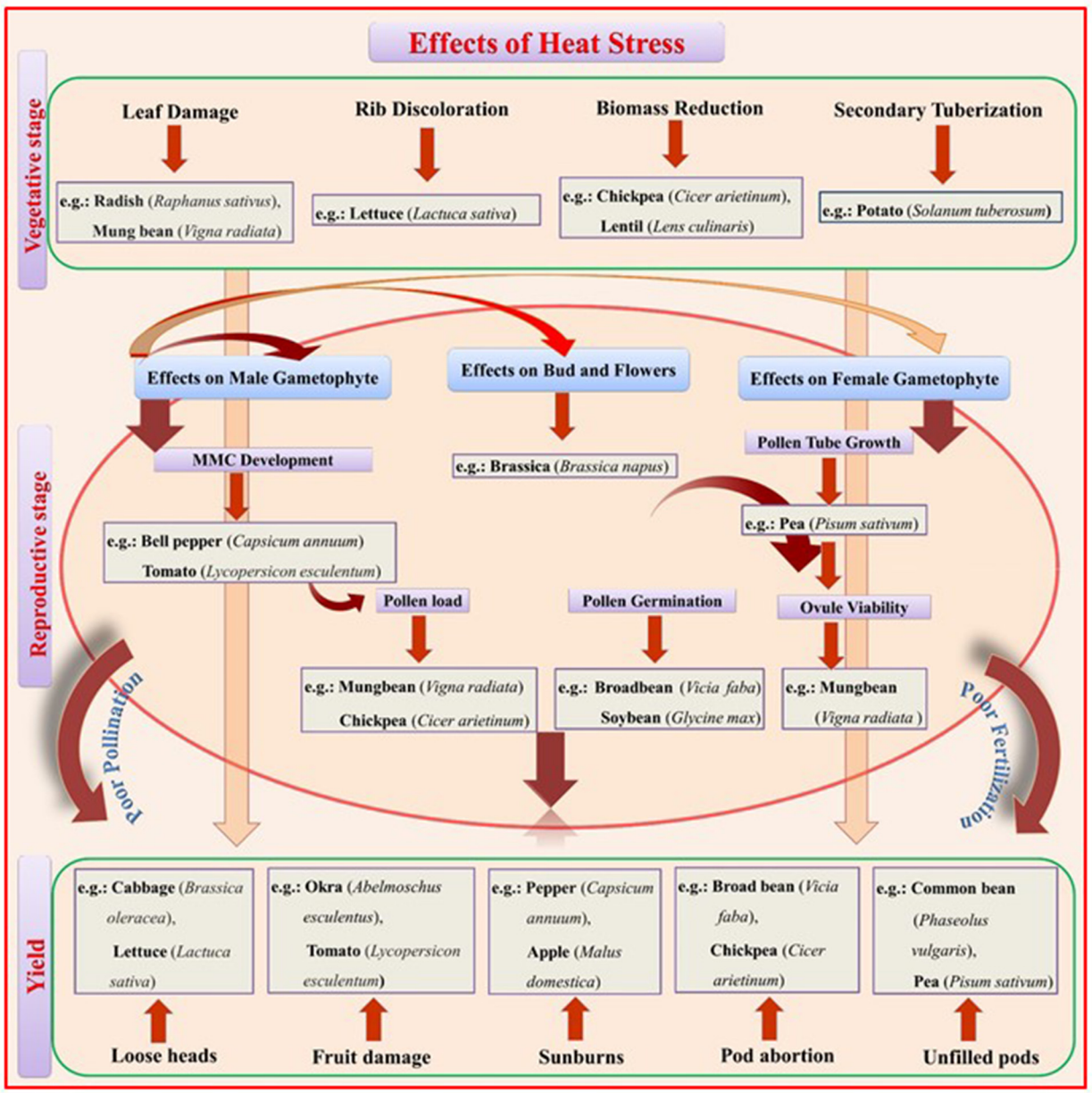

FIGURE 1 | A schematic representation of the effects of heat stress (HS) on vegetative and reproductive growth stages that reduce yield. Heat stress at the vegetative stage promotes leaf damage, rib discoloration in leafy vegetables, biomass reduction in food legumes, and secondary tuberization in potato. Heat stress at the reproductive stage negatively affects the overall route from Microspore Mother Cell (MMC) development to fruit setting/seed filling through pollination and fertilization. The male gametophyte is more prone to heat stress, leading to poor pollen germination, pollen load, and pollen tube growth inside the style and inability to fertilize the ovule at the required rate.

(Guilioni et al., 1997), tomato (Sato et al., 2004), common bean (Omae et al., 2012), and mungbean (Sharma et al., 2016).

Male Gametophyte Development and Function

Threshold temperatures needed to impose damages in reproductive tissues are less than the one needed to cause injury to vegetative tissues. Male gametophytes are more sensitive to HS than female gametophytes, with lower threshold temperatures than vegetative tissues. HS damage can occur pre-pollination or post-pollination, impairing fertilization and ultimately reducing seed set (Sage et al., 2015). Pre-pollination events that are highly susceptible to high temperature are (1) meiosis I and meiosis II of the microspore mother cell (Young et al., 2004), (2) development and subsequent dissolution of the tapetum layer (Farooq et al., 2017), and (3) exine and intine formation (Nahar et al., 2016). Post-pollination events affected by HS are (1) pollen load, (2) pollen germination, (3) pollen tube growth, and (4) fertilization (Hedhly et al., 2009; Sita et al., 2017).

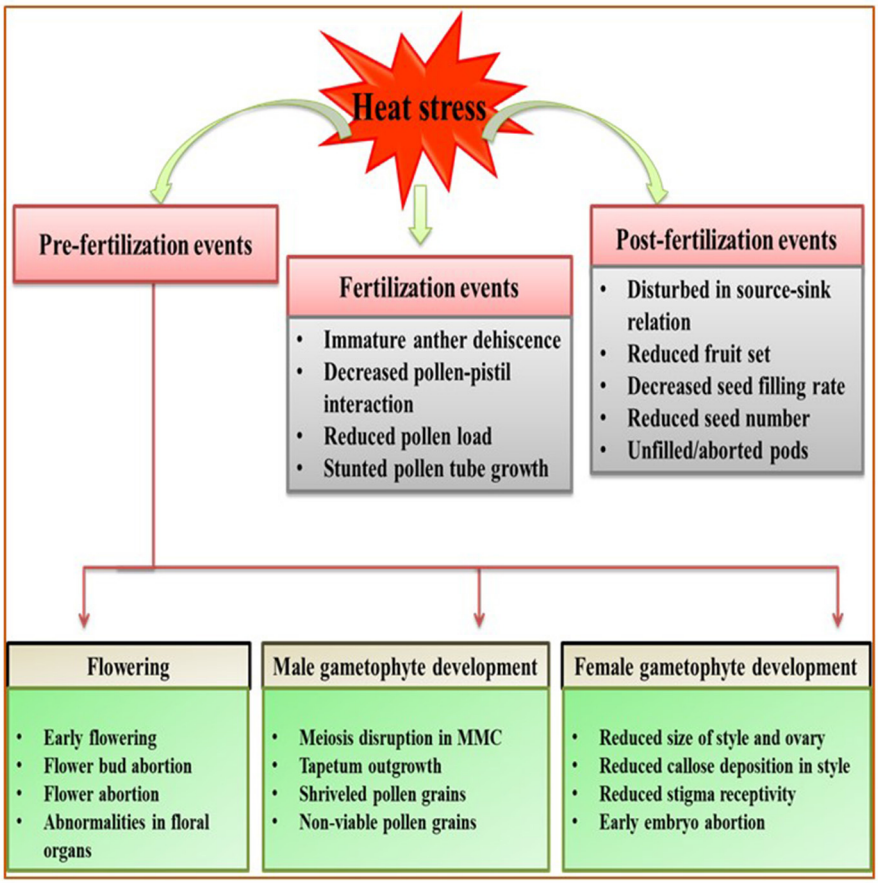

FIGURE 2 | Generalized overview of the effects of heat stress (HS) on the reproductive stage of plants, broadly categorized into three events: pre-fertilization, fertilization, and post-fertilization. Heat stress affects the flowering stage by promoting early flowering and flower bud/flower abortion. During male gametophyte development, heat stress disrupts meiosis and decreases tapetum growth, resulting in shriveled and non-viable pollen grains. During female gametophyte development, heat stress reduces style and ovary size and callose deposition, reduces stigma receptivity, and causes early embryo abortion. Moreover, immature dehiscence and malformed pollen grains result in poor pollination and fertilization. Heat stress during post-fertilization decreases the seed filling rate and disturb source–sink relations, potentially reducing yield manifold.

The sensitivity of male gametophytes to HS varies according to plant species (Li et al., 2018).

HS reduced fertility of microgametophytes in brassica (Rao et al., 1992) and impaired meiosis in tomato, damaging pollen germination and pollen tube growth (Foolad, 2005). In soybean, HS reduced pollen production, germination, tube elongation, and impaired pollen development (no apertures and disturbed exile ornamentation) (Salem et al., 2007; Nahar et al., 2016; Djanaguiraman et al., 2019). In capsicum, HS produced shrunken and empty microspores without an exine layer (Erickson and Markhart, 2002). Shriveled pollen grains under HS may be due to decreased starch accumulation in anther walls and pollen grains reducing soluble sugars for their development (Pressman et al., 2002).

Female Gametophyte Development and Function

Female gametophytes are relatively more tolerant to HS than male gametophytes (Hedhly, 2011). HS impairs megaspore mother cell development by impeding meiosis, reducing pistil size, reducing stigma receptivity due to poor pollen adhesion, reducing stigmatic papillae for holding pollen grains, interrupting nutrient transport from style to pollen impeding pollen tube germination and growth, as noticed in chickpea (Kaushal et al., 2016), bean (Porch and Jahn, 2001) and cowpea (Ahmed et al., 1992). HS, reduced callose deposition in lentil styles (Bhandari et al., 2017), reduced the amount of attractants from ovule synergids cells that misguide the pollen tube (Saini et al., 1983) to severely affect the fertilization. Furthermore, HS damages the embryo sac and causes early embryo abortion, likely arresting fertilization; for instance, in tomato, HS exposure (40°C for 3 h) for 4 days before anthesis resulted in aborted embryos with degenerated eggs and synergids (Iwahori, 1965). Abnormalities in embryo sac development have also been observed in brassica, reducing seed set and yield (Polowick and Sawhney, 1988). HS also reduced ovule viability in common beans (Ormrod et al., 1967; Suzuki et al., 2001). Unlike, male

TABLE 3 | Effect of heat stress on reproductive tissues of some vegetable crops.

Crop	Heat stress	Effect	References
Brassica (*Brassica napus*)	35/23°C	Reduced *in-vitro* pollen germinability, pollen viability, and thinner pollen tubes with stunted & convoluted morphology.	Young et al., 2004
		Microspore and pollen development are sensitive to heat stress.	Sato et al., 2002
Bell pepper (*Capsicum annuum*)	33°C	Pollen development (during megaspore mother cell (MMC) meiosis) is greatly reduced. Reduced pollen viability, reduced anther dehiscence, reduced mature pollen grains, slightly swollen and deformed (affect pollen morphology) and without exine layer.	Erickson and Markhart, 2002
Broad bean (*Vicia faba*)	34/26°C	Pollen germination	Bishop et al., 2016
Broccoli (*Brassica oleracea* var. *italica*)	35°C	Arrested the development of flower buds	Björkman and Pearson, 1998
Chickpea (*Cicer arietinum* L.)	40/25°C	Pollen germination, pollen tube growth Pod set	Devasirvatham et al., 2013
Common bean (*Phaseolus vulgaris*)	33/27°C 33/29°C	Anther indehiscence and pollen sterility Degeneration of tapetal cells.	Gross and Kigel, 1994
Cowpea (*Vigna unguiculata*)	33/30°C	Another development	Ahmed et al., 1992
Mungbean (*Vigna radiata* L.)	>40/28°C	Reduced pollen viability, pollen germination, pollen load, stigma receptivity and ovule viability	Sharma et al., 2016
Okra (*Abelmoschus esculentus*)	45°C	Incomplete dehiscence, shrunken pollen, smaller anther sacs, reduced pollen number, pollen viability, and pollen germination.	Hayamanesh, 2018
Pea (*Pisum sativum*)	36/24°C	Decreased pollen germination, pollen tube growth, pod length, and seed number per pod.	Jiang et al., 2015
Soybean (*Glycine max*)	38/28°C	Decreased *in-vitro* pollen germination.	Djanaguiraman et al., 2013b
Tomato (*Lycopersicon esculentum*)	32/26°C	Reduced number of pollen grains, pollen viability, and pollen germination.	Sato et al., 2002
	31/25°C	Reduced number of pollen grains, pollen viability, and pollen germination.	Firon et al., 2006
	29°C	Decreased fruit number, fruit weight/plant and seed number/fruit	Peet et al., 1998

gametophyte, detailed impacts of HS on female gametophyte organs are, however, barely known. This may be because of the reason that female gametophyte is protected inside the ovary and sheltered and difficult to reach and dissect.

Fertilization

High-temperature stress (>30°C) negatively impacts male and female gametophyte development, leading to poor development and deformities of reproductive tissues, limiting the fertilization process in many plant species (Saini and Aspinall, 1982; Prasad et al., 2017). HS also reported to affect the flower pollination rate in tomato resulting in low fruit set with reduced lycopene content and fruit quality (Alsamir et al., 2021) Indehiscent anthers, non-viable pollen, and poor stigma receptivity are possible causes for fertilization failure and sterility imposition in many crops, including chickpea (Kumar et al., 2013), soybean (Board and Kahlon, 2011), mung bean (Kaur et al., 2015), tomato (Pressman et al., 2002), common bean (Porch and Jahn, 2001), and capsicum (Erickson and Markhart, 2002).

Post-fertilization Events

Fruit/Pod Set

High-temperature stress affects the proportion of flowers forming fruits (fruit set) (Prasad et al., 2000). HS (38/30°C) markedly decreased fruit weight (51.6%), fruit diameter (25%), fruit length (30%), and seed number per fruit (57%) in sweet pepper compared with normal temperature (33/21°C) (Thuy and Kenji, 2015). Peet et al. (1998) reported that high temperature (29°C) decreased fruit number (10%), total fruit weight/plant (6.4%) and seed number/fruit (16.4%) in male fertile tomatoes compared to optimum temperature (25°C). The high temperature impaired pollen development and release, leading to reduced fruit set in male-fertile tomatoes compared with male-sterile lines. Similarly, fruit set and fruit size in tomato plants declined at 29/23°C compared to 24/18°C (Saha et al., 2010). HS seriously damaged fruit set in tomatoes exposed to 40°C for 4 h before anthesis and reduced the pollen germination from 79.5% (at 30/17°C) to 30% and pod set from 63% (at 30/17°C) to 14.9% (Rudich et al., 1977). In Common bean, high temperature (32/27°C) reduced the pod set from 17 to 97%, seed set by 39–98%, and seeds/pod by 42 to 73% compared to control temperature (22/17°C) (Gross and Kigel, 1994). Similar finding on bean plants exposed to even higher temperatures (40/30°C) had fewer filled pods, parthenocarpic pod development, sickle-shaped pods, reduced seed size, and fewer seeds/pod and total seeds than control condition (Prasad et al., 2002; Soltani et al., 2019). In peas, high temperature (32°C for 6 h) at the reproductive stage increased the abortion rate of reproductive

organs (flower buds and young pods) from 20 to 50% which reduce seed yield (Bueckert et al., 2015).

Seed Development and Seed Filling

Seed formation and seed filling are the last phases of the life cycle of seed plants; and; HS drastically affects seed development and the seed-filling phase, increasing the fraction of abnormal and shriveled seeds (Sehgal et al., 2018). In common bean, a linear relationship between temperature and grain weight was recorded resulting in a significant decrease in seed weight, i.e., 0.07 g per °C when temperature was raised beyond 31/21°C (Prasad et al., 2002). Seed development starts from cell division and, when seed cells are fully formed, storage reserves start to accumulate (Egli, 1998). Direct effects of HS on division and size of endosperm cells are well-documented (Commuri and Jones, 2001). Reduced division and size of endosperm cells results in accumulation of fewer carbohydrates, proteins, lipids, and starch accumulate in developing seeds. HS also accelerates the rate and duration of seed filling, resulting in abnormal seeds and significant yield losses (Farooq et al., 2017). Not only yields, HS affects seed quality characteristics, reducing seed number and size, degrading nutrient composition, and decreasing seed viability, through impaired nutrient uptake, assimilate partitioning, and translocation (Prasad et al., 2008). Starch, proteins, and lipids are the principal reserves transferred from the main plant to developing seeds (Alencar et al., 2012), but HS limits their synthesis and translocation during seed filling, affecting grain quality (Farooq et al., 2017), and could be due to decreased enzyme activity. The activity of starch synthesizing enzymes, such as starch synthase, sucrose synthase, and invertase, decrease under HS, as reported in pea (Smith and Denyer, 1992) and chickpea (Kaushal et al., 2013). Similarly, HS disrupts seed storage proteins, such as β-glycocynin and globulin 11S in soybean (Hashizume and Watanabe, 1979; Iwabuchi and Yamauchi, 1984), and sucrose-synthesizing enzymes and proteins that aid in sucrose translocation. Reduced sucrose synthase activity affects the sucrose and starch ratio, decreasing the transfer of soluble carbohydrates to developing ovules, as reported in pea (Jeuffroy et al., 1990) and cowpea (Ismail and Hall, 1999). Reduced crop duration and seed filling has been correlated with an inefficient light capture ability (canopy growth rate) in small plants, decreasing the photosynthetic rate and thus seed size, as reported in soybean (Board and Kahlon, 2011). Prasad et al. (2002) reported a linear relationship between temperature and grain weight in common bean, with seed weight decreasing by 0.07 g per °C at temperatures above 31/2.

PHYSIOLOGICAL ASPECTS AND CELLULAR FUNCTIONS UNDER HEAT STRESS

Membranes

HS disrupts the organization of the plasma membrane by increasing unsaturated fatty acids, thus making the membrane more fluid (Hofmann, 2009), and influencing the cellular functions by initiating a signal cascade (Firmansyah and Argosubekti, 2020; Hassan et al., 2021). HS also accelerates the kinetic energy and movement of various molecules through the membrane. Further, protein denaturation and altered tertiary and quaternary structure of membrane proteins increase membrane fluidity (Savchenko et al., 2002). Thus, HS disturbs primary processes of plant-like photosynthesis and respiration due to increased permeability or solute leakage from cells (**Figure 3**). Therefore, cell membrane thermostability trait used to evaluate HS on plants and identify heat-tolerant and heat-sensitive genotypes; for example, in soybean (Martineau et al., 1979), potato (Chen et al., 1982), and cowpea (Ismail and Hall, 1999). The effectiveness of cell membrane thermostability assays depends on the tissue type and stress type used for plant adaptation. It is also unknown whether membrane thermostability is linked to other plant characteristics that confer heat tolerance, such as growth and yield.

Photosynthesis

Photosynthesis is highly sensitive to HS and photosynthetic activity reduces drastically under HS. Studies have detailed the affected photosynthetic mechanisms that ultimately reduce the photosynthetic capacity of plants (Berry and Bjorkman, 1980; Sharkey, 2005). Thylakoid reactions, Rubisco activity, and photosynthetic pigments are generally disturbed by HS. HS primarily affects the physical state and structure of the thylakoid membrane by triggering thylakoid leakiness and unstacking thylakoids, damaging the D1 protein of PSII (Sharkey, 2005). To counterbalance these reactions, zeaxanthin synthesis increases, affecting the normal state of thylakoids (Havaux, 1996). HS disturbs the electron flow between the two photosystems (PSI and PSII) and reduces the photosynthetic efficiency of plants. HS also accelerates the phosphorylation of light-harvesting complex (LHCII) and disconnects it from PSII core complex, thus decreasing its turnover rate, but increasing the turnover rate of PSI (Wise et al., 2004). HS dephosphorylates core proteins (D1, D2, and CP43), deactivating PSII (Yamamoto et al., 2016). HS alters the fluorescence induction parameters, measured as the Fv/Fm ratio; this ratio helps to determine the quantum efficiency of PSII and indicates the rate of linear electron flow and overall photosynthetic performance of plants (Jamil et al., 2007). HS decreased chlorophyll a fluorescence, PII quantum yield, photochemical quenching, and increased respiration rate in soybean (Djanaguiraman et al., 2013a).

Along with thylakoid reactions, HS triggers the deactivation of Rubisco (Crafts-Brandner and Salvucci, 2000). Rubisco being dual enzyme catalyses the carboxylation of ribulose−1-5-bisphosphate in the photosynthetic Calvin cycle and oxygenation in the photorespiratory pathway; the ratio between two reactions governs the photosynthetic efficiency of plant. But the elevated temperature inhibits the CO_2 fixation and increases the oxygenase activity and reduces photosynthetic rate (Crafts-Brandner and Salvucci, 2000). Rubisco activation is not only associated with pH and Mg^{2+} concentration of stroma but also with Rubisco activase (RA); an ATPase. RA induces the activation of the Rubisco by increasing the proportion of its active sites and brings conformational changes that allow CO_2 and Mg^{2+} for activation and carbamylation. High temperature can disturb the pH and Mg^{2+} concentration of

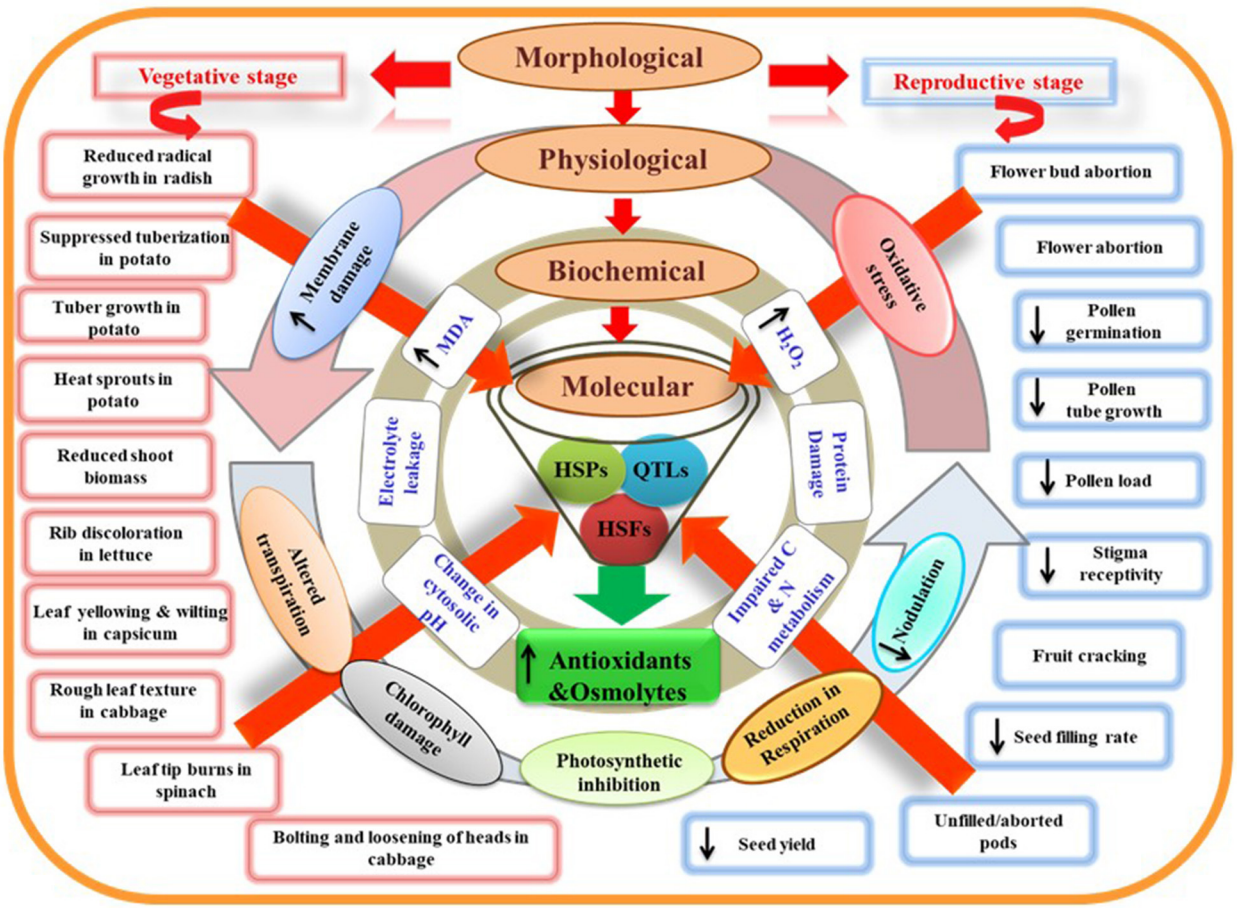

FIGURE 3 | Model representing morphological, physiological, biochemical, and molecular characteristics of plants under heat stress. Morphological damages at vegetative and reproductive stages can be visualized as direct measures of plant stress. At the physiological level, these damages are associated with leaky plasma membrane, altered transpiration, chlorophyll damage, reduced photosynthesis, respiration, and nodulation rate. Disturbed physiological processes can promote oxidative stress damage observed through stress indicators like increased *malondialdehyde* (*MDA*) and hydrogen peroxide (H_2O_2) content. Protein damage and impaired carbon and nitrogen metabolism due to impaired enzymatic activities further exaggerate stress levels at the biochemical level. Heat shock proteins (HSPs), heat shock factors (HSFs), and quantitative trait loci (QTLs) related to heat stress responses of plants may play a key role in the plant adaptation. HSPs and HSFs have a central role in regulating the activity of various genes that amplify the production of antioxidants and osmolytes and are helpful governing thermotolerance.

stroma, interfering with the carbamylation step of Rubisco activation (Weis, 1981a,b) and also caused RA dissociation because of its poor structural stability and heat labile nature (Demirevska-Kepova and Feller, 2004). Few reports have noticed that heat stress affects the photosynthesis through heat sensitivity of Rubisco and RA activity, for instance in tomato, heat stress (40°C for 8 h for 6 days to 3 weeks old plant) decreased the accumulation of Rubisco enzyme's isoforms (Parrotta et al., 2020), as in pea (Haldimann and Feller, 2005), potato (Cen and Sage, 2005) and spinach (Zhao Q. et al., 2018).

Pea plants exposed to HS reduced chlorophyll biosynthesis due to the destruction of various enzymes involved in biosynthetic pathways (Dutta et al., 2009; Aleem et al., 2021). HS decreased the activity of first enzyme of the biosynthetic pathway, 5-aminolevulinate dehydratase, in cucumber (Tewari and Tripathy, 1998). Decreased chlorophyll content, Chl a/b ratio, and chlorophyll/carotenoid ratio have been reported in many crops under HS (Aien et al., 2011) (**Table 4**). Similarly, HS stress causes pre-mature leaf senescence in soybean leaves which results in decreased photosynthesis primarily due to decreased chlorophyll content, higher reactive oxygen species, lower antioxidants, and increased thylakoid membrane damage (Djanaguiraman and Prasad, 2010). HS increased ethylene production in leaves which was one of the reasons of premature leaf senescence in soybean (Djanaguiraman and Prasad, 2010). Detailed anatomical studies showed that HT stress significantly increased the thicknesses of the palisade and spongy layers and the lower epidermis (Djanaguiraman et al., 2013a). In addition, HT stress damaged the plasma membrane, chloroplast membrane, thylakoid membranes; mitochondrial membranes, cristae, and matrix were distorted which led

TABLE 4 | Effect of heat stress on photosynthesis in some vegetable crops.

Crop species	Temperature	Effect	References
Broad bean (*Vicia faba*)	42°C	Decreased content of Chl a, Chl b, and carotenoids	Hamada, 2001
Cabbage (*Brassica oleracea* var. *capitata*)	40°C	Decrease in F_v/F_m values and photosynthetic efficiency	Chang et al., 2016
Cauliflower (*Brassica oleracea* var. *botrytis*)	40°C	Significant reduction in chlorophyll fluorescence F_v/F_m Inhibition of CO_2 fixation and damage to photosynthetic electron transport at site of PS II	Lin et al., 2015
Chickpea (*Cicer arietinum* L.)	40/30°C	Reduced chlorophyll content	Kaloki et al., 2019
Common bean (*Phaseolus vulgaris*)	45°C	Partially-reversible inactivation of PS-II and dissociation of light harvesting complex from reaction center of PS-II Destruction of PS-II reaction center and formation of quenching species	Costa et al., 2003
Cowpea (*Vigna unguiculata*)	30/25°C	Reduced rate of photosynthesis	McDonald and Paulsen, 1997
Cucumber (*Cucumis sativus* L.)	33–48°C	Decline in PS II activity and photochemical quenching Decreased net photosynthetic rate	Ding et al., 2016
	42°C	Chlorophyll biosynthesis	Tewari and Tripathy, 1998
Mungbean (*Vigna radiata*)	>40/28°C	Decline in PS II activity	Sharma et al., 2016
Okra (*Abelmoschus esculentus*)	>39°C	Adverse effects on the photosynthetic apparatus	Hayamanesh, 2018
Pea (*Pisum sativum*)	>40°C	Decreased photosynthetic electron transport Complete suppression of photosynthetic electron transfer	Haldimann and Feller, 2005
	45°C	Decreased CO_2 assimilation and O_2 evolution	Georgieva et al., 2000
Potato (*Solanum* spp.)	25°C	Decreased photosynthetic rate Decreased Chl a+b and carotenoid content	Aien et al., 2011
	38°C	Rapid and irreversible loss of PS II	Aien et al., 2011
Soybean (*Glycine max*)	38/28°C 38/30°C	Decrease in leaf photosynthetic rate by 20.2% Significantly affects net photosynthesis and total chlorophyll content Decreased chlorophyll content, photosynthetic rate,	Nahar et al., 2016
	39/20°C	Severely damaged PSII site	Li et al., 2009
Spinach (*Spinacia oleracea*)	40°C	Inhibition of oxygen evolution Cleavage of D1 protein of PSII	Yoshioka et al., 2006
Tomato (*Solanum lycopersicum*)	36/38°C	Decreased F_v/F_m values and PS II damage Decreased net photosynthetic rate Decreased chlorophyll content	Zhou et al., 2017

to decreased photosynthesis (Djanaguiraman et al., 2013a) (**Figure 3**).

Nitrogen Content, Fixation and Nodulation

Nitrogen is one of the main nutrients required by the plant for proper growth, development and productivity. It is the constituent of various important organic compounds like amino acids, proteins, nucleic acids, enzymes, and the chlorophyll molecule (Christophe et al., 2011). Nitrogen content in the plant measured as nitrate, ammonium ions, and proteins. Besides performing basic roles in plants, its metabolism is also very crucial for heat tolerance because it increases the osmolyte content and antioxidant enzyme activity (Ru et al., 2022). Studies have also shown their role in promoting the HSP production (Heckathorn et al., 1996). Osmolytes like proline and quaternary ammonium compounds, being nitrogen rich and accumulate in plants under heat stress conditions (Rivero et al., 2004). Ammonium ion and proline accumulation confer heat tolerance to tomato and promoting higher biomass production (Rivero et al., 2004). During the reproductive period, nitrogen concentration successively increases when temperatures rise for example in pea, when high temperature occurs during or after flowering seed N concentration is increased (Larmure et al., 2005). Similarly, in soybean, seed N concentration increases during the reproductive period at temperature 40/30°C (Thomas et al., 2003). Increases in the accumulation of proteins; level of globulin protein storage causing a reduction of the albumin/globulin content in mature seeds (Hurkman et al., 2009). In pea, the final level of vicilin storage proteins was higher under heat stress (Bourgeois et al., 2009). However, in tomato roots, it has been reported that HS disturbs enzymes involve in nitrogen metabolism (nitrate and ammonium assimilation)

thereby decreasing total protein content and level of nutrient uptake and assimilation (Giri et al., 2017). Further, studies on the contrasting genotypes of brassica revealed that HS (40/30°C for 7 days) negatively affected the activities of nitrogen assimilation enzyme including Glutamate synthase (GOGAT), glutamine synthetase (GS), glutamate dehydrogenase (GDH), more in heat sensitive genotype (WS-6) as compared to heat tolerant genotype (WS-1). These enzymes help in possessing better photosynthetic nitrogen use efficiency (Yuan et al., 2017).

Symbiotic nitrogen fixation in leguminous crops depends on the presence of appropriate *Rhizobium* species in the vicinity of root zone, however, almost all processes starting from rhizobial survival to host infection and nitrogen fixation depend mainly on the environmental factors, such as soil temperature (Bordeleau and Prévost, 1994). High temperature interferes with almost all processes of symbiotic nitrogen fixation, directly as well as indirectly, soil temperature affects not only the rhizobial survival in the root zone but also the exchange of molecular signals between two symbiotic partners (Alexandre and Oliveira, 2013). Rhizobial strains have an optimum soil temperature (25–30°C) for their growth and nitrogen fixing ability and Rhizobia are greatly affected by high soil temperature. However, optimum temperature varies with the crop species, for instance, in soybean, weak rhizobia were formed at 40°C and no rhizobia were isolated at 45°C (Chen et al., 2002). HT interferes directly with nodule development as it hampers nodule development and increases nodule senescence (Aranjuelo et al., 2007). HS affects indirectly the nitrogen fixation by inhibiting the formation of root hairs, infection thread formation, reducing the nodulation sites, adherence between bacteria and root hair (bacterial infection), and bacteroid formation (Zahran, 1999; Hungria and Vargas, 2000; Alexandre and Oliveira, 2013).

Elevated temperature also affects nodule growth rate, nodule size, and nodule fixation ability, as reported for common bean exposed to HS (35 and 38°C/8 h/day) at the flowering stage (Hungria and Franco, 1993). Another study showed that at 47°C temperature no nodules were formed in common bean (Karanja and Wood, 1988). Studies have shown that nodulation ability varies inversely with temperature, and legume species differ in their temperature endurance; for instance, common bean is more sensitive to temperature stress than cowpea and soybean for nitrogen fixation (Piha and Munns, 1987). In cowpea, the optimum temperature for nodule growth and development is 30–36°C; temperatures above 40°C lead to fewer or no nodules (Day et al., 1978). In common bean, nodules that formed at high temperature (≥35°C) were inefficient and unable to fix nitrogen (Hungria and Franco, 1993). Piha and Munns (1987) noted that nodules formed at 35°C were small and had low nitrogenase activity. The optimum temperature for nodule growth is 20°C for pea and 25–30°C for soybean (Michiels et al., 1994). HS decreased nodulation ability in mungbean (Sharma et al., 2016). In common bean, HS affected nitrogen fixation due to decreased activity of enzymes involved in nitrogen metabolism, such as dinitrogenase complex, glutamine synthetase (GS), and glutamine synthase (GOGAT), decreasing the concentration of ureids-N in nodules and xylem sap (Hungria and Kaschuk, 2014). Prasad et al. (2000) observed that high soil temperatures (35°C) significantly decreased number of nodules and nodule dry weight per plant compared to optimum soil temperature (25°C) in peanut.

C.N ratio: Plant growth and defense are both fuelled by compounds synthesized from a common pool of carbon and nitrogen, implying the existence of a competition for carbon and nitrogen allocation to both metabolisms. The ratio of carbon to nitrogen (C: N) of an organ is often regarded as a convenient indicator of growth and quality. Almost a century ago, plant nutrition was considered a crucial factor in controlling flowering time. According to Klebs (1913), a high endogenous carbon: nitrogen ratio promotes flowering, while a low carbon: nitrogen ratio promotes vegetative growth. Inferred from the fact that (a) conditions favoring photosynthetic CO_2 fixation generally accelerate flowering and (b) high nitrogen intake (fertilizers) might delay or reduce reproductive development in some plants (Bernier et al., 1981). The flowering percentage increased when NH_4NO_3 concentration decreased from 16.5 to 8 g l^{-1}, in tomato plant (Dielen et al., 2001). Royer et al. (2013) revealed that C:N ratio in the pool of resources in the total plant, were correlated with the concentrations of diverse compounds of the primary and secondary metabolisms in young tomatoes. Under HS, Peet et al. (1997) found that in tomato plants, the carbon and nitrogen metabolism get imbalanced, and stem and petiole elongation consume too much nutrients, which in turn reduces the dry matter storage of the plant, affecting tomato quality and yield. Soil mixed with dry powder of *Sesbania* plant (leaves + tender stems; C: N ratio 15.4) plays effective role in enhancing resistance and resilience (stability) of soil microbial activity against heat stress (Kumar et al., 2014). Heat stress may accelerate leaf senescence and increase respiration rate which consequently decreases plant N and C availability for seeds and shorten the duration of seed filling period in soyabean (Egli and Wardlaw, 1980). Thus, balanced C:N ratio plays an important role in plant physiological process. Similarly, Larmure et al., 2005 demonstrated that the lower seed N concentration in pea plant at the average temperature range (13–23°C) can be explained by prolonged duration of the seed-filling associated with the lower seed N concentration, higher C availability for the seeds. Because the rate of seed N accumulation per degree-day mainly depends on N availability to seed filling, the rate of N accumulation was higher at 25/20°C than at lower temperature. HS reduces seed size and modifies the C:N ratio in the period of seed formation in pea (Guilioni et al., 2003).

Antioxidants and Oxidative Stress

Severe HS generates ROS, such as hydrogen peroxide (H_2O_2) and superoxide radical (O_2^-), as byproducts of the aerobic metabolism, which adversely affect cellular metabolism, such as lipid membrane peroxidation, and damage nucleic acids and proteins (Bita and Gerats, 2013). Plants respond to ROS production by activating enzymatic and non-enzymatic ROS scavenging systems (Bita and Gerats, 2013). The main ROS scavenging enzymes are superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) glutathione reductase (GR), whereas non-enzymatic chemical are ascorbic acid (ASC) and glutathione (GSH) (Suzuki et al., 2012). SOD helps scavenge O_2^- whereas CAT and POX degrade H_2O_2. Elevated levels of these antioxidants are crucial in

imparting thermotolerance in plants (Awasthi et al., 2014). In soybean, ROS accumulation (mainly H_2O_2 and O_2^-) due to HS is associated with decreased enzyme activities of various antioxidants (Djanaguiraman et al., 2005, 2013a). Similarly, GR and CAT activities decreased in common bean under oxidative stress (Babu and Devaraj, 2008). Likewise, decreased APX and GR expression occurred in mungbean exposed to HS (Sharma et al., 2016). However, relationship between antioxidant enzymes and HS is far more complex in tomato where activity of SOD, APX increased and CAT activity decreased (Zhou et al., 2014). This complexity was also evident in capsicum where, NADPH oxidase and CAT activity increased at high temperature (Gulen et al., 2012). In chickpea, tolerant genotypes had higher SOD, CAT, APX, and GR activity than sensitive genotypes under HS (40/30°C and 45/35°C) (Kumar et al., 2013). Moderate HS increases the expression of various enzymatic antioxidants, while severe HS suppresses it (Wilson et al., 2014).

DEFENSE RESPONSES

In addition to antioxidants, plants endure HS by activating major defense mechanisms which are mainly comprised of increased production of heat shock proteins (HSPs) and compatible solutes (Sakamoto and Murata, 2002; Wahid et al., 2007; Mittler et al., 2012; Khan and Shahwar, 2020). HSPs are the molecular chaperones that protect the misfolded proteins from irreversible aggregation, sorting, translocation, and degradation, important for establishing cellular homeostasis in normal and stressed conditions (Vierling, 1991). There are five classes of HSPs categorized according to their molecular weight: HSP100, HSP90, HSP70, HSP60, and Small HSP (sHSP), and located in the cytoplasm as well as cellular orgenelles, nucleus, chloroplast, mitochondria, and endoplasmic reticulum (Wang et al., 2004). Different chaperone families though have a peculiar role but coordinate cellular homeostasis. Chaperones also maintain crosstalk with signaling molecules, antioxidants (acerbate peroxidase), and osmolytes (trehalose, proline, glycine betaine) (Wang et al., 2004; Kang et al., 2022). Various reports have confirmed accumulation of all HSP families in different vegetables and food legumes under HS, with greater accumulation of sHSPs than other HSPs, as reported for spinach (Guy and Li, 1998), tomato (Preczewski et al., 2000), soybean (Ortiz and Cardemil, 2001), common bean and cowpea (Simões-Araújo et al., 2003), potato (Ahn et al., 2004), cabbage (Park et al., 2013), pea (Talalaiev and Korduym, 2014), faba bean (Kumar et al., 2015), capsicum (Li et al., 2015), chickpea (Meena et al., 2017), and broccoli (Lin et al., 2019). Accumulation of these proteins helps plants to re-establish homeostasis under HS conditions. Hence, the expression level of HSPs and HSFs could be manipulated genetically to improve heat tolerance ability. Overexpression of HSPs facilitates transformed cells to endure HS better than non-transformed cells (Grover et al., 2013); for instance, overexpression of sHSP (HSP21) in transgenic tomato imparts stable PSII, shielding photosynthesis from temperature-dependent oxidative stress and accumulating more carotenoids under HS (Neta-Sharir et al., 2005). Furthermore, overexpression of HSFs facilitates the expression of HSPs; for example, overexpression of HSFA1 in transgenic soybean enhanced the expression of GmHSP70 leading to thermotolerance (45°C) (Zhu et al., 2006). Similarly, overexpression of transcription factor (CaWRKY40) enhanced thermotolerance in capsicum (Dang et al., 2013).

The role of various osmolytes, including proline and glycine betaine, in imparting heat tolerance is well-documented (Sakamoto and Murata, 2002). Osmolytes are low molecular weight compounds that can buffer cellular redox potential under HS. Proline is a well-studied osmolyte, concentration of which increases by several-fold under stress conditions. A heat-tolerant cabbage genotype accumulated more proline (and soluble sugars and antioxidants) than a sensitive genotype (Song et al., 2019). Similarly, Paul et al. (2014) even suggested using increased proline and soluble sugars in potato under HS can used as markers for selecting heat-tolerant genotypes. Increasing HS gradually increased proline and soluble sugar contents in lettuce seedlings, indicating heat tolerance (Han et al., 2013). The role of proline in thermotolerance was also confirmed using exogenous proline applications. Kaushal et al. (2011) noted that exogenous treatment of proline induced thermotolerance in chickpea by protecting the enzymes involved in carbon and antioxidant metabolism. Glycine betaine is another compound that confers heat tolerance; Aien et al. (2011) suggested that glycine betaine imparts heat tolerance in potato genotypes under HS conditions.

Heat Avoidance

Heat avoidance through transpiration cooling is the best strategy adopted by plants to minimize the losses (Julia and Dingkuhn, 2013) Under moderately HS conditions, plants can accelerate growth to promote plant thermonastic responses and architectural changes to move susceptible parts away from soil heat flow or to improve evaporative cooling (Havko et al., 2020). In soybean, tomato, or cabbage, moderately high ambient temperature induces hypocotyl elongation, and tomato displays leaf hyponasty (Quint et al., 2016; Casal and Balasubramanian, 2019; Vu et al., 2019). Pea canopies architecture and leaf type as traits of heat resistance can avoid heat and maintain a lower canopy temperature as leafed cultivars have greater leaf surface area and likely greater transpirational cooling, assuming soil moisture availability and an adequate root system (Tafesse et al., 2019). Another study showed that the leaf movement capacity in beans was shown to function in direct sunlight avoidance and benefited the plant by protecting it against photoinhibition and by maintaining leaf temperatures lower than the air temperature (Pastenes et al., 2004). Thus, as novel donors with higher heat tolerance or escape provides, there is an ample evidence for systematic exploration of wild species and accessions (Prasad et al., 2017) for introducing these traits.

IDENTIFICATION OF TOLERANT GENOTYPES AND IMPROVING ADAPTATION AND MITIGATION TO HS

Physiological Approaches

Heat tolerance is a polygenic trait greatly influenced by environmental changes (Blum, 2018). HS effects are stage-specific, with the response at one stage differing from the

response at another. Breeders employ various techniques to minimize the impact of an unpredictable environment on crops. Conventional breeding is the oldest but most prevalent method, primarily based on selecting phenotypic plant characters (Acquaah, 2015). In recent decades, new techniques have emerged based on morpho-physiological plant characters merged with conventional breeding methods to screen superior varieties. These methods exploit inbuilt plant properties to cope with HS and assist in selecting heat-tolerant genotypes. Screening germplasm of various vegetable crops using various physiological traits linked to heat tolerance would be useful for breeding programs focused on developing HS tolerant genotypes. Although there are several methods or traits used for screening, some of the most common are discussed.

Stay-Green Assay

The stay-green character is the plant's ability to retain chlorophyll and remain green for longer to sustain photosynthesis, especially during seed filling (Thomas and Howarth, 2000). However, the adverse impacts of HS cause leaves structural changes and chlorophyll degradation and it ultimately induces premature, leaf senescence (Djanaguiraman and Prasad, 2010; Jha et al., 2014). Moreover, the onset of HS during seed filling affects various physiological processes, including increased leaf senescence (chlorophyll loss), altered source–sink relationship, and decreased assimilation of reserve food material in developing seeds, limiting plant yield (Luche et al., 2015). Therefore, delayed leaf senescence may be associated with heat tolerance, enabling plants to maintain their photosynthetic ability (Lim et al., 2007). High chlorophyll and carotenoid contents in leaves improve the photochemical efficiency of plants and reduces ROS concentration in plants such as tomato (Zhou et al., 2015) and pea (Tafesse, 2018).

In addition, the stay-green character positively correlates with canopy temperature depression. Stay-green genotypes have lower canopy temperatures due to transpirational cooling than non-stay-green genotypes (Kumari et al., 2013). In addition to these modifications, HS also causes plant morphological and architectural modifications like leaf hyponasty (measured through leaf angles), leaf petiole elongation, small and thin leaves, that are helpful for the plants to keep their canopies cool. For instance, the cucumber species have hyponastic leaves (Park et al., 2019) and reduced leaf size is found in potato (Tang et al., 2018) and capsicum species (Utami and Aryanti, 2021) under heat stress conditions. These processes involve various signaling cascades that mediate the developmental shaping for environment adaptation in plants (Gil and Park, 2019). This trait is also associated with grain yield and quality and abiotic stress tolerance (Kamal et al., 2019). Hence, the stay-green trait is essential for improving crop yield and useful for imparting heat tolerance (Joshi et al., 2007; Kusaba et al., 2013), and thus may be an important genetic trait for improving crop yield under HS.

Canopy Temperature Depression

Canopy temperature depression (CTD) is usually measured as the difference between air and canopy temperature, indicating the plant's ability to lower its foliar temperature by transpirational cooling, as measured by an infrared thermometer. CTD also reflects plant water status and is influenced by the plant's ability to extract water and the transpiration difference between air and plant. Accordingly, CTD has been used to select heat-tolerant and drought-tolerant genotypes. Plants that can maintain cooler canopies during seed filling can tolerate high-temperature stress (Munjal and Rana, 2003). Heat-tolerant varieties of capsicum (Gajanayake et al., 2011) have been selected based on the stay-green trait. In soybean, there is a direct relationship between CTD, canopy greenness, photosynthetic rate, and yield (Kumar et al., 2017). Thus, the CTD trait can be used as a critical genetic trait for crop improvement aimed at increased yields at the vegetative stage.

Cell Membrane Thermostability

HS is amounts of sensed by cell membranes of leaf tissues, weakening cell membrane integrity/rigidity due to an increased degree of unsaturated fatty acids that increase membrane fluidity. This may change membrane permeability and disturb the selective transport of molecules across the membrane, affecting cellular homeostasis (Marcum, 1998). HS can directly affect membrane integrity through photochemical modifications during photosynthesis or ROS (Bita and Gerats, 2013). Cell membrane thermostability (CMT) can be evaluated with an electrolyte leakage test for screening crops for heat tolerance. The method is simple, quick, and inexpensive compared with whole-plant screening and can be used to assess plant tissue responses at the vegetative stage (Yeh and Lin, 2003). Electrolyte leakage is measured using a conductivity meter, with higher conductivity values indicating higher membrane damage (Nyarko et al., 2008). The CMT test has been used to screen heat-tolerant varieties of many crops, including soybean (Martineau et al., 1979), potato (Nagarajan and Bansal, 1986), cowpea (Ismail and Hall, 1999), cabbage (Nyarko et al., 2008), cauliflower (Aleem et al., 2021) chickpea (Kumar et al., 2013), mungbean (Sharma et al., 2016), and cucumber (Ali et al., 2019).

Chlorophyll Fluorescence

Chlorophyll fluorescence—expressed as the Fv/Fm ratio (Fv: variable fluorescence; Fm: maximum fluorescence)—is used to detect the state of PSII function in terms of the energy absorbed by PSII in chlorophyll and damage to photosynthetic apparatus by excess light *in vivo* (Maxwell and Johnson, 2000). Chlorophyll fluorescence is a rapid, reliable, and inexpensive procedure for predicting photosynthetic performance under HS. Reduced Fv/Fm values indicate damage to the light-harvesting complex (Moradpour et al., 2021). Chlorophyll fluorescence has been used to select heat-tolerant varieties of sweet pepper (Hanying et al., 2001), common bean (Stefanov et al., 2011), chickpea (Kaushal et al., 2013), mungbean (Kaur et al., 2015), tomato (Zhou et al., 2015; Poudyal et al., 2018), and okra (Hayamanesh, 2018). Makonya et al. (2019) showed that tolerant chickpea genotypes maintain higher Fv/Fm during HS than sensitive genotypes, and Fv/Fm positively correlates with grain yield in the field. Killi et al. (2020) reported the retention of PSII function at elevated temperature positively correlated with antioxidant

activity, confirming the applicability of this trait for selecting heat-tolerant varieties.

Relative Water Content

Relative water content indicates the hydration status of plants and reflects the balance between leaf water supply and transpiration rate. Hence, it can measure leaf water deficit and the degree of damage under HS (Mullan and Pietragalla, 2012). High transpiration increases water loss, which can cause tissue dehydration and wilting (Mazorra et al., 2002). Therefore, genotypes that can maintain turgid leaves will minimize HS effects and have numerous physiological advantages. Gowda et al. (2011) suggested using RWC as selection criteria for improving yield under HS. High temperature (40–42°C) at the vegetative and reproductive stage gradually reduced the RWC of capsicum genotypes, more so at the reproductive stage (Puneeth, 2018). RWC has been used to select heat-tolerant genotypes of mungbean (Sharma et al., 2016), capsicum (Puneeth, 2018), common bean (Chavez-Arias et al., 2018), lentil (Sita et al., 2017), tomato (Zhou et al., 2018), cucumber (Ali et al., 2019), and potato (Handayani and Watanabe, 2020) where genotypes with high RWC under HS were rated as heat tolerant.

Stomatal Conductance

Stomatal conductance measures the rate of carbon dioxide entering or water vapor exiting stomata. This change in transpiration rate facilitates changes in leaf temperature and water potential (Farquhar and Sharkey, 1982). Leaf stomatal conductance is often recognized as an important trait for evaluating differences in response to changing environments. It can be used to determine trait such as photosynthetic CO_2 uptake, leaf temperature, and water loss (Vialet-Chabrand and Lawson, 2019). Decreased stomatal activity under a changing environment can significantly affect plant growth and biomass (Way and Pearcy, 2012). *In vivo* stomatal conductance can be measured with a steady-state leaf porometer and gas exchange. HS increases *in vivo* adaxial stomatal conductance relative to the control (Sharma et al., 2016). Low stomatal responses under stress can limit photosynthetic rate and cause unnecessary transpiration, decreasing plant water use efficiency and productivity (Matthews et al., 2018). This phenomenon has been used to select heat-tolerant genotypes of sweet pepper (Hanying et al., 2001); tomato (Camejo et al., 2005; Abdelmageed and Gruda, 2009), chickpea (Kaushal et al., 2013), and mungbean (Kaur et al., 2015). While many studies have successfully used one of the traits above to select heat-tolerant genotypes, combining multiple traits would reflect heat tolerance better than relying on a single trait.

Reproductive Function, Gamete Viability and Fruit-Set

Fruit yield in vegetables crops is a function of fruit numbers and fruit size. There is a strong and positive correlation between fruit-set and gamete viability (Prasad et al., 2017). Gamete functions (pollen and ovule) is the most important factor for fruit-set under HS. In tomato, fruit-set has been shown to correlate with pollen viability (Firon et al., 2006). In general, heat tolerant genotypes maintain higher pollen viability compared to heat susceptible genotypes (Dane et al., 1991). Gamete functions depend on its viability, which can be evaluated by viability assays like staining, *in-vitro* and *in-vivo* germination of pollen, and ovule function. Genotypes are known to differ in gamete viability under HS stress. Singh et al. (2015) concluded from their research on tomato that traits like fruit-set and pollen viability could be used as a strategy to screen genotypes for HS. In general, the combination of gamete viability and fruit-set provide tolerance to HS (Paupière et al., 2017b; Pham et al., 2020). Similarly observations were also made on peppers (Aloni et al., 2001; Reddy and Kakani, 2007).

Cardinal temperatures (Tmin, Topt, and Tmax) for pollen grain germination can be used to screen germplasm for HT stress tolerance. Results from *in-vitro* studies showed that genotypes varied in response to temperature for cardinal temperatures, and the differences in cardinal temperatures were mainly responsible for tolerance/susceptibility of genotypes to HT stress in soybean (Djanaguiraman et al., 2019) and peanut (Kakani et al., 2002). The genotypes having higher ceiling temperature (Tmax) for pollen germination values tend to be HT tolerant in most cases. Cardinal temperature for pepper were different among susceptible and tolerant cultivars (Reddy and Kakani, 2007) and can be used to identify temperature tolerant or sustainable genotypes of pepper (Gajanayake et al., 2011). All the aforementioned traits based on leaf function are used collectively to select heat tolerant cultivars. Though many studies have successfully employed one trait for selection of heat tolerant genotypes, a combination of these traits reflects a better status of heat tolerance rather than relying on a single trait.

OMICS APPROACHES

Genomics

Various modern genome-based technologies can be used to introduce genetic variations for HS tolerance into plants. Under high-temperature stress, plants activate a complex chain of molecular responses, including heat-stress-responsive genes that control primary and secondary metabolism, transcription, translation, and lipid signaling, or protein modifications, including phosphorylation HS transcription factors (HSFs) that regulate differential expression of HSPs (Janni et al., 2020). HSPs and HSFs are key players in the acquisition of the HS response. HSFs are mainly involved in sensing and relaying the HS signal to activate the response (Mittler et al., 2012). Genome-wide associated studies (GWAS) have been conducted on a few vegetable crops to search for novel genes and transcription factors associated with heat tolerance. Genomic studies on cabbage (*Brassica rapa* ssp.) disclosed the role of differentially expressed long non-coding (lncRNAs), mRNAs, and microRNAs. Their expression is associated with phytohormones such as salicylic acid (SA) and brassinosteroids (BRs), possibly involved in heat tolerance. Of these, 25 lncRNAs were co-expressed with ten heat-responsive genes (Wang A. et al., 2019). NAC, a large family of transcription factors, was analyzed in cabbage; 188 genes were identified that play a major role in resistance to high-temperature stress (Ma et al., 2014). Analysis of the potato

Hsp 20 gene family revealed 48 putative Hsp20 (StHsp20) that accumulated under heat treatment. Different levels of these transcripts were upregulated during different HS exposures. The transcription of HSPs are regulated by HSFs that play an important role in imparting thermotolerance in plants (Zhao P. et al., 2018). Guo et al. (2015) characterized 35 putative Hsp 20 genes (CaHsp20) located on 12 chromosomes in thermotolerant (R9) and thermosensitive (B6) lines of pepper in four tissues (roots, stem, leaves, and flowers). Under high temperature stress (40°C), most of the CaHsp20 genes had higher expression in both lines, more so in the thermosensitive line. Chidambaranathan et al. (2018) identified 22 Hsfs in the desi (ICC4958) and kabuli (CDC Frontier) genomes of chickpea (15-day-old seedlings; heat treatment of 35 ± 2°C). Field analysis was undertaken to compare the expression pattern at the podding stage. HS at the seedling and pod development stages upregulated the expression of *CarHsfA2, A6a, A6c*, and *B2a*, indicating their role in conferring HS tolerance in chickpea. Yang et al. (2016) recorded 26 HSF (Sly HSF) genes in tomato, with HS (38°C) increasing the expression of most, especially SlyHSF-05/07/13/18/20/23/24. Expression of the SlyHSF-18 gene increased manifold compared to the control, indicating its strong response and correlation to high temperature sensitivity. Moreover, SlyHSF-02 was the main regulator for activating the heat response and acquiring thermotolerance in tomato.

Transcriptomics

Transcriptomics refers to the study of the transcriptome [entire set of transcripts (mRNA, tRNA, and rRNA, miRNA, siRNA, snRNA, snoRNA, and lncRNA)] expressed in a cell, tissue, organ, or organism. It represents all RNA synthesized, including protein-coding, non-coding, spliced, polyadenylated, and RNA-edited transcripts (Imadi et al., 2015). Transcriptomics reveals the molecular mechanism underlying the phenotype and explains how genes are expressed and interconnected (Jha et al., 2017). High throughput methods (microarray, RNA sequencing, RT-PCR) are used to analyze the expression level of multiple transcripts in different conditions. Several transcriptome studies in vegetable crops under HS have revealed the molecular basis for heat tolerance.

Transcriptome analysis in heat-stressed spinach (42°C for 15 days) revealed the expression of 4,145 transcripts (2,420 upregulated and 1,725 downregulated) in heat-tolerant and heat-sensitive genotypes (Guo et al., 2020). An enrichment analysis showed that the major metabolic difference between tolerant and sensitive genotypes was carbohydrate metabolism (Guo et al., 2020). Similarly, transcriptome analysis revealed 23,000–30,000 expressed genes in soybean seeds and differentially expressed genes (DEGs; 5–44% of expressed genes) (Gillman et al., 2019). The DEGs were measured at high temperature in mature, imbibed, and germinated seeds in a heat-tolerant (PI 587982A) and conventional high-yielding variety (S 99-11986), with 7,789 DEGs common between genotypes, 11,833 common between mature and imbibed seeds, and 13,344 common between imbibed and germinated seedlings (Gillman et al., 2019). In capsicum, seedling transcriptomics revealed 3,799 DEGs in R597 (heat-tolerant genotype) and 4,010 DEGs in S590 (heat-sensitive genotype), related to hormones, HSPs, transcription factors, and calcium and kinase signaling (Li et al., 2015). Further, R597 had higher expression of transcription factors and hormone signaling genes than S590 (Li et al., 2015). Transcriptomic analysis of heat-tolerant PS-1 and heat-sensitive H-24 tomato genotypes under HS (40°C for 1 h) revealed upregulated genes associated with protease inhibitors, HSPs, and transcription factors, manifold higher in the tolerant genotype than the sensitive genotype (Sadder et al., 2014).

Proteomics

Proteomic analysis in heat-stressed radish leaves (advanced inbred line NAU-08Hr-10) revealed eleven deferentially expressed proteins, of which four belonged to HSPs, four to energy and metabolism, two to redox homeostasis, and one to signal transduction (Zhang et al., 2013). Comparative proteome analysis of heat-tolerant (JG 14) and heat-sensitive (ICC16374) chickpea genotypes under HS during anthesis revealed that 482 heat-responsive proteins (related to photosynthesis, energy metabolism, and signaling molecules) were synthesized in higher amounts in the heat tolerant genotype compared to the sensitive genotype (Parankusam et al., 2017). Proteomics of spinach (50-day-old) exposed to 37/32°C for 24, 48, or 72 h identified heat-stress-responsive proteins in heat-tolerant (Sp75) and heat-sensitive (Sp73) lines (Li et al., 2019). The abundance pattern indicated that HS inhibited photosynthesis, initiated ROS scavenging pathways, and sped up carbohydrate and amino acid metabolism. A comparative proteomic study showed that heat-sensitive genotypes have a lower ability for photosynthetic adaptation, osmotic homeostasis, and antioxidant enzyme activities than heat-tolerant genotypes (Li et al., 2018). Ahsan et al. (2010) used a proteomics approach to study the tissue-specific protein expression pattern in heat-stressed soybean seedlings (40 ± 2°C for 12 h), identifying 61, 54, and 35 differentially expressed proteins in roots, leaves, and stem, respectively. Many of the proteins related to HSPs and the antioxidant system were upregulated.

Metabolomics

Recent metabolite profiling has focused on important metabolites that govern temperature stress tolerance (Guy et al., 2008). Wang J. et al. (2019) studied the metabolism of heat-tolerant (17CL30) and heat-sensitive (05S180) capsicum cultivars; the tolerant genotype accumulated 94 differentially accumulated metabolites (DEM) while the sensitive genotype accumulated 108 DEM. Both genotypes shared common metabolites, but they were more highly expressed in tolerant genotypes. Metabolite profiling of tomato anthers exposed to 38°C for 2 h revealed that flavonoids (alkaloids and flavonoids in young microspores) protect against HS (Paupière et al., 2017a,b). A metabolomics study on heat-stressed soybean seeds revealed 275 metabolites that comprised antioxidants, including ascorbate precursors, tocopherol, flavonoids, phenylpropanoids, which were more enriched in tolerant than sensitive genotypes (Chebrolu et al., 2016).

MOLECULAR BREEDING

Of late, molecular breeding has emerged as one of the important tools to identify progeny plants possessing the targeted genes/QTLs including the presence of several genes or ascertain the amount of genome of recurrent parent in a plant. Molecular breeding relies on molecular markers and hence the outcome, unlike the phenotyping, is not influenced by environmental factors. The molecular breeding has been exploited successfully in crop breeding and has led to the development of crop varieties possessing resistance to diseases or varieties with resistance genes pyramids (Janni et al., 2020). Molecular breeding methods to improve heat tolerance include (i) transfer of quantitative trait loci, (ii) marker-assisted selection. Other methods include marker assisted recurrent selection, marker-assisted pyramiding, and single nucleotide polymorphism. These methods pave the way for breeding stress tolerance in plants (Collard and Mackill, 2007). These methods pave the way for breeding stress tolerance in plants (Collard and Mackill, 2007).

Quantitative Trait Loci

QTL is a stretch of genomic regions on a chromosome that is linked to a quantitative trait. Usually, this stretch contains several genes and each QTL contribute partially to the trait in question; and hence, several QTLs together govern a trait. In molecular breeding, whole QTL is transferred to the recurrent parent utilizing markers flanking to the QTLs and sometimes using markers present within the QTL region. The exploitation of molecular breeding for QTLs transfers in breeding programs, a QTL must be well-defined and demonstrated to be linked to a particular trait (Collard and Mackill, 2009). Heat tolerance is a polygenic trait governed by several genes (Golam et al., 2012) and several QTLs. Unprecedented advances in genomics, especially molecular marker development, have identified numerous QTLs contributing to HS tolerance by dissecting various traits ranging from phenological, physiological, biochemical, reproductive biology to yield and yield-related traits (Lucas et al., 2013; Wen et al., 2019; Song et al., 2020; Jha et al., 2021; Vargas et al., 2021) in various vegetable crops, including bottle gourd *(Lagenaria siceraria),* cowpea (*Vigna unguiculata* [L.] Walp.), common bean, chickpea, chili, and tomato (**Table 5**). In broccoli (*Brassica oleracea* var. *italica*), five QTLs were identified under HS—QHT_C02, QHT_C03, QHT_C05, and QHT_C07 from the heat-tolerant parent and QHT_C09 from the heat-sensitive parent, with a positive epistatic co-relation between QHT_C03 and QHT_C05 for heat tolerance and APX activity was co-located with QHT_C03 (Branham et al., 2017). Likewise, QTLs such as QHT_C02, QHT_C05, and QHT_C09 were co-located with the AP2 gene governing floral development under HS (Aukerman and Sakai, 2003). Similarly, the meristem identity gene (TFL) was associated with QHT_C02 (Duclos and Björkman, 2008). Subsequently, two novel QTLs contributing to heat tolerance were uncovered by phenotypic evaluation of double haploid-based mapping population for two consecutive summer seasons and by employing QTL-seq approach in broccoli (Branham and Farnham, 2019). Recently, subjecting genome wide association (GWAS) study of one hundred forty two lines unearthed a total of fifty seven significant marker trait associations for various physiological and yield related traits under heat stress in *Brassica rapa* (Chen et al., 2022). In tomato, Xu et al. (2017) mapped 13 QTLs for heat tolerance linked with reproductive traits, including pollen viability, pollen number, style protrusion, anther length, style length, flower per inflorescence, and inflorescence number. These QTLs showed additive effects and no epistatic interaction. Likewise, six QTLs linked to fruit set in tomato at high temperatures were identified (Grilli et al., 2007). Based on evaluating recombinant inbred lines and introgression lines developed from *Solanum lycopersicum* var. "MoneyMaker" × *S. pimpinellifolium* across multi environments under high temperature stress enabled in identification of 22 QTLs related to reproductive traits (flower number fruit number and fruit set proportion) on LG1, 2, 4, 6, 7, 10, and 11 explaining phenotypic variation from 4 to 13% (Gonzalo et al., 2020). In combination of phenotypic assessment of leaf cell membrane stability by applying heat stress in F_2 derived mapping population with QTL-seq approach in F_2 derived mapping population assisted in uncovering a total of seven QTLs *qHT1. 1*, *qHT2. 1*, *qHT2. 2*, *qHT5. 1*, *qHT6. 1*, *qHT7. 1*, and *qHT8. 1* conferring heat tolerance in bottle gourd (Song et al., 2020). Likewise, employing conventional QTL mapping and QTL-seq analysis allowed in identifying a total of five major QTLs *qHII-1-1, qHII-1-2, qHII-1-3, qHII-2-1*, and *qCC-1-5* (*qREC-1-3*) related to heat injury index under heat stress in tomato (Wen et al., 2019). The authors performed the functional validation of the underlying selected four potential candidate genes *SlCathB2, SlGST*, *SlUBC5*, and *SlARG1*. To decipher genetic basis of heat tolerance in cucumber, QTL analysis of mapping population developed from "99281" (heat-tolerant) × "931" (heat-sensitive) population phenotypically evaluated during summer 2018, 2019, and 2020 allowed to identify one major QTL *qHT1.1* on LG1 (Liu et al., 2021). There were 98 genes underlying this QTL. Of these identified genes, expression of *Csa1G004990* candidate gene was higher in "99281" than "931" genotype rendering it heat tolerant. In order to shed light into the functional role of HSP20 contributing to heat tolerance, in *Cucurbita moschata*, genome wide bioinformatic analysis enabled in unveiling 33 *HSP20* genes across the genome (Hu et al., 2021). Functional validation of CmoHSP20-7, 13, 18, 22, 26 and 32 genes indicated their possible role in heat tolerance in *Cucurbita moschata* (Hu et al., 2021).

In cowpea, five QTLs governing pod set at high temperature, namely Cht-1, Cht-2, Cht-3, Cht-4, and Cht-5, with CB 27 line of cowpea donating alleles for four QTLs (Cht-1, Cht-2, Cht-3, Cht-4) and IT82E-18 contributing alleles for Cht-5 (Lucas et al., 2013). Combinations of any of the four QTLs with Cht-5 positively correlated with heat tolerance in cowpea. Further, the presence of all five QTLs in the same line had the strongest positive correlation with heat tolerance (Lucas et al., 2013). Recently, four QTLs were identified in chickpea that conferred heat tolerance for filled pods (qfpod03_6), grain yield (qgy03_6), total seed number (qvs05_6), and pod set (q% podset08_6) using recombinant inbred lines produced from ICC 4567 (heat-sensitive) × ICC 15614 (heat-tolerant) lines (Paul et al., 2018). One QTL (qTBP5.2) was detected in lettuce, governing the tip-burn resistance trait, therefore beneficial in breeding programs

TABLE 5 | List of selected QTLs contributing to heat tolerance in vegetable crops.

Crop	Mapping population	Trait used	Name of gene/ QTL	Type of marker	Linkage groups	Phenotypic variation	References
Bottle gourd (*Lagenaria siceraria*)	L1 × L6	Relative electrical conductivity	*qHT1.1, qHT2.1, qHT2.2, qHT5.1, qHT6.1, qHT7.1, and qHT8.1*	SNP	1, 2, 5, 6, 7, 8	–	Song et al., 2020
Cowpea (*Vigna unguiculata*)	CB27 x IT82E-18, RIL 141	–	*Cht−1, Cht−2, Cht−3, Cht−4, Cht−5*	SNP	2, 3, 6, 7, 10	11–18%	Lucas et al., 2013
	IT93K-503-1 x CB46, RIL 113; IT84S-2246 x TVu146, RIL 136	Seed coat browning	*Hbs-1, Hbs-2 and Hbs-3*	SNP	1, 3, 8	6–77%	Pottorff et al., 2014
Common bean (*Phaseolus vulgaris*)	IJR × AFR298, RIL	Reproductive trait and yield and yield traits	32 QTLs	SNP	1, 2, 3, 4, 5, 8, 9, 10	7.8–36%	Vargas et al., 2021
Chickpea (*Cicer arietinum*)	DCP 92-3 × ICCV92944 RIL(184)	Phenological, physiological and yield related traits	77 QTLs	SNP	LG1–LG8	5.9–43.5%	Jha et al., 2021
	DCP 92-3 × ICCV92944F2(206)	Phenological and physiological traits	2 QTLs	SSR	–		Jha et al., 2019
	ICC 4567 × ICC 15614, RILs(292)	Yield and yield traits	4 QTLs	SNP	CaLG05, CaLG06	–	Paul et al., 2018
	GPF2 × ILWC292, RIL	Phenological, physiological and yield related traits	28 + 23 QTLs	SNP	All LG groups except LG8	5.7–13.7%	Kushwah et al., 2021
Chili (*Capsicum annuum*)	AVPP0702 × Kulai, backcross	Reproductive and yield trait	Hsp70 and sHsp gene	SSR	–	–	Usman et al., 2018
Tomato (*Lycopersicon esculentum*)	Nagcarlang × NCHS-1180 F2	Reproductive traits; viz., pollen viability, pollen number, style length, anther length; inflorescence number and flowers per inflorescence	*qPV11, qPN7, qSP1, qSP3, qAL1, qAL2, qAL7, qSL1, qSL2, qSL3, qFPI1 qIN1, qIN8*	SNP	1, 2, 3,7, 8, 11	10.5–38.7%	Xu et al., 2017
	MAGIC population	Yield components, phenology andfruit quality	69 plasticity QTLs	SNP			Bineau et al., 2021
	LA1698 × LA2093	Relative electrical conductivity REC), chlorophyll content (CC) and maximum photochemical quantum	5 major QTLs *qHII-1-1, qHII-1-2,qHII-1-3, qHII-2-1*and *qCC-1-5 (qREC-1-3)*	SNP	1, 2	16.48%	Wen et al., 2019
	Solanum lycopersicum var. "MoneyMaker" × *S. pimpinellifolium* accession TO-937RIL and IL	Reproductive traits viz., flower number, fruit number per truss and percentage of fruit set, stigma exsertion (SE),pollen viability (PV), tip burn	22 QTLs	SNP8K SNP SOLCAP Infinium chip	1, 2, 4, 6, 12	3.6–12.8%	Gonzalo et al., 2020

(Jenni et al., 2013). The information on genomes of crops is expanding rapidly. The sequencing coupled with resequencing will generate more information that will subsequently be used to gather detailed knowledge of QTLs and genomic bases of heat tolerance in crops. The closely-related crops share syntenic relationships and possess similar genomic regions with each other. In the forthcoming years, comparative genomic analysis and advancements in knowledge of molecular biology might allow us to transfer heat tolerant regions from one crop to another, thereby expanding the repository of cold tolerance in crop plants.

MARKER-ASSISTED SELECTION

As mentioned earlier, phenotype-based selection is prone to environmental conditions sometimes leading to erroneous

conclusions especially if trait is complex and conferred by polygenes or QTLs. Under such circumstances, genotype-based selection is more effective, precise and fast as compared to phenotypic selection. Genotype-based selection rather than phenotype-based selection is possible using markers linked to gene of interest. Genotype-based selection utilizes DNA markers that are linked tightly to the gene(s) of interest (Collard and Mackill, 2007). For MAS, first step is to identify markers linked to the gene or QTL using either mapping populations or association mapping where a panel of genotypes is used to identify liked markers. Subsequently, these markers are used to ascertain transfer of the gene to the progeny populations. Different types of markers, such as RFLP (restricted fragment length polymorphism), AFLP (amplified fragment length polymorphism), SSR (single sequence repeat), and SNPs (single nucleotide polymorphisms), can be detected, and the amount of variation in each marker can be determined. Using this approach, gene mapping and identifying gene associations with particular traits are useful for genetic crop improvement (Ruane and Sonnino, 2007).

Paul et al. (2018) identified SNP markers linked to QTLs for heat tolerance traits (50% flowering, podding behavior, total filled pods, % pod set, total seed number, grain yield, biomass, harvest index, 100-seed weight) in chickpea RILs (heat-tolerant ICC 15614×heat-sensitive ICC 4567). Composite interval mapping analysis affirmed two genomic regions (CaLG05 and CaLG06) with four QTLs (grain yield, total seed number, total filled pods, % pod set). A GWAS used 16,877 SNPs to identify marker-trait associations (MTA) in 135 diverse pea lines exposed to >28°C in the field to understand the genetic basis for heat tolerance (Gali et al., 2019). The study identified 32 MTAs and 48 candidate genes associated with various traits, including chlorophyll concentration, photochemical reflectance index, canopy temperature, reproductive stem length, internode length, pod number, with the potential for developing heat-tolerant cultivars (Tafesse et al., 2020). Lin et al. (2006) identified 14 RAPD markers linked to heat tolerance traits (flower number, fruit number, fruit set, yield) in tomato RILs derived from CL5915 (heat-tolerant) and L4422 (heat-sensitive) under HS. Developing heat tolerant *Capsicum annuum* through transferring heat shock protein encoding gene *Hsp70* and *sHsp* from AVPP0702 into Kulai an elite *C. annuum* cultivar by adopting marker assisted back crossing approach is notable illustration of marker assisted breeding for heat tolerance (Usman et al., 2018). Likewise, three non-synonymous SNPs identified in the *qHT2.1* major effect QTL in bottle gourd (Song et al., 2020) and non-synonymous SNP identified in the *QHT_C09.2* QTL regions in broccoli (Branham and Farnham, 2019) contributing to heat tolerance, which could be potentially used as candidate markers for screening heat tolerant bottle gourd and broccoli genotypes.

TRANSGENICS

Altering the genetic makeup of vegetable crops is a possible solution for developing crops that can grow and reproduce well under increasing temperatures. Plants have an inherent ability to endure supra optimal temperatures ("basal thermotolerance" or "acquired tolerance to increasing temperature") (Grover et al., 2013). The level of thermotolerance varies between plant species depending on their genetic makeup and specific expression of defense-related genes, however, levels of thermotolerance vary in different plant species again due to differences in genetic makeup of the plant species. Even within a species, genotypes differ for reaction (tolerance or sensitive) to HS owing to varying genetic makeup. Considerable number of genes/QTLs conferring tolerance to HS has been identified in vegetable crops and these genes/QTLs can be transferred from heat-tolerant genotypes to heat-sensitive genotypes using transgenic approaches to develop genetically modified heat tolerant crops. Genes expressed in heat-tolerant crops can be transferred to heat-sensitive crops using transgenic approaches to develop genetically modified heat-tolerant crops. Candidate genes for development of transgenics for heat tolerance are HSP, compatible osmolyte, and antioxidant levels, and detoxifying pathways (Parmar et al., 2017).

Manipulating HSPs

Many vegetable crops have been manipulated for increased expression of HSPs. For instance, in tomato, overexpression of trehalose-6-phosphate synthase/phosphatase (TPSP) gene derived from *Escherichia coli* increased the expression of HsfA1, HsfA2, and HsfB1, which was linked to escalating Hsp17.8, ER-sHsp and Mt-sHsp levels to impart heat tolerance (Lyu et al., 2018). Similarly, overexpression of small heat shock protein (CaHsp 25.9) improved thermotolerance in Capsicum transgenic lines (R9 and B6) under HS, decreasing MDA content and increasing proline and SOD content (Feng et al., 2019). In transgenic potato lines, overexpression of the A2 HSc70 (Heat-Shock Cognate) allele-maintained tuber yield at elevated temperature (Trapero-Mozos et al., 2018).

Manipulating Antioxidants

HS causes oxidative damage in plants; therefore, developing transgenics with enhanced antioxidative mechanisms may enhance thermotolerance in plants. Antioxidant mechanisms were manipulated in pea by incorporating heat shock factor gene (HsfA1d) from *Arabidopsis thaliana*. Under HS (42°C), transgenic pea plants had five-fold higher expression of HsfA1d than wild pea, decreasing H_2O_2 accumulation, and higher SOD and APX activities and proline content (Shah et al., 2020). Tang et al. (2006) developed transgenic potato plants (SSA plants) expressing Cu/Zn SOD and APX gene in chloroplasts under the control of a SWPA2. The transgenic plants had less damage induced by methyl viologen than non-transgenic plants. In the same study, photosynthetic activity decreased by 29% in non-transgenic plants but only 6% in transgenic plants under HS (42°C for 20 h). Overexpression of cytosolic APX (cAPX) in transgenic tomato (*Lycopersicon esculentum* cv. Zhongshu No. 5) under HS (40°C for 13 h) resulted in several-fold higher APX activity than wild plants, reducing electrolyte leakage (24% in A9 line and 52% in A16 line) compared with wild plants. Similarly, overexpression of cAPX in transgenic tomato increased tolerance HS (Wang et al., 2006).

Cross-Talk Between HSP and Redox Mechanism

Equilibrium between ROS generation and ROS scavenging is disturbed by the high temperature stress (Foyer and Noctor, 2005). One of the best strategies adopted by the plant cells is the production of HSPs on exposure to high temperature (Wang et al., 2004). HSPs positively affect thermotolerance by protecting ROS scavenging system and actively resulting in lower ROS concentration. HSPs also enable protein refolding, preventing aggregation of non-native proteins and stabilize polypeptides and membrane under stress conditions (Scarpeci et al., 2008). It is unclear whether there is specific interaction between HSPs and ROS scavenging machinery but ROS accumulation is reduced *via* HSP induced ROS scavenging activity. Hence the cross-talk between production of HSFs/HSPs and ROS scavenging activity play important role in acclimation (Kang et al., 2022). The communication between ROS and HSFs involve Mitogen Activated Protein Kinase (MAPK). ROS dependent phosphorylation can play vital role in HSF activation (Driedonks et al., 2015). MAPK3 and MAPK6 are the key players which are activated by H_2O_2 and further phosphorylate the HSFs, for instance in tomato, heat induced MAPK transduces the heat stress signal *via* HSFA3 (Link et al., 2002). Induction of heat shock transcription factors HsfA2 and HsfA4 is reported to be regulators of genes associated with ROS mitigation. HsfA4A is the principle candidate to function as H_2O_2 sensor (Scarpeci et al., 2008). At transcriptional level, HSPs are regulated by HSFs that bind to the conserved regulatory element of heat shock element (HSEs) and act as promoter for Hsp genes. Under stress conditions ROS mainly H_2O_2 functions as signal transduction molecule and cause HSF activation. ROS enhances the dissociation of HSP and HSF complex and promote the HSF trimerization and relocate the same to the nucleus leading to activation of the expression of HSPs and other heat responsive genes (Ul Haq et al., 2019) (**Figure 4**).

AGRONOMIC APPROACHES

By employing improved agronomic practices for different crops has improved crop yields. These practices include better soil, water, nutrient, weed, and pest management strategies, selection of varieties, and appropriate planting times and planting densities, and more and more (HanumanthaRao et al., 2016). Agronomic practices control soil temperature by minimizing the evaporation (Ferrante and Mariani, 2018) helping the cultivators with sustained water use, proper fertilizer use, and improved land maintenance, consequently improving crop quality and quantity. In addition, agronomic practice also helps with increased soil physical, chemical and microbial status. These help with water and nutrient availability and plant uptake. Agronomic practices for increasing vegetable crop yields that are efficient, cost-effective, and easily adaptable for HS management are described below.

Land preparation for planting involves tillage, seedbed shaping, and mulching. These practices depend on the soil type, physical and chemical properties. Sandy loam soils are best for raising vegetables such as potato, cauliflower, lettuce, cabbage, and tomato. Tillage includes breaking up/loosening the soil by plow, favoring seed germination, and proper seedling growth. Tillage also helps control weeds, aerate soil, and bury the previous crop's residues; the tillage method varies between crops (Kladivko, 2001). However, the same benefits can be obtained with no-till or minimum tillage practices that minimizes soil disturbance and helps with building of soil organic carbon over time. Mulching is a process of covering the soil with chopped residues; it has many benefits, including reduced soil erosion and water loss, which maintain soil temperature (Mulumba and Lal, 2008). Use of conservation agricultural practices with minimum soil disturbance, grass mulch cover and crop rotations not only significantly increased yield of green pepper but also decreased irrigation water use and runoff, while increasing percolated water in the root zone (Belay et al., 2020). Similarly, improved yields of tomato, cucumber and bitter guard were observed under conservation agriculture (Paudel et al., 2020). Conservation agricultural practices in vegetable production systems has shown to increase soil organic matter and nutrients (Belay et al., 2022). Irrigation increases soil moisture, decreasing soil temperature (by 2°) compared to non-irrigated soil (Lobell and Bonfils, 2008). Water quality and supply varies according to soil type, crop (warm- or cool-season), and weather conditions. Generally, vegetable crops are irrigated at 4–6-day intervals during summer and 14–15-day intervals during winter to reduce the high-temperature effects. Many modern technologies for irrigation are available that minimize water use, such as drip or trickle irrigation and overhead micro-sprinklers.

Variety selection is a successful agronomic approach for achieving high yields under high-temperature stress. Selection characteristics include high yield, disease resistance, maturity group, and grain quality (Pedersen, 2003). Suitable crop genotypes need to be early maturing and high yielding to escape heat by completing their life cycle early and thus perform better under HS (Sekhon et al., 2010). Furthermore, shifting the sowing time (early or late) is another strategy to avoid HS and avoid heat induced yield reduction as has been reported in mungbean (up to 50%) and soybean where yield declined tremendously by delay in the sowing date (Coventry et al., 1993; Miah et al., 2009). The goal of selection of crop duration and time of planting is to avoid HS during sensitive stages of reproductive development. In contrast, late sowing has been used to screen large populations of chickpea (Gaur et al., 2013), mungbean (Sharma et al., 2016), and lentil (Sita et al., 2017) genotypes for heat tolerance, some of which have been released (e.g., chickpea ICCV 92944) (Gaur et al., 2013). Heat-tolerant varieties of some vegetable crops are listed in **Table 6**. Hence, determining the ideal sowing time and selection of heat tolerant varieties is crucial for growth, development, and yield of crops.

Nutrients/Thermo-Protectants

HS can be alleviated by exogenous application of nutrients or thermo-protectants as a seed pretreatment, foliar spray, or by fertilizer application *via* broadcasting, pellet placement, or band placement (Waraich et al., 2012; HanumanthaRao et al., 2016). Macro-nutrients such as N, P, K, Ca, and Mg are

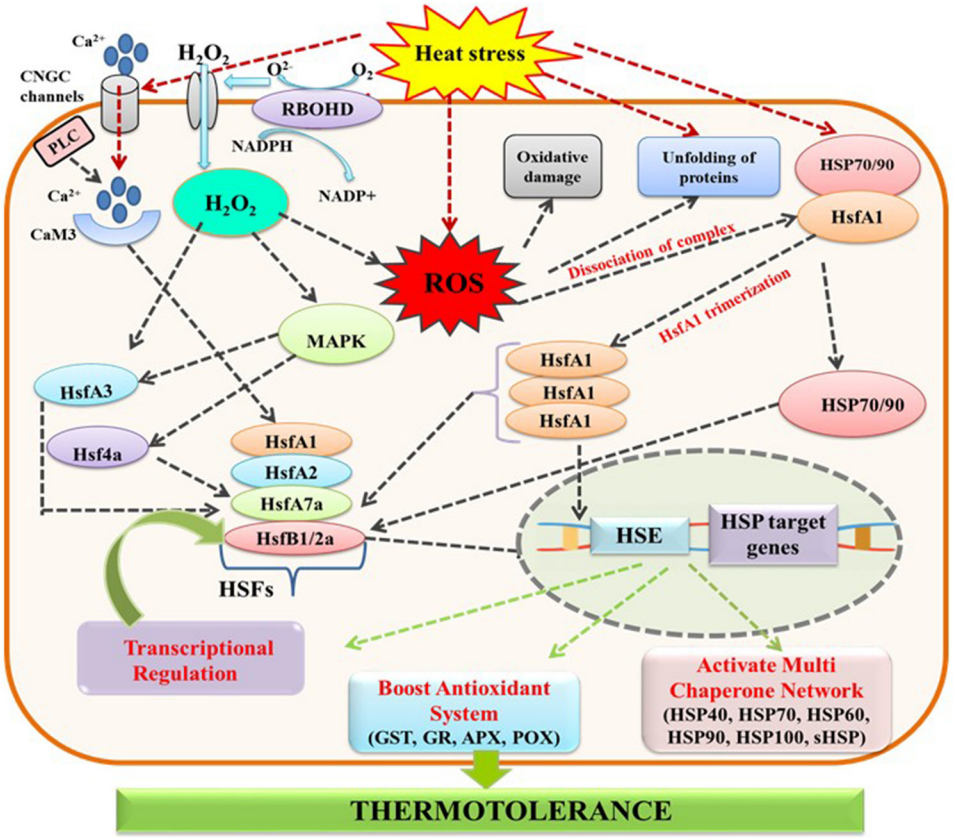

FIGURE 4 | Cross talk between HSPs and redox reaction: -Heat stress imposes damages to plant like increased membrane fluidity, unfolding of proteins, ROS production and dissociation of HSP70/90-HsfA1 complex. To endure HS, Plants activate various mechanisms to preserve their adaptation. First such mechanism is the activation of cyclic nucleotide gated calcium (CNGC) channels that result in the movement of Ca^{2+} ions in to cytoplasm and bind with Calmodulin Protein (CaM3) forming the Ca^{2+}-CaM3 complex and help in the activation of Heat shock factors (HSFs). Second mechanism involves Phosphoinositol signaling pathway that also lead to the influx of more Ca^{2+} in to the cytoplasm and merge with Ca^{2+}-CaM3 pathway. Another mechanism during HS is the activation of ROS signaling network by Respiratory Burst Oxidase Homolog D (RBOHD) that produce O^{2-} which is converted in to H_2O_2 that is involved in the induction of HSFs activation. ROS like H_2O_2 also activate the HSFs complex through mitogen activated protein kinase (MAPK). On activation, HSFs move to the nucleus and activate HSE and HSP target genes. HS also lead to the dissociation, of HSP70/90-HsfA1 complex; on dissociation HsfA1 undergoes trimerization that further activates the HSFs complex in the cytosol and Heat shock element (HSE) in the nucleus. Their activation has many positive effects on the cellular metabolism like transcriptional regulation, activation of antioxidant system and multi chaperone network (HSP60, HSP70, HSP90, HSP100, and sHSP) that may lower down the ROS levels in the cell and help in achieving thermotolerance.

required by plants (>10 mM) and help maintain structural and functional integrity (Waraich et al., 2011). Nutrient deficiencies alter the levels of tolerance to abiotic stresses. During HS, N deficient plants were associated with increased lipid peroxidation, while N supplemented plants tolerated photo-oxidative damage (Kato et al., 2003). Likewise, K deficient plants had reduced translocation of photo-assimilates to the sink organ, whereas K application improved the translocation and utilization of photo-assimilates, maintained cell turgidity, and upregulated enzymatic activity under HS (Mengel et al., 2001; Cakmak, 2005), increasing yield by 1.9-fold in Capsicum and 2.4-fold in tomato (Waraich et al., 2012). Similarly, exogenous application of calcium (2 L/ha) increased lettuce production under HS (Almeida et al., 2016).

Micronutrients such as B and Mn also provide heat tolerance of plants by increasing antioxidant activity and alleviating the damage induced by HS stress (Waraich et al., 2011). Other elements such as Se increased enzymatic activity and decreased membrane damage and ROS production in soybean (Djanaguiraman et al., 2005). Seed pretreatment and foliar application of thermoprotectant molecules such as proline, glycinebetaine, salicylic acid, spermidine, putrescine, GABA, ascorbic acid provides thermotolerance to crop plants (HanumanthaRao et al., 2016). For instance, exogenous application of proline mitigated HS effects in chickpea (Kaushal et al., 2011). Ascorbic acid application to mungbean seedlings under HS in a controlled environment improved seedling growth (Kumar et al., 2011). In cucumber, a 1 mM SA foliar spray provided heat tolerance by increasing CAT activity and thus reducing membrane damage and H_2O_2 levels (Shi et al., 2006). Similarly, Kaur et al. (2009) reported that exogenous application

TABLE 6 | Heat-tolerant varieties of some vegetable crops.

Crop	Trait indicating tolerance	Heat-tolerant varieties	References
Broad bean (*Vicia faba*)	Seed yield	C.52/1/1/1	Abdelmula and Abuanja, 2007
Broccoli (*Brassica oleracea* var. *italica*)		Gypsy and Packman	Farnham and Bjorkman, 2011
Cabbage (*Brassica oleracea* var. *capitata*)	Cell membrane thermostability	Sousyu	Chauhan and Senboku, 1996
		ASVEG#1	Fu et al., 1993
Capsicum (*Capsicum annuum*)		Mr. Lee No. 3 selex, CCA-119A, Susan's Joy, CCA-3288	Dahal et al., 2006
		IIHR Sel.-3	Devi et al., 2017
Cauliflower (*Brassica oleracea* var. *botrytis*)		IIHR316-1, IIHR371-1 and PusaMeghna	Devi et al., 2017
Chickpea (*Cicer arietinum*)		ICCV07110, ICCV92944	Kumar et al., 2013
Common bean (*Phaseolus vulgaris*)	Chlorophyll fluorescence	Ranit and Nerine RS	Petkova et al., 2007
		IIHR-19-1	Muralidharan et al., 2016
Cowpea (*Vigna unguiculata*)		IT93K-452-1, IT98K-1111-1, IT93K-693-2, IT97K-472-12, IT97K-472-25, IT97K819-43 and IT97K-499-38.	Timko and Singh, 2008
Lettuce (*Lactuca sativa*)		S24 and S39	Han et al., 2013
Mungbean (*Vigna radiata*)	Seed yield	NFM-6-5 and NFM-12-14	Khattak et al., 2006
	Biomass, number of flowers, pods and seeds weight/plant	EC693357, EC693358, EC693369, Harsha and ML1299	Sharma et al., 2016
Okra (*Abelmoschus esculentus*)	Yield (fruit number)	L2-11 and L4-48	Hayamanesh, 2018
Potato (*Solanum tuberosum*)	Tuber yield and dry matter	HT/92-621 and HT/92-802	Minhas et al., 2001
Pea (*Pisum sativum*)		IIHR-1 and IIHR-8	Muralidharan et al., 2016
Soybean (*Glycine max*)	Pollen traits	45A-46	Alsajri et al., 2019
	Pollen traits	DG 5630RR	Salem et al., 2007
Spinach (*Spinacia oleracea*)	Seed germination	Ozarka II, Donkey, Marabu, and Raccoon	Chitwood et al., 2016
Tomato (*Lycopersicon esculentum*)		CL1131-0-043-0-6, CL6058-0-3-10-2-2-2 PusaSadabahar, PusaSheetal, Pusa Hybrid-1	Abdul-Baki, 1991* Devi et al., 2017

of SA (10 and 20 μM) to heat-stressed brassica seedlings (40–55°C) improved CAT and POX activities. Pretreatment of SA to mungbean seedlings decreased lipid peroxidation and enhanced antioxidant activity, improving membrane stability (Saleh et al., 2007). In chickpea, a 100 μM SA foliar spray to heat-stressed seedlings (46°C) increased proline content (Chakraborty and Tongden, 2005). Thus, exogenous SA application mitigates the harmful impacts of heat-induced damage by strengthening antioxidative pathways. Foliar spray of Se (8 μM) to cucumber plants exposed to 40/30°C during flower initiation (35–75 DAS) decreased oxidative damage by stabilizing the antioxidative mechanism and increasing ROS scavenging (Balal et al., 2016).

Microorganisms Imparting Thermotolerance

In addition to other factors, plant-associated microorganisms, including plant-growth-promoting rhizobacteria, endophytic bacteria, and symbiotic fungi, play a significant role in imparting thermotolerance in plants (Grover et al., 2011). Many agriculturally important microbes have been discovered that colonize and promote plant growth and aid in nutrient and disease control through various direct and indirect methods (Singh et al., 2016). The interaction between microorganisms and host plants imparting stress tolerance is a complex process and polygenic in nature. Ali et al. (2009) discovered a thermotolerant

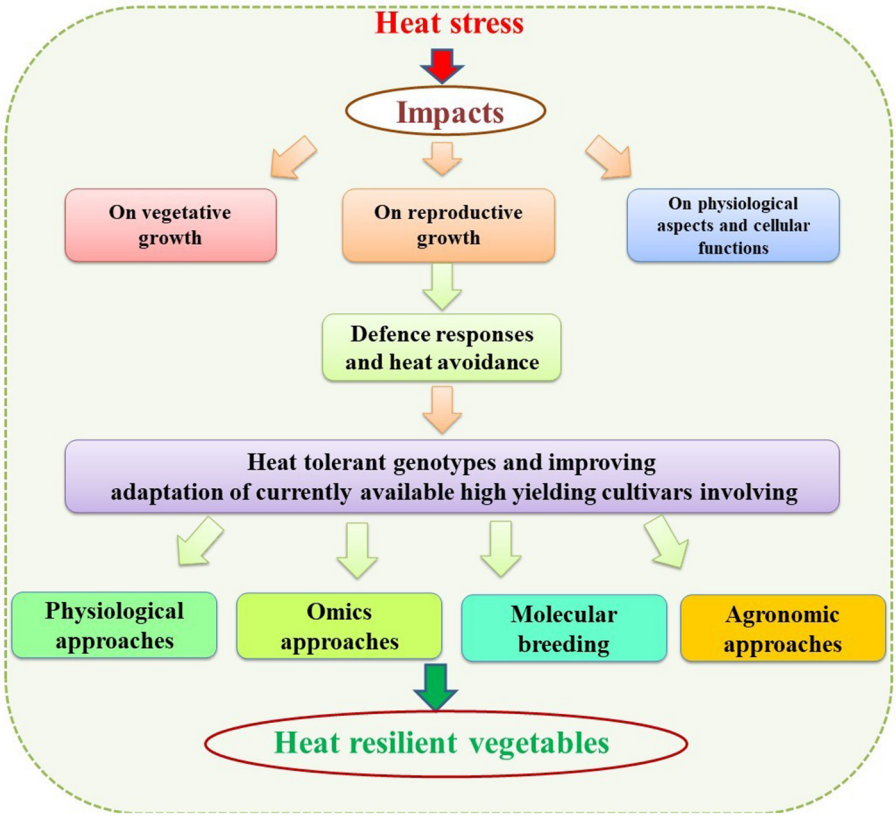

FIGURE 5 | Heat stress has various negative impacts on the plant like reducing vegetative and reproductive growth, interfering with the physiological and cellular functions. To combat such impacts, plant activates multiple responses and heat avoidance mechanisms which can be used to identify heat resilient vegetable crops. Different approaches categorized in this article for this purpose are physiological based, omics based, molecular breeding based and agronomic based. Such possible options will pave the way for improving adaptation and mitigation of heat stress in vegetable crops.

strain of *Pseudomonas* sp. AMK-P6 in sorghum that elicits HSPs synthesis under high-temperature stress, and improves biochemical activities by inducing the synthesis of osmolytes such as proline, sugars, amino acids, and chlorophyll. *Pseudomonas putida* NBRI0987, a thermotolerant strain (<40°C) was isolated from the chickpea rhizosphere (Srivastava et al., 2008). A recent study on different rhizobacterial strains of pigeon pea at high temperature (30, 40, 50°C) showed that S1p1 and S12p6 were the most promising strains for plant growth and development, stimulating auxin production, flavonoid production, and siderophore formation (Modi and Khanna, 2018). It would be worth evaluating the effectiveness of these microbes in vegetable crops for induction of thermotolerance.

Protected Cultivation

Growing vegetables in protected environments on small-scale farms using modern technologies has gained considerable attention for their high yields and quality and regular vegetable supply in the off-season (Sabir and Singh, 2013). Protected cultivation involves manipulating environmental factors such as temperature, humidity, light, water, and soil by designing suitable structures and following appropriate practices (Wittwer and Castilla, 1995). The main practices for protected cultivation are row tunnels, polytunnels, and mulching, which are more beneficial than open-field cultivation with less demand for fertilizers, pesticides, and water (Choudhary et al., 2013). In tomato, using a fogging system for 20 min/h (between 10 a.m. and 4 p.m.) in a hot shade house (>37°C) obtained high fruit yields with fewer physiological disorders (Ro et al., 2021). A similar fogging system improved the antioxidant defense responses in tomato plants (Leyva et al., 2013). Related approaches have been used to cultivate cucumber, capsicum, and lettuce with high yields (Sabir and Singh, 2013).

CONCLUSIONS

Vegetables are a distinct collection of plant-based foods that vary in nutritional diversity and form an important part of healthy diets. They also have great potential for boosting human health. Exposure to high temperatures or HS can directly or indirectly influence the production and quality of fresh vegetables. Several heat-induced morphological damages, such as poor vegetative growth, leaf tip burning, rib discoloration in leafy vegetables, sun burned fruits, decreased fruit size; pod abortion,

and unfilled pods are common, which can render vegetable cultivation unprofitable. Key physiological and biochemical effects associated with crop failure include membrane damage, photosynthetic inhibition, oxidative stress, and reproductive tissue damage. Reproductive stage has extensively been studied and found to be more sensitive to HS as it directly affects yields by reducing processes like pollen germination, pollen load, pollen tube growth, stigma receptivity, ovule fertility, and seed filling, resulting in poorer yields. Hence, sound and robust adaptation strategies are needed to mitigate the adverse impacts of HS to ensure the productivity and quality of vegetable crops.

Most important strategy to manage HS is deployment of heat tolerant cultivars (**Figure 5**). Physiological traits, such as stay-green trait, canopy temperature depression, cell membrane thermostability, chlorophyll fluorescence, relative water content, and stomatal conductance, are especially important in developing high-yielding heat-tolerant varieties/cultivars. Molecular approaches like omics, molecular breeding and transgenics have the potential to enhancing heat tolerance either by transferring heat tolerant genes/QTLs to elite cultivars with the help of molecular markers or elucidating mechanisms of tolerance leading to identification of heat tolerance genes and transferring those across genera or families *via* genetic modifications. Besides these approaches, simple agronomic methods are also important for mitigating HS effects at the grassroots level. Therefore, developing heat-tolerant plant types using physiological, molecular, and breeding-based techniques is essential for sustaining vegetable production systems and human health. Further, these approaches will offer insight into the physiological and molecular mechanisms that govern thermotolerance and pave the way for engineering 'designer' vegetable crops for better health and nutritional security.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Genome-Wide Characterization of the Aquaporin Gene Family in Radish and Functional Analysis of *RsPIP2-6* Involved in Salt Stress

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Aquaporins (AQPs) constitute a highly diverse family of channel proteins that transport water and neutral solutes. *AQPs* play crucial roles in plant development and stress responses. However, the characterization and biological functions of *RsAQPs* in radish (*Raphanus sativus* L.) remain elusive. In this study, 61 non-redundant members of AQP-encoding genes were identified from the radish genome database and located on nine chromosomes. Radish AQPs (RsAQPs) were divided into four subfamilies, including 21 plasma membrane intrinsic proteins (PIPs), 19 tonoplast intrinsic proteins (TIPs), 16 NOD-like intrinsic proteins (NIPs), and 5 small basic intrinsic proteins (SIPs), through phylogenetic analysis. All RsAQPs contained highly conserved motifs (motifs 1 and 4) and transmembrane regions, indicating the potential transmembrane transport function of RsAQPs. Tissue- and stage-specific expression patterns of AQP gene analysis based on RNA-seq data revealed that the expression levels of *PIPs* were generally higher than *TIPs*, *NIPs*, and *SIPs* in radish. In addition, quantitative real-time polymerase chain reaction (qRT-PCR) revealed that seven selected *RsPIPs*, according to our previous transcriptome data (e.g., *RsPIP1-3*, *1-6*, *2-1*, *2-6*, *2-10*, *2-13*, and *2-14*), exhibited significant upregulation in roots of salt-tolerant radish genotype. In particular, the transcriptional levels of *RsPIP2-6* dramatically increased after 6 h of 150 mM NaCl treatment during the taproot thickening stage. Additionally, overexpression of *RsPIP2-6* could enhance salt tolerance by *Agrobacterium rhizogenes*-mediated transgenic radish hairy roots, which exhibited the mitigatory effects of plant growth reduction, leaf relative water content (RWC) reduction and alleviation of O^{2-} in cells, as shown by nitro blue tetrazolium (NBT) staining, under salt stress. These findings are helpful for deeply dissecting the biological function of *RsAQPs* on the salt stress response, facilitating practical application and genetic improvement of abiotic stress resistance in radish.

Keywords: radish, aquaporin, PIPs, *RsPIP2-6*, salt stress

INTRODUCTION

Soil salinization is one of the main abiotic stressors in global agriculture production. Approximately 25% of the global cultivated land area is salinized, and the problem has sequentially deteriorated due to climatic variation and desertification (Tuteja, 2007; Zhu, 2016). Plant growth and development, as well as crop yield, are severely hindered by salt stress. An excessive soil salt content causes vegetable crops to be short, with yellow leaves and brown roots (Chrysargyris et al., 2019; Daničić et al., 2021). In addition, an unsuitable salt environment destroys the plasma membrane structure, greatly increasing membrane permeability and resulting in the destruction of the water balance in plants (Ueda et al., 2016). Osmotic stress and radial water transportation are mainly dependent on aquaporin (AQP) activity (Horie et al., 2011; Chaumont and Tyerman, 2014; Laur and Hacke, 2014; Bouda et al., 2018). AQPs are integral membrane proteins that belong to the ancient superfamily of major intrinsic proteins (MIPs), which are widely distributed in animals, plants, and microbes (Gomes et al., 2009). Increasing evidence has demonstrated that AQPs efficiently transport water and other small molecule substrates and play important regulatory roles in seed germination, tissue expansion, reproductive growth, fruit ripening, water movement, and maintenance of cellular water homeostasis in plants (Eisenbarth and Weig, 2005; Chen et al., 2013; Moshelion et al., 2015; Shivaraj et al., 2017; Zargar et al., 2017). In addition, when plants are exposed to abiotic stress, AQPs quickly respond and regulate water transport, reducing H_2O_2 accumulation and membrane damage by enhancing the antioxidant system in plants (Hu et al., 2012).

The typical AQPs are composed of four monomers, and each monomer contains six transmembrane domains (TM1–TM6) and five connecting loops (LA–LE), forming independent transmembrane pores localized on the intra-(LB, LD) or extracytosolic (LA, LC, LE) sides of the membrane (Afzal et al., 2016; Ozu et al., 2018). Through folding and linking, two Asn-Pro-Ala (NPA) motifs form a narrow channel to control the permeability of water (Murata et al., 2000), which plays a vital role in water molecules across the membrane. Based on protein sequence similarity and subcellular localization, AQPs are divided into eight subfamilies, including plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs), uncategorized X intrinsic proteins (XIPs), GlpF-like intrinsic proteins (GIPs), hybrid intrinsic proteins (HIPs), and large intrinsic proteins (LIPs) (Danielson and Johanson, 2008; Hussain et al., 2020). Among them, PIPs are the subfamily with the most members that can be categorized into two phylogenetic subgroups, PIP1s and PIP2s, according to the length of the N- and C-termini of PIPs (Tyerman et al., 1999). PIP2s exhibit strong water permeability when expressed in *Xenopus* oocytes, whereas PIP1s generally have much lower or even no water channel activity (Fetter et al., 2004). PIP1 and PIP2 aquaporins may interact to increase water permeability (Hachez et al., 2013). *PIP* expression levels are complexly regulated by various physiological and environmental stressors, including plant hormones and abiotic stress (Kapilan et al., 2018), especially under drought and salt stress (Srivastava et al., 2016). Overexpression of *PIP* genes can improve salt tolerance of transgenic plants in several plants, such as sugarcane (Tang et al., 2021), barley (Alavilli et al., 2016), soybean (Zhou et al., 2014), *Leymus chinensis* (Ma and Liu, 2012), durum wheat (Ayadi et al., 2011), and rice (Guo et al., 2006). *PIP* genes might function as regulators of plant salt tolerance.

Radish (*Raphanus sativus* L.) is an important root vegetable crop belonging to the *Brassicaceae* family. Soil salinization and secondary salinization causing salt stress seriously affect the yield and quality of radish taproots. However, little information on the *AQP* gene family is available on radish. In the present study, a genome-wide analysis of the identification of *AQP* genes was performed, and its evolutionary relationships, structural characteristics, promoter analysis, and chromosomal distribution were systematically characterized. Moreover, the transcript profiles of *RsPIPs* in different developmental stages and tissues are detected and seven selected genes are also performed for differentially responsive genes under salt stress. Furthermore, the biological function of *RsPIP2-6* was validated by *Agrobacterium rhizogenes*-mediated transgenic radish hairy roots in the face of salt stress. These results provide fundamental insights for the genetic improvement of salt tolerance traits and for revealing the salt stress response mechanism of radish.

MATERIALS AND METHODS

Genome-Wide Identification of Aquaporin Genes in Radish

The gene and protein sequence information for radish were obtained from the public genome database (RGD[1]). The candidate AQP proteins that included the Asn-Pro-Ala (NPA) domain (PF00230) were identified through Pfam.[2] The hidden Markov model (HMM) search was then processed using HMMER 3.0[3] to retrieve the sequences, and SMART[4] and CDD[5] were employed to remove proteins with incomplete AQP conserved domains, ensuring the reliability of all radish aquaporin members (RsAQPs). Following this, Clustal W[6] was conducted for multiple sequence alignment, and all AQP protein sequences, including radish and *Arabidopsis*, were imported to generate the phylogenetic tree using MEGA 5.0 with neighbor-joining (NJ) and the bootstrap value set to 1000. The *Arabidopsis* AQP protein sequences were downloaded from the TAIR database.[7]

Chromosome Localization, Protein Properties, Gene Structure, and Promoter *Cis*-Elements Analysis

The structural intron and exon characteristics of the *RsAQP* family genes were determined using Gene Structure Display

[1]http://radish-genome.org/
[2]http://pfam.xfam.org
[3]http://hmmer.janelia.org/
[4]http://smart.embl-heidelberg.de/
[5]https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi
[6]https://pir.georgetown.edu/pirwww/search/multialn.shtml
[7]https://www.arabidopsis.org/

Server 2.0.[8] The chromosome localization of *RsAQPs* was plotted using MapChart software.[9] The ExPASy ProtParam tool[10] was used to analyze the RsAQP protein properties, including the number of amino acids (AAs), molecular weight (MW), theoretical isoelectric point (pI), hydrophilicity index (HI) and instability index (II). The conserved motifs of the RsAQP family were identified using the MEME Suite 5.4.1.[11] Moreover, transmembrane prediction was detected using Hidden Markov Models Server v.2.0.[12] Additionally, the promoter region (1500 bp sequence upstream of the translation initiation sites) of *RsAQP* genes was extracted and analyzed in the PlantCARE database for the identification of potential *cis*-acting elements (Lescot et al., 2002).

Expression Analysis of *RsAQP* Genes

The published RNA-seq data of five tissues (cortical, cambium, xylem, root tip, and leaf) at six stages (7, 14, 20, 40, 60, and 90 days after sowing) were used to analyze the expression patterns during radish development (Mitsui et al., 2015). Based on the reads per kilobase per kilo (RPKM) values, the heatmap was generated by TBtools[13] (Chen et al., 2020). The expression profiles of the identified *RsAQP* genes under salt stress were extracted and performed from our previous transcriptome data (Sun et al., 2016).

Plant Materials, Growth Conditions, and Salt Treatments

Two previously screened advanced inbred radish lines, namely the salt-sensitive ('NAU-TR12') and the salt-tolerant ('NAU-TR17') genotypes, were used in this study (Zhang et al., 2021). The seeds were rinsed and sterilized before germinating on moist filter paper in the dark for 2 days. Subsequently, seedlings were transferred into plastic pots and cultured at 25°C day/18°C night with 16 h light/8 h dark, 60% relative humidity and 12,000 lx light. After 3 (young seedling stage) and 8 (taproot stage) weeks, these seedlings were transferred into the plastic container with a half-strength Hoagland nutrient solution (Xu et al., 2013). During a 1-week slow seeding period, the plants were treated with 150 mM NaCl solution and the NaCl-free nutrient solution was used as a control (CK). Three biological replicates were employed in each treatment, and each replicate included 20 seedlings. Different tissues (such as leaf and root) were harvested in triplicate at 0, 6, 12, and 24 h after a continuous time under NaCl treatment. Then, the samples were immediately frozen in liquid nitrogen and subsequently stored at −80°C for further use.

RNA Extraction and RT-qPCR Analysis

Total RNA extraction was performed with an RNAprep Pure Plant Kit (Tiangen, Beijing, China), and cDNA was synthesized using a PrimeScriptTM RT reagent kit (Takara, Dalian, China) according to the manufacturer's instructions. RT-qPCR analysis was carried out on the LightCycler® 480 System (Roche, Mannheim, Germany). All primers used for RT-qPCR are listed in **Supplementary Table 3**. *RsActin* was employed as the internal standard to normalize expression. The relative expression level was normalized to the *RsActin* gene and calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Three replicates were performed in this study.

The relative expression levels of the salt stress samples were compared to those of the controls. The gene fragments for RT-qPCR were isolated among young and taproot thickening periods from two radish varieties: 'NAU-TR12' (salt-sensitive) and 'NAU-TR17' (salt-tolerant).

Agrobacterium rhizogenes-Mediated Transformation System of Radish

The coding sequence (CDS) of *RsPIP2-6* was amplified with the primer pair *RsPIP2-6OE*-F/*RsPIP2-6OE*-R. The PCR fragments were then inserted between *Xba*I and *Kpn*I restriction sites (**Supplementary Table 1**). The plant expression vector pCambia1300 with the 35S promoter included a green fluorescent protein (GFP) tag. The recombination vector containing *RsPIP2-6* was transformed into *A. rhizogenes* strain MSU440.

RsPIP2-6-transformed radish hairy root composite plants were obtained by infection, according to Wei et al. (2016). The germinating radish seeds were sown on vermiculite and cultured at 25°C day/18°C night with 16 h light/8 h dark, 60% relative humidity and 12,000 lx light. After 4 days, seedlings with consistent growth were selected, and the original roots of the radishes were cut off. The growing tip and 0.5–1 cm elongated hypocotyl (composite plants that contained the transformed hairy roots with a wild-type shoot) were retained for *A. rhizogenes* infection. *Agrobacterium rhizogene* harboring *RsPIP2-6*-GFP (*OE*) or the empty vector (pCambia1300-GFP: *EV*) in 50 mL LB liquid medium plus 50 mg/L streptomycin and 100 mg/L kanamycin were incubated overnight at 28°C on a rotary shaker at 200 rpm until the OD_{600} reached 0.8–1.0 (Qin et al., 2021). Bacterial cells were centrifuged at 5000 rpm for 5 min and re-suspended in MS liquid medium (OD_{600} = 0.8–1.0) containing 100 μM acetosyringone (AS) and infected in the dark for 40–60 min (Huang et al., 2022). Subsequently, the composite plants were planted into a substrate (peat:vermiculite = 2:1) and treated with 150 mM NaCl at four leaves and one shoot period for 6 days. Three biological replicates were employed in each treatment. Each sample of at least six seedlings was harvested for salt treatment in the experiment, and three seedlings were randomly selected and photographed.

Chlorophyll Fluorescence Measuring and Histochemical Staining

Chlorophyll fluorescence was analyzed using a chlorophyll fluorometer (IMAG-PAM). Three leaves and one shoot of soil-grown *OE* and *EV* seedlings were treated with 0 or 150 mM NaCl for 6 h before being subjected to chlorophyll fluorescence determination. The seedlings were dark-adapted for at least 30 min before measurements. Fv/Fm was averaged from equal circles of interesting areas on the leaves (Zhou et al., 2022). Chlorophyll fluorescence images and chlorophyll fluorescence

[8]http://gsds.cbi.pku.edu.cn/
[9]https://mapchart.net/greece.html
[10]https://www.expasy.org/
[11]https://meme-suite.org/meme/
[12]https://services.healthtech.dtu.dk
[13]https://github.com/CJ-Chen/TBtools

parameters of the samples were measured synchronously using Imaging PAM software. Each sample of at least 9 seedlings was used for chlorophyll fluorescence determination, and one leaf was randomly selected photo. In addition, histochemical staining was conducted with NBT, as previously described by Alvarez et al. (1998), and RWC in leaves was determined according to Hu et al. (2016). Three replicates were employed in each treatment, and each replicate included at least three seedlings.

Statistical Analysis

All experiments in this study were performed with at least three repetitions. The significance of differences determined by one-way ANOVA followed by Duncan's test among treatment means using IBM SPSS Statistics 25 (IBM Corp., United States) was defined as significant when $P < 0.05$, as indicated in the figure legends.

RESULTS

Identification and Characterization of *RsAQPs* in Radish

The homology search resulted in 62 putative AQP protein sequences obtained in radish. After removing the sequence with an incomplete NPA domain, 61 non-redundant and complete aquaporin members were identified from the radish genome database (**Table 1**). All members were correspondingly named according to the classification of model plant *Arabidopsis* from the TAIR database.[14] Based on physical and chemical property analyses, the protein sizes of RsAQPs varied from 122 to 553 AAs, and 55 members (90.16% of all RsAQPs) were concentrated at 20–35 kDa. The theoretical pI values ranged from 4.96 to 10.07, and the MWs ranged from 12.76 to 61.49 kDa. Additionally, the average instability coefficient (IC) was 29.58, and most (58 members, 95.08%) were structurally stable, with an IC less than 40.00. Furthermore, all proteins except RsNIP6-3 were predicted to be hydrophobic.

Phylogenetic Analysis of *RsAQP* Genes

To systematically classify the subfamily of RsAQPs and reveal the evolutionary relationship with the aquaporin members of *Arabidopsis* (AtAQP), a phylogenetic tree was constructed using the neighbor-joining method with the amino acid sequences (**Figure 1**). By homologue comparative analysis of the protein sequences between RsAQPs and AtAQPs, the 61 RsAQPs were separated into four distinct subfamilies according to their grouping with AtAQPs, covering RsPIPs, RsTIPs, RsNIPs, and RsSIPs. Among them, RsPIPs were the most abundant subfamily, containing 21 members, which were further divided into 2 subgroups containing 7 RsPIP1 members and 14 RsPIP2 members. There were 19 members involved in RsTIPs and 5 members in RsSIPs, which were clustered into 5 and 2 subgroups, respectively. The orthologous sequence of AtNIP3-1 was not identified in radish.

[14]http://www.arabidopsis.org

Gene Structure and Conserved Domain Analysis of RsAQPs

Exon–intron organization analysis of the 61 RsAQPs showed that the number of introns ranged from zero to seven, and the same subfamily generally contained similar gene structures (**Figures 2A,B**). Specifically, the RsSIP subfamily contained two introns, while the RsPIP subfamily displayed three introns, except for *RsPIP1-7* and *RsPIP2-10*, which had two and one introns, respectively. Most of the *RsTIPs* had two introns, except *RsTIP1-5* and *RsTIP1-6*, which lacked introns. The structure of the RsNIP subfamily was relatively complex, with the number of introns varying from one to seven.

A total of 15 conserved motifs were generated from 61 RsAQPs (**Figure 2C**), and the motif compositions were similar in the same subfamily. Among these, motifs 1 and 4 were involved in all RsAQP proteins, suggesting that these motifs were the basic region of RsAQPs. However, some motifs were unique and were only detected in specific subfamilies. For instance, motifs 7, 10, and 15 were detected only in RsPIPs, whereas motifs 9 and 12 were uniquely distributed in RsNIPs and RsTIPs, respectively. These special motifs might be the characteristic domains of RsPIPs, RsTIPs, and RsNIPs. In addition, some motifs were covered in different subfamilies. For example, motifs 2, 5, and 6 could be discovered in RsPIPs, RsTIPs, and RsNIPs, while motifs 3 and 8 were both distributed in RsPIPs and RsTIPs. The diversity of motif compositions in the RsAQPs family reflected their evolutionary processes and contributed to their functional differentiation.

Promoter *Cis*-Element Prediction and Transmembrane Region Analysis

Various *cis*-acting elements, including stress-, development-, and hormone-responsive elements, were widely distributed in the promoter regions of the *RsAQP* genes (**Figure 3**). By calculating the number of different *cis*-elements, the light-responsive element was the most frequent in the *RsAQP* promoter, followed by MeJA-responsive and abscisic acid-responsive elements. Notably, defense and stress elements were distributed in all RsAQP subfamilies. The wound-responsive element only existed in the *RsPIP* and *RsTIP* promoters, while the element involved in seed-specific regulation was only present in the *RsSIPs*. Moreover, none of the elements involved in cell cycle regulation were contained in the *RsNIPs* and *RsSIPs* (**Table 2**). These results suggest that the transcriptional regulation of different types of *RsAQP* genes was diverse, indicating the diversity of RsAQP functions. Furthermore, other *cis*-elements involved in osmotic stress, such as MBS (CAACTG), ABRE (ACGTG) and ABA (TAACCA), were also observed in R*sAQP* promoters. This suggests that these aquaporin members may be regulated by various factors in radish, including drought and ABA, which need to be experimentally demonstrated in further studies. Moreover, all RsAQPs contained transmembrane regions that varied from 3 to 12 (**Supplementary Table 1**), and more than half (33 RsAQPs) comprised six typical transmembrane domains.

TABLE 1 | Identification and characterization of AQP proteins in radish.

Protein name	Gene ID	Number of amino acids	Molecular weight	Theoretical pI	Instability index	Aliphatic index	Hydropathy index
RsPIP1-1	Rs265710	286	30667.57	8.86	31.02	96.92	0.365
RsPIP1-2	Rs218100	286	30614.59	9.01	34.55	96.92	0.378
RsPIP1-3	Rs605220	286	30527.6	9.16	31.22	97.62	0.419
RsPIP1-4	Rs212290	286	30527.6	9.16	31.22	97.62	0.419
RsPIP1-5	Rs000570	286	30588.65	9.02	32.08	94.55	0.386
RsPIP1-6	Rs480800	286	30620.65	9.03	32.63	94.5	0.376
RsPIP1-7	Rs159240	287	30749.77	8.99	29.48	92.16	0.359
RsPIP2-1	Rs359040	283	21453.85	6.71	32.14	95.54	0.445
RsPIP2-2	Rs359080	283	30119.75	6.51	30.33	95.51	0.501
RsPIP2-3	Rs359050	285	30232.87	6.95	30.86	96.21	0.505
RsPIP2-4	Rs612380	285	30232.87	6.95	30.86	96.21	0.505
RsPIP2-5	Rs120730	283	30039.7	6.51	29.65	97.6	0.525
RsPIP2-6	Rs257780	287	30461.24	6.5	34.15	99.62	0.563
RsPIP2-7	Rs404730	285	30099.91	7.62	26.34	103.75	0.522
RsPIP2-8	Rs079440	283	30067.94	8.53	28.56	100.04	0.475
RsPIP2-9	Rs137470	285	30061.83	6.88	28.35	102.95	0.505
RsPIP2-10	Rs123510	288	30907.89	8.97	25.25	102.95	0.477
RsPIP2-11	Rs260210	202	21453.85	6.71	32.14	95.54	0.445
RsPIP2-12	Rs151510	281	29853.65	8.82	26.49	96.9	0.427
RsPIP2-13	Rs044090	282	29837.71	8.83	29	97.62	0.493
RsPIP2-14	Rs430170	281	29810.69	8.99	31.62	96.23	0.471
RsTIP1-1	Rs204560	251	25610.7	6.02	26.19	107.73	0.797
RsTIP1-2	Rs176140	253	25832.86	5.61	25.55	110.71	0.816
RsTIP1-3	Rs316110	253	25734.72	5.32	30.58	111.9	0.834
RsTIP1-4	Rs316050	253	25734.72	5.32	30.58	111.9	0.834
RsTIP1-5	Rs105440	252	25903.02	5.12	16.56	106.51	0.817
RsTIP1-6	Rs480080	252	25943.02	5.13	20.46	104.96	0.808
RsTIP2-1	Rs232070	248	24886.86	5.32	26.03	110.6	0.956
RsTIP2-2	Rs301510	249	25020.13	5.32	29.77	114.1	1.001
RsTIP2-3	Rs301530	249	25020.13	5.32	29.77	114.1	1.001
RsTIP2-4	Rs282040	248	24852.92	5.3	23.21	113.39	0.993
RsTIP2-5	Rs037700	217	22021.56	6.03	20.97	110.65	0.811
RsTIP2-6	Rs180310	138	14082.46	5.12	29.4	114.49	0.808
RsTIP2-7	Rs321260	145	14486.85	4.96	23.69	125.79	1.084
RsTIP2-8	Rs060660	465	46575.37	5.05	22.76	119.18	1.082
RsTIP3-1	Rs455830	267	28168.67	7.2	25.9	111.16	0.606
RsTIP3-2	Rs299110	267	28468.07	6.54	31.22	112.66	0.581
RsTIP3-3	Rs013400	268	28676.32	6.49	28.67	112.54	0.568
RsTIP4-1	Rs194740	249	26195.44	5.3	23	112.81	0.726
RsTIP5-1	Rs345340	255	26402.72	6.71	25.98	96.35	0.759
RsNIP1-1	Rs597390	297	31511.65	8.62	31.71	107.68	0.446
RsNIP1-2	Rs051540	297	31511.65	8.62	31.71	107.68	0.446
RsNIP1-3	Rs162110	289	30633.6	8.86	29.28	105.92	0.469
RsNIP2-1	Rs255960	282	30253.84	8.66	40.63	111.12	0.242
RsNIP2-2	Rs444150	324	34586.75	5.75	34.68	94.78	0.318
RsNIP2-3	Rs249950	323	34727.96	6.42	41.97	101.73	0.326
RsNIP4-1	Rs186920	283	30281.59	7.66	33.43	105.05	0.575
RsNIP4-2	Rs510390	278	29678.07	8.6	31.55	111.12	0.745
RsNIP4-3	Rs580980	283	30086.34	8.21	30.21	110.88	0.689
RsNIP4-4	Rs552680	283	30120.35	6.81	31.12	112.26	0.707
RsNIP5-1	Rs090820	301	31073.22	8.66	35.28	96.31	0.537
RsNIP6-1	Rs103230	305	31823.04	8.26	33.09	99.87	0.429
RsNIP6-2	Rs103190	242	24968.15	7	27.15	102.85	0.594
RsNIP6-3	Rs103210	553	61490.11	5.85	31.64	90.98	−0.265

(Continued)

TABLE 1 | (Continued)

Protein name	Gene ID	Number of amino acids	Molecular weight	Theoretical pI	Instability index	Aliphatic index	Hydropathy index
RsNIP7-1	Rs222440	127	13465.47	5.68	39.16	108.9	0.54
RsNIP7-2	Rs222590	122	12759.07	8.8	44.72	122.21	0.829
RsSIP1-1	Rs221110	239	25576.16	9.68	27.73	101.8	0.687
RsSIP1-2	Rs291150	255	27481.6	10.07	26.94	96.9	0.459
RsSIP2-1	Rs536450	237	25738.68	9.75	29.26	122.49	0.75
RsSIP2-2	Rs374490	238	26159.29	9.7	24.86	117.06	0.656
RsSIP2-3	Rs515300	237	25815.76	9.61	20.08	115.11	0.664

Chromosomal Localization Analysis of RsAQPs

A total of 57 *RsAQPs* (93.44%) were successfully located on nine chromosomes of radish through MapChart analysis, except for *RsSIP2-3*, *RsNIP4-2*, *RsNIP4-3*, and *RsNIP4-4* (**Figure 4** and **Supplementary Table 2**). At least two members were mapped on each chromosome. Interestingly, some *RsAQPs* were located in clusters in certain chromosomal regions, especially on chromosomes 2 and 6. Among them, chromosome 6 possessed the largest number of *RsAQP* genes, followed by chromosomes 4 and 5, and the fewest number of *RsAQP* genes were found on chromosomes 7 and 8.

Spatial and Temporal Expression Patterns of RsAQPs

The expression profiles of the 61 *RsAQP* genes among different tissues (cortical, cambium, xylem, root tip, and leaf) and developmental stages (40, 60, and 90 days) were determined in the publicly available RNA-seq data (Mitsui et al., 2015) and presented in the heatmap (**Figure 5**). In total, the expression levels of *RsPIPs* and *RsTIPs* were significantly higher than those of *RsNIPs* and *RsSIPs* in all tissues. For the *RsTIP* subfamily, *RsTIP1-1* to *RsTIP1-4*, *RsTIP2-2*, and *RsTIP2-3* showed high expression within roots and leaves, while other *RsTIP* members were expressed at extremely low levels. However, most *RsPIPs* showed high transcript levels in the leaves and roots of the radish, especially *RsPIP2s*. For example, *RsPIP2-1*, *RsPIP2-2*, *RsPIP2-3*, *RsPIP2-4* and *RsPIP2-5* maintained relatively high expression levels at the middle stage of the roots, while the expression patterns of *RsPIP2-6* were relatively higher at the earlier and later stages (**Figure 5A**).

In the tissues for 40, 60, and 90 days, the expression levels of *RsPIPs* and *RsTIPs* were also significantly increased compared to *RsNIPs* and *RsSIPs*. For the *RsTIP* subfamily, *RsTIP1-1* to *RsTIP1-4* and *RsTIP2-1* to *RsTIP2-4* were expressed at high levels. In the *RsPIP* subfamily, *RsPIP1-3*, *RsPIP1-4*, *RsPIP1-6*, *RsPIP2-13*, and *RsPIP2-14* were highly expressed in the cortex, cambium, xylem, root tip, and leaf. *RsPIP2-6* was mainly expressed in the cortex, cambium and xylem, while *RsPIP2-1* was intensively expressed in the cambium and xylem (**Figure 5B**). These *RsPIP* genes might play critical roles in the development of radish roots.

Expression Profiles of *RsPIPs* in Different Stages and Varieties Under Salt Stress

Based on our previous RNA-seq data in radish taproots and the variation of the expression levels under salt stress (Xie et al., 2015; Sun et al., 2016), seven *RsPIPs* (*RsPIP1-3*, *1-6*, *2-1*, *2-6*, *2-10*, *2-13*, and *2-14*) were selected to further determine their expression patterns by RT-qPCR under different salt exposure durations in two radish varieties (**Figure 6** and **Supplementary Tables 3, 4**). At the seeding stage, almost all seven *RsPIP* genes were significantly upregulated under salt stress in the salt-tolerant variety 'NAU-TR17,' however, they did not show obvious variation in the salt-sensitive variety 'NAU-TR12' (**Figure 6A**). The salt-responsive expression profiles of these genes were screened at the taproot thickening period in 'NAU-TR17.' As shown in **Figure 6B**, the *RsPIP2-1* and *RsPIP2-6* genes exhibited sharp growth at 6 and 24 h, especially for *RsPIP2-6*, with a 250-fold increase.

Agrobacterium rhizogenes-Mediated Overexpression of *RsPIP2-6* Confers Salt Tolerance in Radish With Transgenic Hairy Roots

Agrobacterium rhizogenes-mediated transformation was employed to determine the biological gene function of *RsPIP2-6* in radish when exposed to salt stress, based on the transcript expression level. *RsPIP2-6*-overexpressing hairy roots were successfully obtained, and transgenic positive hairy

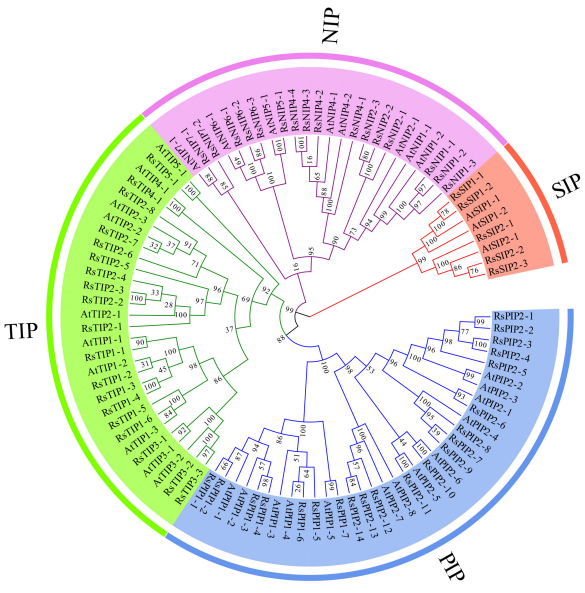

FIGURE 1 | Phylogenetic relationship between the members of RsAQP and AtAQP.

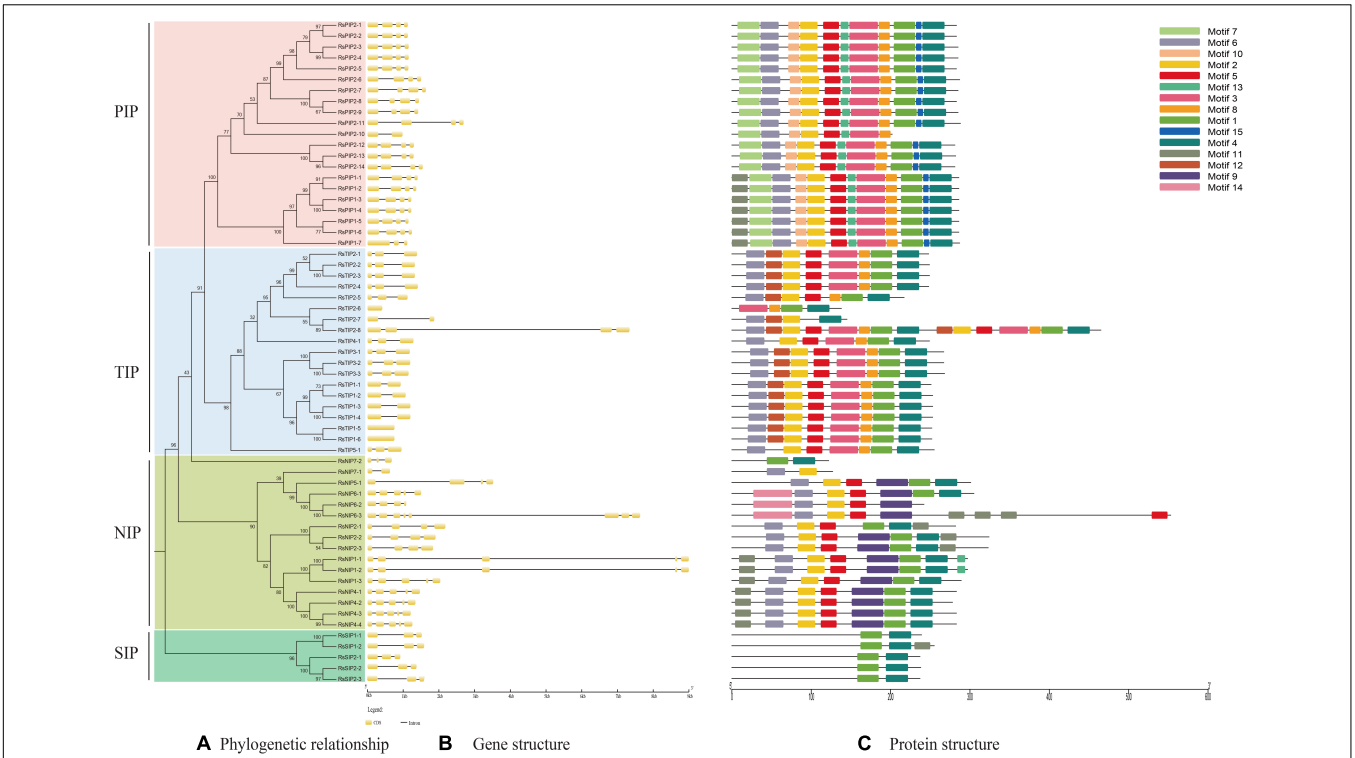

FIGURE 2 | Conserved motifs and gene structure distribution of RsAQP proteins. **(A)** Phylogenetic tree of RsAQP proteins. **(B)** Exon–intron structure of *AQP* genes in radish. **(C)** Conserved motif distribution of RsAQP proteins.

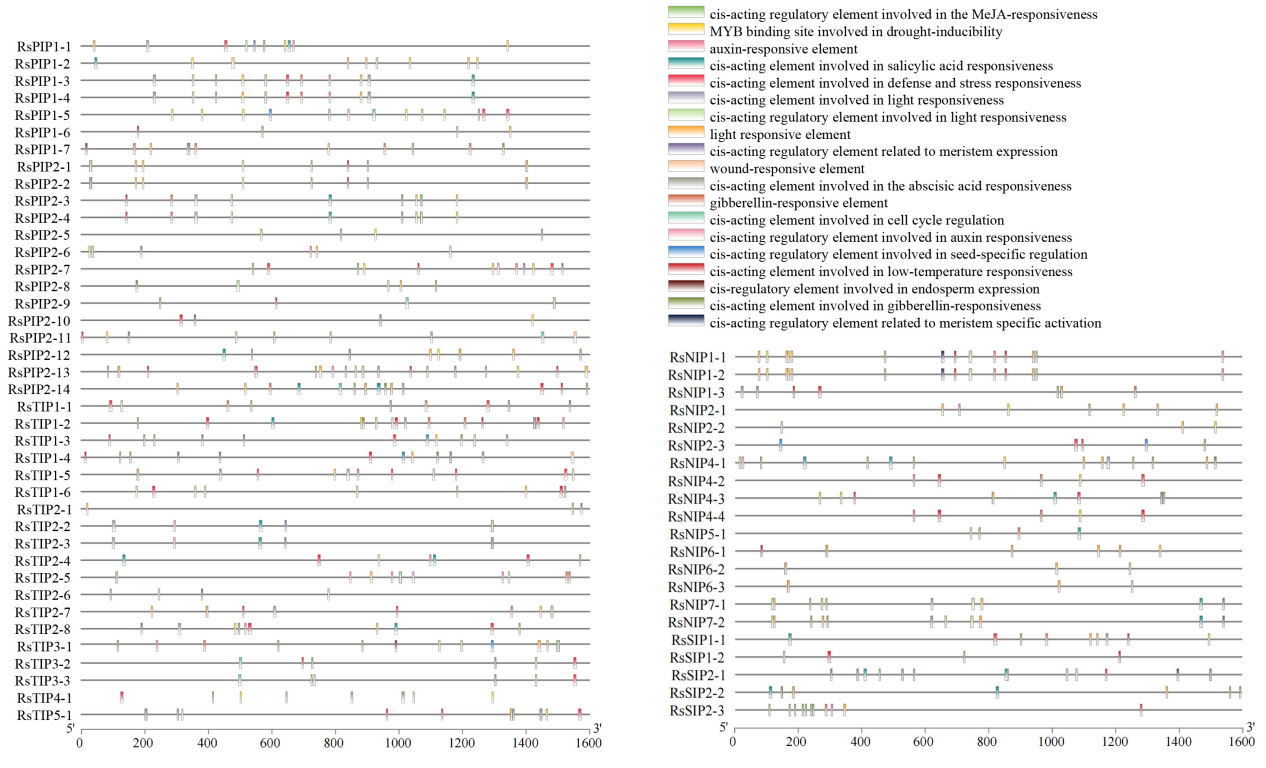

FIGURE 3 | Promoter *cis*-element prediction of *RsAQP* genes.

TABLE 2 | Number of occurrences of each *cis*-acting element in the *RsAQP* promoter.

Responsive elements	*Cis*-element	Occurrences	Total
Hormone	MeJA-responsive element	162	352
	Auxin-responsive element	28	
	Salicylic acid-responsive element	28	
	Abscisic acid-responsive element	102	
	Gibberellin-responsive element	32	
Stress	Drought-inducibility	30	105
	Defense and stress-responsive element	36	
	Wound-responsive element	3	
	Low-temperature-responsive element	36	
Development	Light-responsive element	230	266
	Meristem expression element	22	
	Cell cycle regulation element	7	
	Seed-specific regulation element	4	
	Endosperm expression element	3	

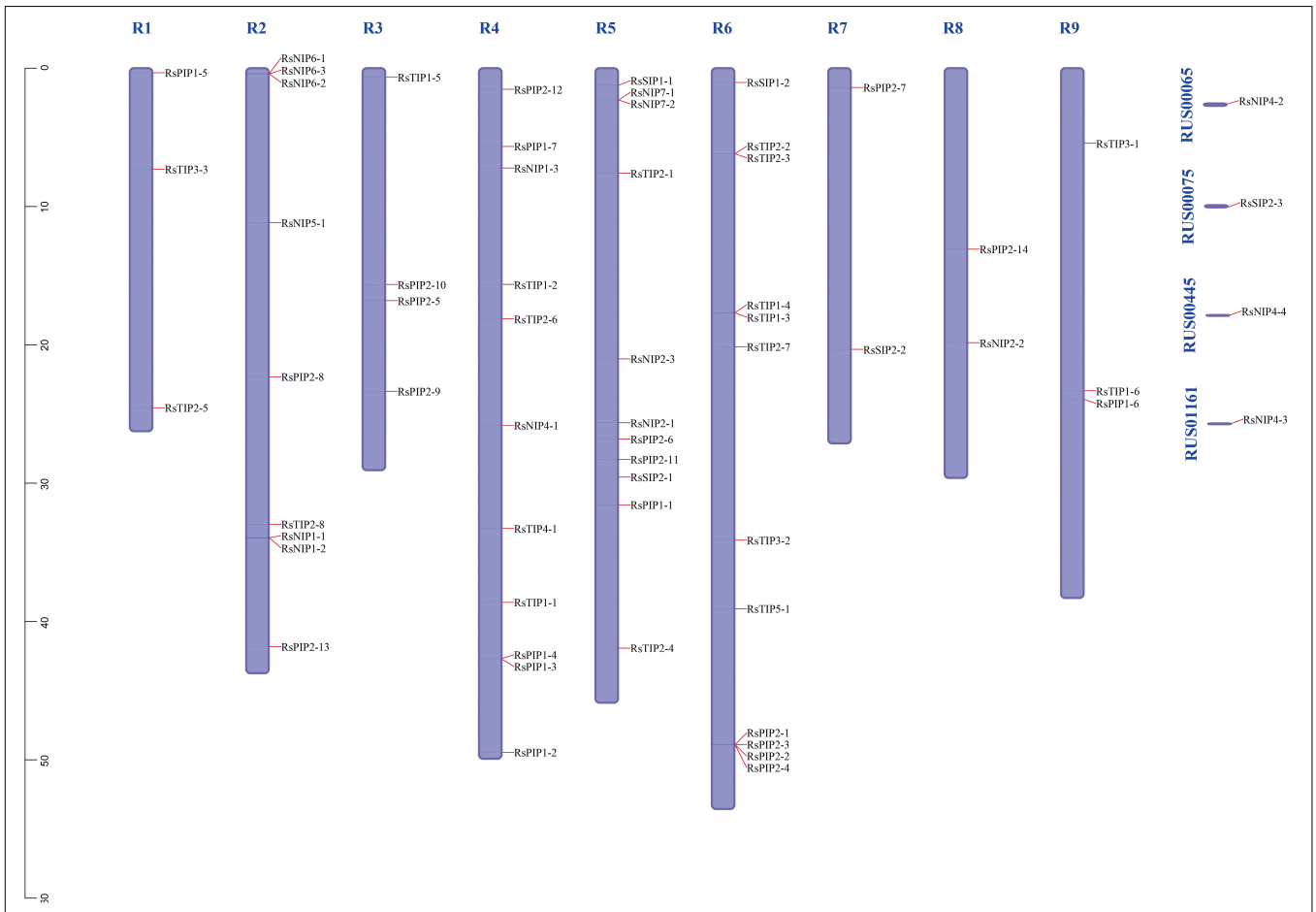

FIGURE 4 | Chromosomal distributions of *RsAQP* genes.

roots were identified by PCR, GFP signal detection and RT-qPCR (**Figures 7A–C**). The composite plants of *OE* with high expression in hairy roots were used for functional verification, while transgenic hairy root *EV* were used as a control. As shown in **Figure 7D**, no significant phenotypic differences were observed between the *EV* and *OE* plants under normal conditions. After exposure to 150 mM NaCl solution for 6 days, the leaves of *EV* plants were severely withered and yellowed or were dead and had a lower RWC in the leaves, while *OE* plants still grew vigorously and had a higher leaf RWC (**Figures 7E,F**).

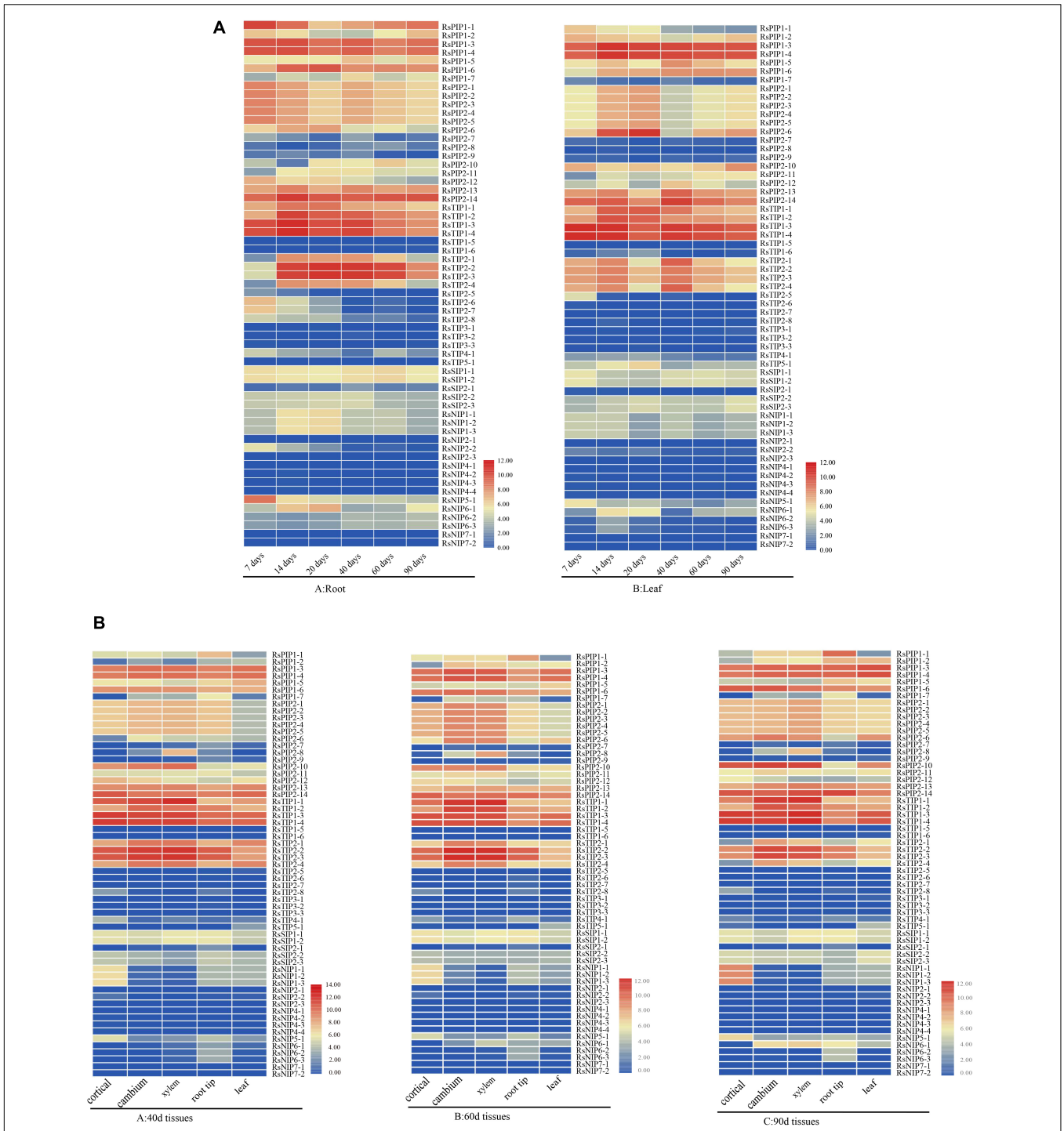

FIGURE 5 | Expression profiles of *RsAQP* genes in different stages and tissues. **(A)** *RsAQP* gene expression heatmap in six stages (7, 14, 20, 40, 60, and 90 days) of two tissues (root and leaf). **(B)** *RsAQP* gene expression heatmap in three stages (40, 60, and 90 days) of five tissues (cortical, cambium, xylem, root tip, and leaf).

Additionally, the survival rate of *EV* plants was reduced to 55.5%, while *OE* exhibited a reduction of 88.8% compared to their untreated conditions. Interestingly, the lateral root numbers of *OE* were significantly more plentiful than *EV*. The FluorCam chlorophyll fluorescence imaging system showed that the fluorescence intensity of *EV* plants markedly decreased in comparison to transgenic plants during salt stress (**Figure 7G**), indicating that photosynthetic capacity (Fv/Fm) had a downward trend. The photosynthetic capacity of transgenic plants was higher than that of *EV* plants, which indicated that *OE* could

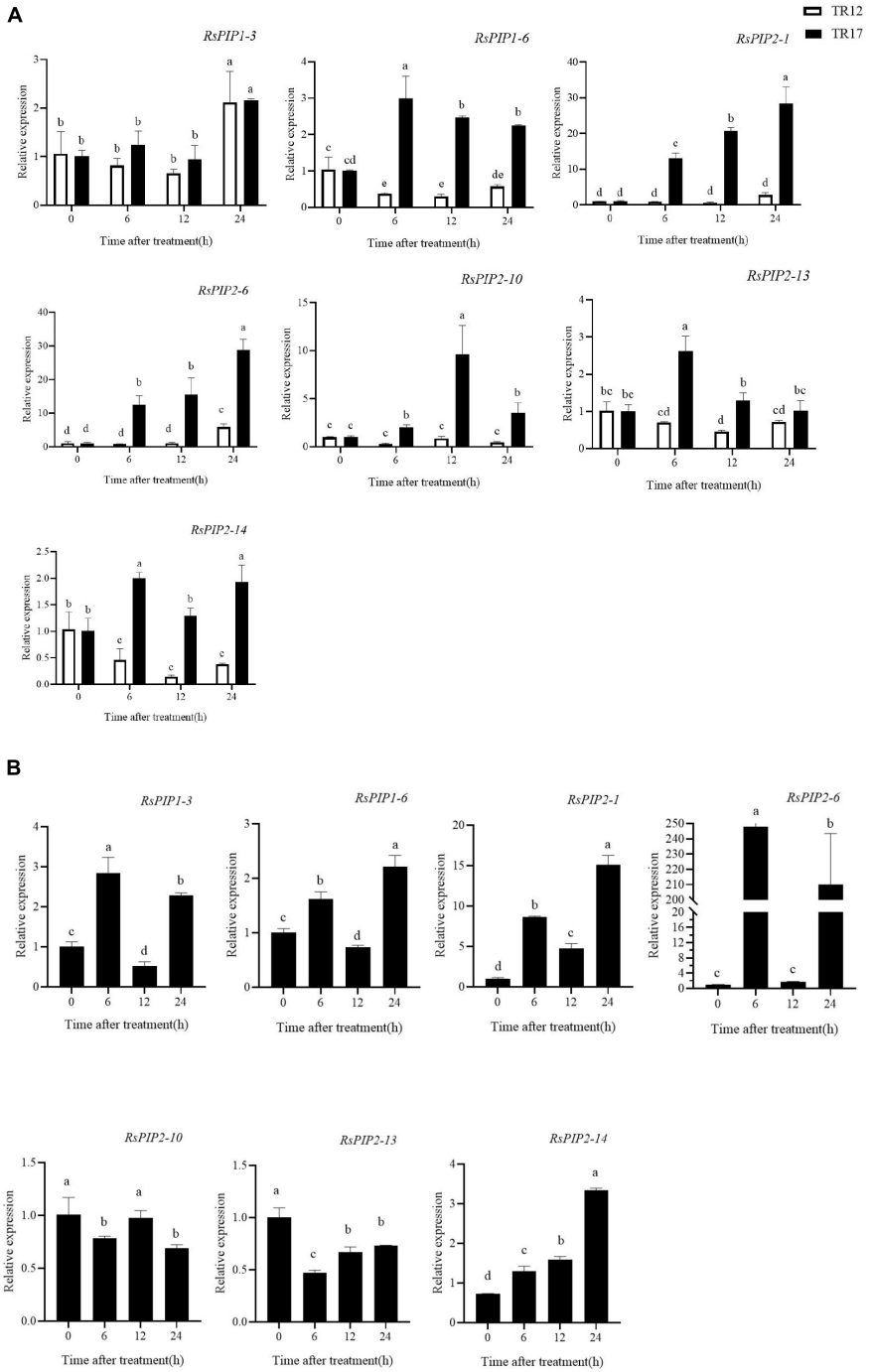

FIGURE 6 | Expression levels of *RsPIP* genes under NaCl treatment in young roots and the taproot thickening period. **(A)** Expression levels of *RsPIP* genes in young roots for the indicated time (h) under 150 mM NaCl treatment. **(B)** Expression levels of *RsPIP* genes in the taproot thickening period for the indicated time (h) under 150 mM NaCl treatment. *RsActin* was used as an internal control for qRT-PCR. The relative expression levels of the *RsPIP* genes were calculated based on the comparative threshold cycle (Ct). Statistical analysis was processed using GraphPad Prism 8. The significant difference was analyzed using IBM SPSS Statistics 25, values with different lowercase letters indicate a significant difference at $p < 0.05$ according to Duncan's multiple range tests. Each bar shows the mean ± SE of the triplicate assay.

alleviate the damage caused by salt stress on photosynthesis and could improve the salt tolerance of radish (**Figure 7H**). NBT staining showed that *EV* exhibited more severe damage in comparison with *OE* roots under salt stress (**Figure 7I**). Taken together, these results indicate that *RsPIP2-6* might be a positive regulator in radish against salt stress.

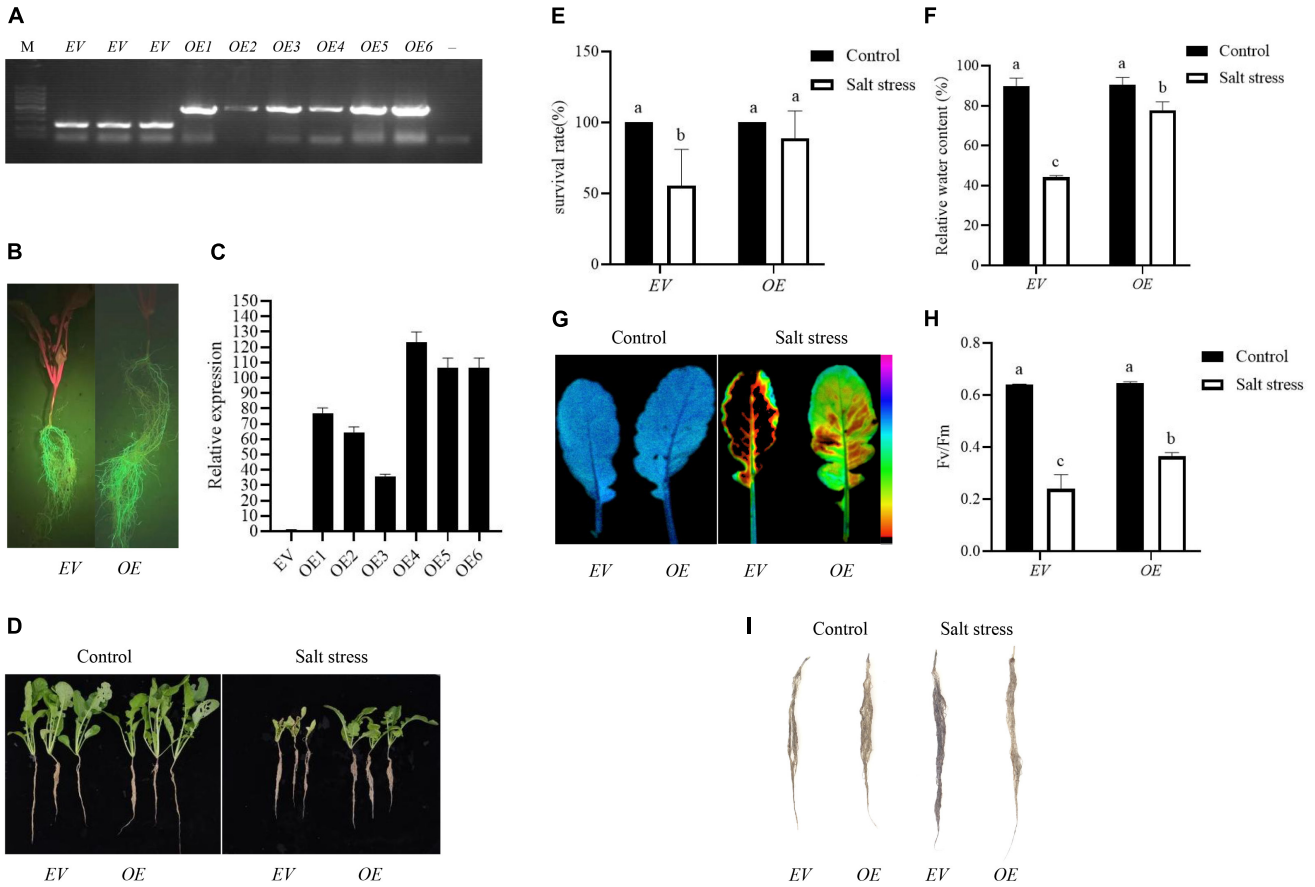

FIGURE 7 | Overexpression of *RsPIP2-6* positively regulated salt tolerance in radish. **(A)** PCR identification of *RsPIP2-6*-GFP (1300-GFP-F/*RsPIP2-6*-GFP-R primers) and 1300-GFP (1300-GFP-F/1300-GFP-R primers) in transgenic radish hairy roots. M: DL5000 marker; –, ddH_2O; *EV*: radish hairy root containing empty vector (pCambia1300-GFP), *OE*: radish hairy root overexpressing *RsPIP2-6*. **(B)** Green fluorescent protein (GFP) fluorescence in the hairy roots of radish. **(C)** Relative expression levels of *RsPIP2-6* in *EV* and *OE*s. **(D)** Phenotypes of *EV* and *OE* seedlings with 0 or 150 mM NaCl (150 mM for 6 days). **(E)** Statistical analysis of the survival rates of *EV*s and *OE*s with 0 or 150 mM NaCl. **(F)** Relative water content of *EV*s and *OE*s in radish with 0 or 150 mM NaCl. **(G)** Lipid peroxidation visualized by autoluminescence imaging. The color palette indicated luminescence intensity from low (purple) to high (black) values. **(H)** Fv/Fm rate in *EV*s and *OE*s radish with 0 or 150 mM NaCl. **(I)** Histochemical staining with NBT in the hairy roots of radish *EV*s and *OE*s with 0 or 150 mM NaCl. Each bar shows the mean ± SE of the triplicate assay, values with different lowercase letters indicate a significant difference at $p < 0.05$ according to Duncan's multiple range tests.

DISCUSSION

Characterization of *AQP* Gene Family Members in Radish

The AQPs, as a class of multifunctional proteins, not only participate in maintaining cellular water homeostasis in plants but also in other physiological activities, such as seed germination, growth and development, transport of nutrient elements, heavy metal elements, CO_2 transport, and stomatal movement, especially abiotic stress tolerance (Martinez-Ballesta and Carvajal, 2014). Accurate annotation of the *AQP* gene was an important starting point for future research on the gene function of analysis. An increasing number of *AQP* genes have been identified in many plants *via* genome sequencing. The *AQP* gene family has 39 members in *Arabidopsis* (Johanson et al., 2001), 42 in apple (Liu et al., 2019), 59 in *Brassica rapa* (Kayum et al., 2017), 33 in rice (Nguyen et al., 2013), 76 in tobacco (De Rosa et al., 2020), 47 in tomato (Reuscher et al., 2013), and 40 in chickpea (Deokar and Tar'an, 2016). However, the number and molecular characteristics of *AQP* family genes in radish are largely unclear. In the present study, 61 *AQP* genes were identified by whole genome analysis of *AQP*-encoding genes in radish. A higher number of *RsAQP* genes might indicate specific amplification, with higher evolution and more meticulous functional division. The RsAQP family was divided into four subfamilies (PIP, TIP, NIP, and SIP) based on their homology to AtAQPs. Interestingly, there were generally more members of each subfamily of radish than *Arabidopsis*, but no homologous genes of *AtNIP3-1* were identified in radish. The gene number of the *PIP* subfamily was significantly higher than that of other subfamilies in most plants, including radish, which indicated that *PIPs* had a more complex evolutionary process. Additionally, all AQPs in *B. rapa* functional analysis showed that most PIP subfamily proteins exhibited a high degree of

identity with abiotic stress-related AQP proteins from other plant species (Kayum et al., 2017). The phylogenetic relationship of RsAQPs was also supported by both their gene structures and conserved motifs. From an evolutionary perspective, the increasing number of genes might be due to gene replication events, including segmental and tandem duplication (Bancroft, 2001). Gene structure analysis showed that each subfamily displayed a similar exon–intron organization in *Arabidopsis* and radish (Jiang et al., 2020). Nineteen *RsPIP* genes contained three introns, aside from *RsPIP1-7* and *RsPIP2-10*. *RsTIPs* possessed introns, with numbers varying from zero to three, which was also similar to *AtTIPs*. Introns are related to gene evolution, which has been proposed to affect gene expression (Rose, 2008). More and longer introns exist in more highly expressed genes (Ren et al., 2006). The gain/loss of exons and introns might be the result of chromosomal rearrangements and fusions and can potentially lead to the functional diversification of multiple gene families (Xu et al., 2012).

The expression of *AQP* genes is regulated by various stressors in plants, such as drought, salt, and cold (Feng et al., 2018; Pawłowicz and Masajada, 2019). Promoter analysis revealed that the RsAQP gene promoters contained *cis*-elements in response to multiple hormones, stress, and development (**Table 2**). Subsequently, the expression of seven *RsPIP* genes was upregulated under salt exposure, indicating that they might play a crucial role in the response to salt stress. Similar results were also observed in soybean (Zhou et al., 2014), *Arabidopsis* (Feng et al., 2018), and *Canavalia rosea* (Lin et al., 2021). The distribution of *RsAQP* in linkage groups showed tandem duplicated pairs, such as *RsPIP2-1*, *RsPIP2-2*, *RsPIP2-3*, and *RsPIP2-4*, on the R6 chromosome, which might have been caused by gene duplication during evolution. Tandem duplications are a common phenomenon in nature, such as leucine-rich repeat domains in asparagus with both tandem genes and duplication across multiple chromosomes (Die et al., 2018). Conserved motif analysis showed that all RsAQP proteins shared the typical AQP domain. Motifs 1 and 4 were distributed in the four subfamilies (PIP, TIP, NIP, and SIP), indicating that they were highly conserved and might be the characteristic domain of the RsAQP family. Motifs 9 and 12 were distributed only in the TIP and NIP subfamilies, respectively.

Expression Divergence of *RsAQP* Genes

The expression level of *AtPIP2* was downregulated under salt stress in the roots of *Arabidopsis* (Boursiac et al., 2005), while *OsPIP2* was upregulated in rice (Guo et al., 2006). In the present study, *RsPIP2-6* increased dramatically compared to other *RsPIP* genes in the taproot thickening period of 'NAU-TR17' under salt stress. Therefore, *RsPIP2-6* might be a critical candidate gene for salt tolerance. Each specific isoform, as well as the plant genotype, might influence transcriptional aquaporin regulation under salt stress in broccoli plants (Muries et al., 2011). *FaPIP1;2* and *FaTIP1;1* transcript levels increased after salt treatment in a highly salt-tolerant genotype, whereas *FaPIP2;1* remained a relatively stable transcript level (Pawłowicz et al., 2017). The transcription level of the *PIP2;4* gene increased, while the *PIP1;2*, *TIP1;1*, and *TIP2;2* genes were reduced under salinity stress in *Piriformospora indica* (Ghorbani et al., 2019). The seedlings and reproductive stages were more vulnerable to salt stress than the vegetative stages, while the roots were more sensitive than other organs (Nam et al., 2015). These studies suggested that AQPs from different species had a high sequence homology, whereas they retained functional and regulatory specificity. These different, even contradictory, transcriptional regulations of *AQPs* might be caused by the tissue location of *AQPs*, plant species and growth phase, and salt concentration and duration of treatment.

The high efficiency of genetic transformation is an indispensable factor in gene function verification and germplasm improvement in radish. However, the efficiency of *A. tumefaciens*-mediated transformation in radish is extremely low, which greatly hinders gene function analysis (Muto et al., 2021). Therefore, the high-throughput production of transgenic plants in the short run is important for gene function research, especially for plants with a "bottleneck" to plant regeneration (Jian et al., 2009). To date, a fast and efficient transformation technique with *A. rhizogenes* has been widely used for functional genomics in plants (An et al., 2017; Che et al., 2019; Qin et al., 2021). In radish, only two reports have been successful in developing transgenic plants using the *A. rhizogenes*-mediated method (Tanaka et al., 1985; Balasubramanian et al., 2018). Here, *A. rhizogenes*-mediated transformation using composite plants as explants was performed to determine the overexpression of *RsPIP2-6* in radish. As a result, *RsPIP2-6*-transformed plants grew more vigorously, with a higher survival rate and a lower degree of damage compared with empty vector-transformed plants under salt stress. In a recent report, overexpression of *IbPSS1* improved salt tolerance in transgenic sweet potato lines obtained from an *A. rhizogenes*-mediated transformation system (Yu et al., 2020). *GmLecRlk*-overexpressing soybean lines have significantly enhanced salt tolerance by *A. rhizogenes* (Zhang et al., 2022). Similar to the above results, *RsPIP2-6* could also improve radish tolerance to salt stress using the *A. rhizogenes*-mediated transformation system. This finding provides a new idea for the breeding of genetically modified radish.

CONCLUSION

In this study, 61 *RsAQP* genes were identified and characterized based on radish genome data. Furthermore, phylogenetic analysis, gene structure, conserved motifs, promoter *cis*-elements, chromosome distribution, and RNA-seq expression analysis of RsAQP were conducted. The expression profiles of *RsPIPs* in different stages and tissues under salt stress indicate that *PIPs* might play a vital role in maintaining the water potential homeostasis of radish exposed to salt stress. In addition, overexpression of *RsPIP2-6* could enhance salt tolerance by *Agrobacterium rhizogenes*-mediated transgenic radish hairy roots, which showed enhanced tolerance to salt stress. These results provide a beneficial resource for the

evolution and function of *RsAQPs* and provide a basis for the breeding and genetic engineering of radish.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

XY and YW conceived and designed the study. JY and RT contributed to data collection and bioinformatics analysis. KL, XS, and MN were responsible for sample collection and RT-qPCR analysis. XY and XS drafted the manuscript and prepared the figures. LX and LL were contributed to revising the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.860742/full#supplementary-material

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