DAMPS ACROSS THE TREE OF LIFE, VOLUME 1: PLANTS

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DAMPS ACROSS THE TREE OF LIFE, VOLUME 1: PLANTS

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Arabinoxylan-Oligosaccharides Act as Damage Associated Molecular Patterns in Plants Regulating Disease Resistance

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Immune responses in plants can be triggered by damage/microbe-associated molecular patterns (DAMPs/MAMPs) upon recognition by plant pattern recognition receptors (PRRs). DAMPs are signaling molecules synthesized by plants or released from host cellular structures (e.g., plant cell walls) upon pathogen infection or wounding. Despite the hypothesized important role of plant cell wall-derived DAMPs in plant-pathogen interactions, a very limited number of these DAMPs are well characterized. Recent work demonstrated that pectin-enriched cell wall fractions extracted from the cell wall mutant impaired in *Arabidopsis Response Regulator 6* (*arr6*), that showed altered disease resistance to several pathogens, triggered more intense immune responses than those activated by similar cell wall fractions from wild-type plants. It was hypothesized that *arr6* cell wall fractions could be differentially enriched in DAMPs. In this work, we describe the characterization of the previous immune-active fractions of *arr6* showing the highest triggering capacities upon further fractionation by chromatographic means. These analyses pointed to a role of pentose-based oligosaccharides triggering plant immune responses. The characterization of several pentose-based oligosaccharide structures revealed that β-1,4-xylooligosaccharides of specific degrees of polymerization and carrying arabinose decorations are sensed as DAMPs by plants. Moreover, the pentasaccharide 3^3-α-L-arabinofuranosyl-xylotetraose (XA3XX) was found as a highly active DAMP structure triggering strong immune responses in *Arabidopsis thaliana* and enhancing crop disease resistance.

Keywords: arabinoxylan, cell wall, damage-associated molecular pattern (DAMP), plant immunity, pattern triggered immunity

INTRODUCTION

Plants are sessile organisms that need to develop robust disease resistance mechanisms to efficiently defend from pathogens and pests. Activation of plant defense responses requires the perception of molecules from the pathogen ("non-self" signals) and from the plant ("damaged-self" signals) that trigger specific resistance responses through diverse molecular monitoring systems (Atkinson and Urwin, 2012). Among these monitoring mechanisms are pattern- and effector-triggered immunity (PTI and ETI) (Dodds and Rathjen, 2010). PTI is based in the recognition by pattern recognition receptors (PRRs) of microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) from microorganisms or of plant-derived damage-associated molecular patterns (DAMPs) (Boller and Felix, 2009). MAMPs and DAMPs structures, with different biochemical composition (e.g., proteins, carbohydrates, lipids, and nucleic acids) have been identified, thus reflecting the diversity of immunogenic structures recognized by plants (Boutrot and Zipfel, 2017). In comparison with the high number of MAMPs characterized so far, much less DAMPs derived from plants have been identified to date (Choi and Klessig, 2016; Duran-Flores and Heil, 2016; Bacete et al., 2018; De Lorenzo et al., 2018; Li et al., 2020).

The plant cell wall is a dynamic and highly regulated structure mainly consisting of carbohydrate-based polymers, essential for growth, and development (Srivastava et al., 2017). Cellulose is the main load-bearing component in all plant cell walls, whereas different types of hemicelluloses and pectins are found in different plant phylogenetic groups (Carpita and Gibeaut, 1993; Carpita and McCann, 2000). Xylans are a diverse group of hemicelluloses with the common feature of a backbone of β-1,4-linked xylose residues (Scheller and Ulvskov, 2010). Monocot xylans usually contain many arabinose residues attached to the backbone and are known as arabinoxylans (AXs). Arabinofuranose substitutions are, in principle, less frequent in dicot xylans, but exceptions are found (Darvill et al., 1980; Fischer et al., 2004; Naran et al., 2008). Instead, dicot xylans are more commonly substituted with α-1,2-linked glucuronosyl and 4-*O*-methyl glucuronosyl residues known as glucuronoxylans which are the dominating non-cellulosic polysaccharides in the secondary walls of dicots. This variability in the fine structure of wall polymers exists not only among phylogenetic groups of plants, but also even between different tissues of a given plant. Cell wall heterogeneity may have had an evolutionary impact in the diversity of mechanisms that pathogens have evolved to breach plant cell walls, including the secretion of numerous cell wall-degrading enzymes (CWDE), such as cellulases, polygalacturonases, or xylanases (Annis and Goodwin, 1997). The functional integrity of cell walls is controlled by cell wall integrity monitoring systems (Bacete et al., 2018; Vaahtera et al., 2019). These systems trigger countervailing responses to cell wall restructuring which occurs upon pathogen infection, abiotic stress, and cell expansion during growth and development. The plant cell wall integrity pathway is strongly involved in the regulation of growth, immune responses and resource allocation between development and immunity (Hamann et al., 2009; Wolf et al., 2012; Engelsdorf et al., 2018). Alterations in cell wall composition or integrity by genetic or chemical means have a significant impact on plant resistance to different pathogens and/or abiotic stresses, since they typically lead to the activation of defensive signaling pathways, some of which are regulated by hormones (Miedes et al., 2014; Mélida et al., 2015; Nafisi et al., 2015; Houston et al., 2016; Bacete et al., 2020). For example, enhanced resistance to pathogens has been observed in *Arabidopsis thaliana* (Arabidopsis) mutants defective in specific cellulose synthases, enzymes involved in xylan decoration and in lignin biosynthesis (Ellis et al., 2002; Hernández-Blanco et al., 2007; Delgado-Cerezo et al., 2012; Xu et al., 2014; Escudero et al., 2017; Gallego-Giraldo et al., 2020; Molina et al., 2020).

Given both the complexity of the plant cell wall and the fact that many pathogens secrete a wide range of CWDE, it would be expected that the breakdown products of cell wall polymers could act as DAMPs that regulate immune responses. Confirming this hypothesis, pectic oligogalacturonides (OGs) were first cell wall DAMPs to be characterized (Nothnagel et al., 1983). OGs are derived from homogalacturonan, the main component of pectins, as a result of the activity of CWDE released by the pathogens during the colonization process (Ridley et al., 2001; Benedetti et al., 2015; Voxeur et al., 2019). Also, the overexpression or inactivation of genes encoding enzymes involved in the control of pectin structure [e.g., pectin methyl esterases (PME) and PME inhibitors], results in the modification of the degree of OGs release upon infection, and alterations of disease resistance phenotypes (Ferrari et al., 2008; Raiola et al., 2011; Lionetti et al., 2017; Benedetti et al., 2018; De Lorenzo et al., 2019). Another group of cell wall-derived carbohydrates recently characterized as DAMPs in Arabidopsis are cellulose-derived oligomers (β-1,4-glucans), which trigger signaling cascades sharing many similarities with the responses activated by OGs (Aziz et al., 2007; Souza et al., 2017; Johnson et al., 2018; Locci et al., 2019). With around 20 different monosaccharide moieties building the polysaccharides of the plant cell wall, other carbohydrate-based cell wall molecules in addition to OGs and cello-oligosaccharides should have been selected by plants as DAMPs. In line with this hypothesis, recent works have also nominated xyloglucan and mannan cell wall-derived oligosaccharides as plant DAMPs (Claverie et al., 2018; Zang et al., 2019), and β-1,3-glucan oligosaccharides present in plant callose but also in fungal cell walls, as dual DAMPs/MAMPs (Mélida et al., 2018).

Thus, growing evidences have awarded the cell wall with prominent novel roles in plant immunity. In this line, we have recently proposed a novel link between the cytokinin signaling pathway, cell wall composition control, and disease resistance responses through Arabidopsis Response Regulator 6 (ARR6) protein (Bacete et al., 2020). Cytokinins have emerged as an important hub integrating defense responses mediated by other hormones, and have been shown to regulate the activation of immune responses (Choi et al., 2011). In Arabidopsis, cytokinins

are perceived by Arabidopsis Histidine Kinase receptors, that are two-component system proteins which initiate a downstream phosphotransfer cascade that leads to the phosphorylation of Arabidopsis Response Regulator (ARR) proteins (To et al., 2007). We showed a novel function for ARR6, as impairment of *ARR6* gene affect plant cell wall composition, which impact plant-pathogen interactions, and might lead to the accumulation of differential or increased levels of DAMPs in *arr6* in comparison to wild-type plants that would favor a "defense-ready" state instead of a resting one. Remarkably, pectin-enriched cell wall fractions extracted from *arr6* cell walls triggered, when applied to wild-type Arabidopsis plants, more intense immune responses than those activated by similar wall fractions from wild-type plants, suggesting that *arr6* pectin fraction is enriched in wall-derived DAMPs. In an effort toward a better understanding of plant mechanisms involved in cell wall-mediated immunity, we have further purified *arr6* pectin fraction. Results from such purifications suggested that pentose-based oligosaccharides co-extracted with pectins (using calcium chelators) could play a role as plant DAMPs. Afterwards, we purified several pentose-based oligosaccharides, generated by enzymatic digestion from a natural material source rich in that type of hemicelluloses, that were biochemically analyzed and tested for their capacity to induce PTI hallmarks (Boller and Felix, 2009; Boudsocq et al., 2010; Ranf et al., 2011). Using this strategy, we identified AX-oligosaccharides as a novel group of DAMPs active on plants and characterized 3^3-α-L-arabinofuranosyl-xylotetraose (XA3XX) as a highly active structure triggering strong immune responses in Arabidopsis and enhancing crop disease resistance.

RESULTS

Low Molecular Weight Pectic Fractions From Arabidopsis Enriched in Oligopentoses Contain Active Plant DAMPs

In a previous work, we hypothesized that the molecular basis of the differential disease resistance responses in the Arabidopsis *arr6-3* mutant allele could be associated with the enhanced and differential presence of carbohydrate-based DAMPs in the pectin-enriched fractions derived from their cell walls (Bacete et al., 2020). These DAMPs, when released, would activate immune responses, thus triggering disease resistance. Pectic fractions isolated from *arr6-3* cell walls triggered more intense Ca^{2+} influxes and MAPK phosphorylation than the fractions from wild-type plants (Bacete et al., 2020), thus they were selected for further analyses in order to characterize the putative DAMPs responsible for the observed differential immune responses in *arr6-3* plants. Pectin-enriched fractions from *arr6-3* and Col-0 plants, extracted with 1,2-cyclohexylenedinitrilotetraacetic acid (CDTA) from purified cell walls, were further fractionated by size exclusion chromatography to obtain samples containing carbohydrates with distinct molecular weights. Four sub-fractions (CDTA-A to CDTA-D) were obtained, containing molecules with different theoretical sizes: *i)* CDTA-A: >270 kDa; *ii)* CDTA-B: 270-25 kDa; *iii)* CDTA-C: 25-5 kDa; *iv)* CDTA-D: <5 kDa (**Figure 1A**). These masses are estimated as the Sepharose column was calibrated with commercial dextrans of known weight-average relative molecular mass, which may not display similar conformations as pectic polymers. Total sugar quantifications showed that even after long dialysis procedures, the CDTA-D fractions contained very low amounts of carbohydrates and were most likely composed of the solvent used to obtain this fraction (CDTA; Mort et al., 1991), and therefore, they were excluded for further analyses. CDTA-A, -B, and -C were tested for their capacity to trigger intracellular Ca^{2+} entry, an early immune response, in Col-0^{AEQ} sensor lines (Ranf et al., 2012). CDTA-C sub-fractions from both Col-0 and *arr6-3* retained most of the activity of the complete CDTA-pectin fractions (**Figures 1B, C**), whereas CDTA-A or -B from *arr6-3* did not present any activity and CDTA-B from Col-0 still presented some activity (**Figures 1B, C**). Thus, we concluded that potential active DAMPs were most abundant in the *arr6-3* CDTA-C sub-fractions.

Neutral sugar analyses by GC/MS revealed that CDTA-C sub-fractions were still very complex in terms of monosaccharide composition, challenging further predictions about the identity of novel DAMPs that they would contain (**Figure 1D**). However, the enrichment of *arr6-3* CDTA-C sub-fractions in arabinose and xylose (**Figure 1D**) was in line with MALDI-TOF/TOF mass spectrometry analyses, which showed only the presence of pentose oligosaccharides (m/z shifts of 132) with degree of polymerization (DP) up to 17 (**Figure 1E**). Other oligosaccharide signatures were not found in the MALDI-TOF/TOF, clearly indicating that pentose-containing carbohydrates could be novel DAMPs present in the PTI-active, CDTA-extractable pectin-enriched fractions of Arabidopsis. However, further fractionation of CDTA-subfractions resulted non-viable due to their complexity in terms of composition and polydispersity combined with the low yields obtained. Thus, we decided to investigate the capacity of different oligosaccharides containing arabinose and xylose which could be obtained from commercial AXs using specific glycosyl hydrolases (GH).

Arabinoxylan Oligosaccharides With Different DP Trigger Calcium Influxes in Arabidopsis

In order to investigate whether pentose-based structures could be sensed by Arabidopsis, we decided to analyze in a first instance the capacity to trigger Ca^{2+} influxes of different commercial polymeric structures (**Figure 2A**). As previously described, polysaccharides often need to be solubilized to smaller entities in order to trigger early immune responses in plants (Mélida et al., 2018). In this regard, partial solubilization by heating of water-dissolved polysaccharides can help to expose ligands which may not be accessible in their insoluble counterparts. Heat-solubilized xylan from beech and AX from wheat triggered subtle calcium influxes compared to chitin, but still represented good candidates as pentose-DAMP sources (**Figure 2A**). Based on these results, we selected wheat AX as the polymeric source to

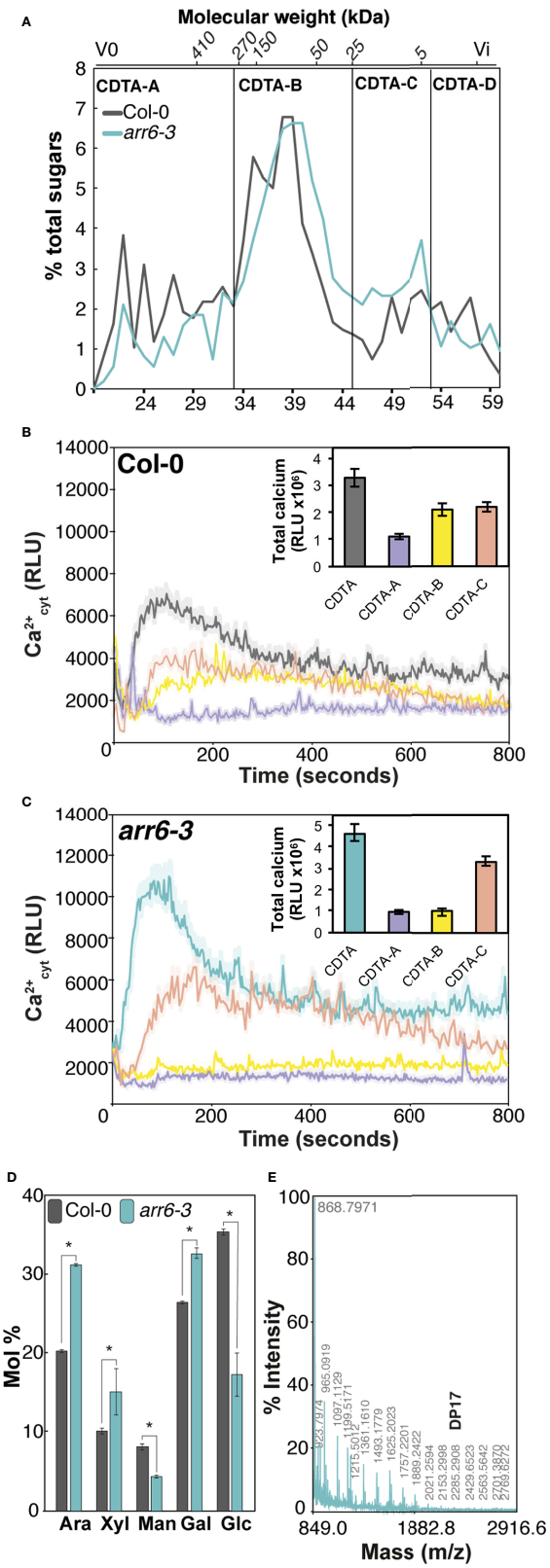

FIGURE 1 | Continued

FIGURE 1 | Pectin-CDTA sub-fractions between 25 and 5 kDa retain most of the activity triggering Ca^{2+} influxes and are enriched in pentose oligosaccharides. **(A)** Size exclusion chromatography (SEC) elution profile (Sepharose CL-6B) of pectin fractions (CDTA extract) from wild type (Col-0) and *arr6-3* plants. The Sepharose column was calibrated with commercial dextrans of known weight-average relative molecular mass and the elution fraction number (bottom) of some of them is indicated on the top of the chromatogram. The elution profiles were monitored by total sugar quantification (phenol-sulfuric method). Sub-fractions were defined as: [A] > 270 kDa, [B] 270–25 kDa, [C] 25–5 kDa, [D] <5 kDa. Profiles are representative of ten independent preparations. **(B, C)** Ca^{2+} influx kinetics triggered by CDTA sub-fractions A-C from Col-0 **(B)** and *arr6-3* **(C)** plants in Col-0^{AEQ} seedlings. Elevations of cytoplasmic calcium concentrations over 800 s were measured as relative luminescence units (RLU). Data are means (n=8) from one experiment representative of three independent ones with similar results. The total areas-under-the-curves were integrated and their average values ± SD (n=8) are represented at the right side of each panel. **(D)** Monosaccharide composition (Mol % ± SD, n=3) of Col-0, and *arr6-3* CDTA-C sub-fraction. Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose. Statistically significant differences between genotypes according to Student's t-test (*$p < 0.05$). **(E)** MALDI-TOF mass spectrum of CDTA-C sub-fraction. M/z shifts are coherent with the presence of pentose oligosaccharides of different degree of polymerization (DP). The spectrum shown (*arr6-3*) is representative of all analyzed pectin I-C sub-fractions (n≥10).

be hydrolyzed to oligosaccharides given that arabinose decorations could mean an advantage compared to non-decorated xylans when enzymatic hydrolysis is used to generate different oligosaccharides of desired DP (McCleary et al., 2015). Wheat AX was hydrolyzed with an endo-xylanase (GH11) from *Neocallimastix patriciarum* and 6 AX-oligosaccharide fractions (#1 to #6) were purified through two rounds of size exclusion chromatography (**Figure 2B**). Purified fractions contained pentose-oligosaccharides ranging from DP 2 to 9 as demonstrated by HPLC-ELSD and MS/MS (**Figure 2C** and **Supplementary Figure S1**). Interestingly, we found that Ca^{2+} burst in treated plants was activated by all fractions except #6, which contained mainly a disaccharide and minor amounts of a trisaccharide (**Figure 2D** and **Supplementary Figure S1**). Since fraction #5 also contained a trisaccharide but triggered intense Ca^{2+} influxes, it seems that a DP above 2 is required by Arabidopsis perception machinery in case of pentose-based oligosaccharides. Together with fraction #5, fraction #4 resulted the most active and according to HPLC-ELSD and MS/MS these contained pentose oligosaccharides ranging from DP 3 to 5 (**Figure 2D** and **Supplementary Figure S1**).

In view of the results obtained with our purified oligosaccharides, we decided to investigate well-defined and highly-pure commercial structures which most likely resemble those from our purifications (McCleary et al., 2015). These included the pentasaccharides 3^3-α-L-arabinofuranosyl-xylotetraose (XA3XX), 2^3-α-L-arabinofuranosyl-xylotetraose (XA2XX), $2^3,3^3$-di-α-L-arabinofuranosyl-xylotriose (A2,3XX), and the tetrasaccharide 2^3-α-L-arabinofuranosyl-xylotriose (A2XX) (**Figure 3A**). Readouts from two early PTI events, such as Ca^{2+} influxes and production of reactive oxygen species (ROS), upon plant treatment with these oligosaccharides indicated that the different pentasaccharides tested were able to trigger immune responses on Arabidopsis seedlings and plants, the responses induced by XA3XX being the most intense ones

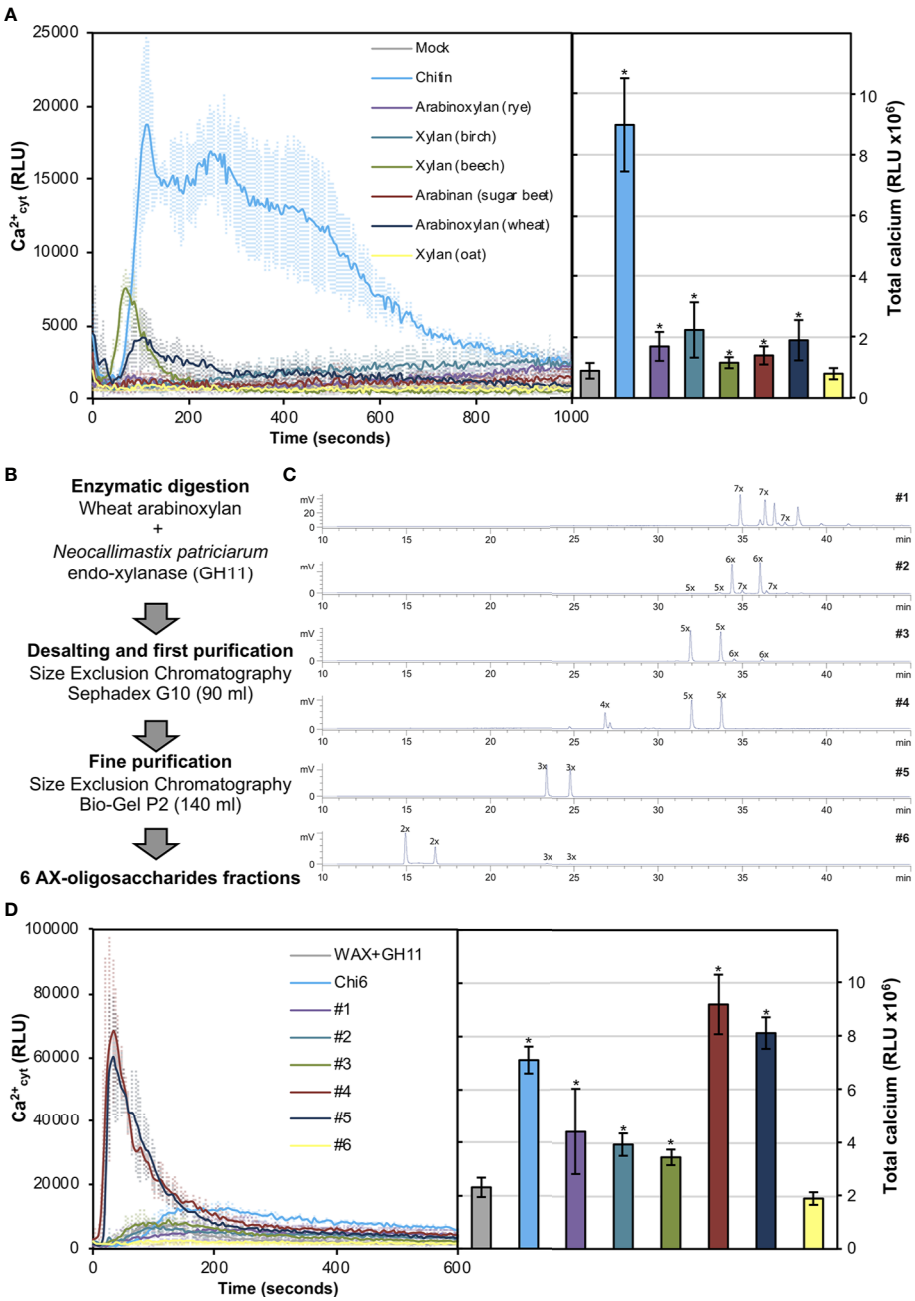

FIGURE 2 | Pentose-based oligosaccharides trigger cytoplasmic calcium elevations. **(A)** Calcium influx measured as relative luminescence units (RLU) over the time in 8-d old Arabidopsis Col-0^{AEQ} seedlings after treatment with 0.5 mg/ml of chitin and arabino-xylan polysaccharide preparations. The total areas-under-the-curves were integrated and their values are represented at the right side of the panel. Data represent mean ± SD (n=8) from one experiment representative of three independent ones with similar results. Statistically significant differences according to Student's t-test (*p<0.05) compared to negative control (mock) are shown. **(B)** Preparation and fractionation pipeline of pentose-based oligosaccharides from wheat arabinoxylan (AX). **(C)** HPLC-ELSD chromatograms of purified oligosaccharide preparations. Peaks are labeled as nX, where "n" correspond to the number of pentoses contained. Double peaks correspond to alpha-/beta-anomeric isomers at the reducing end of each detected oligosaccharide. **(D)** Calcium influxes after treatment with 0.5 mg/ml of the GH11-digested wheat AX before chromatographic purifications (WAX+GH11), chitohexaose (Chi6) and the purified pentose-based oligosaccharides (#1–6). The total areas-under-the-curves were integrated and their values are represented at the right side of the panel. Data represent mean ± SD (n=8) from one experiment representative of three independent ones with similar results. Statistically significant differences according to Student's t-test (*p < 0.05) compared to negative control (WAX+GH11) are shown.

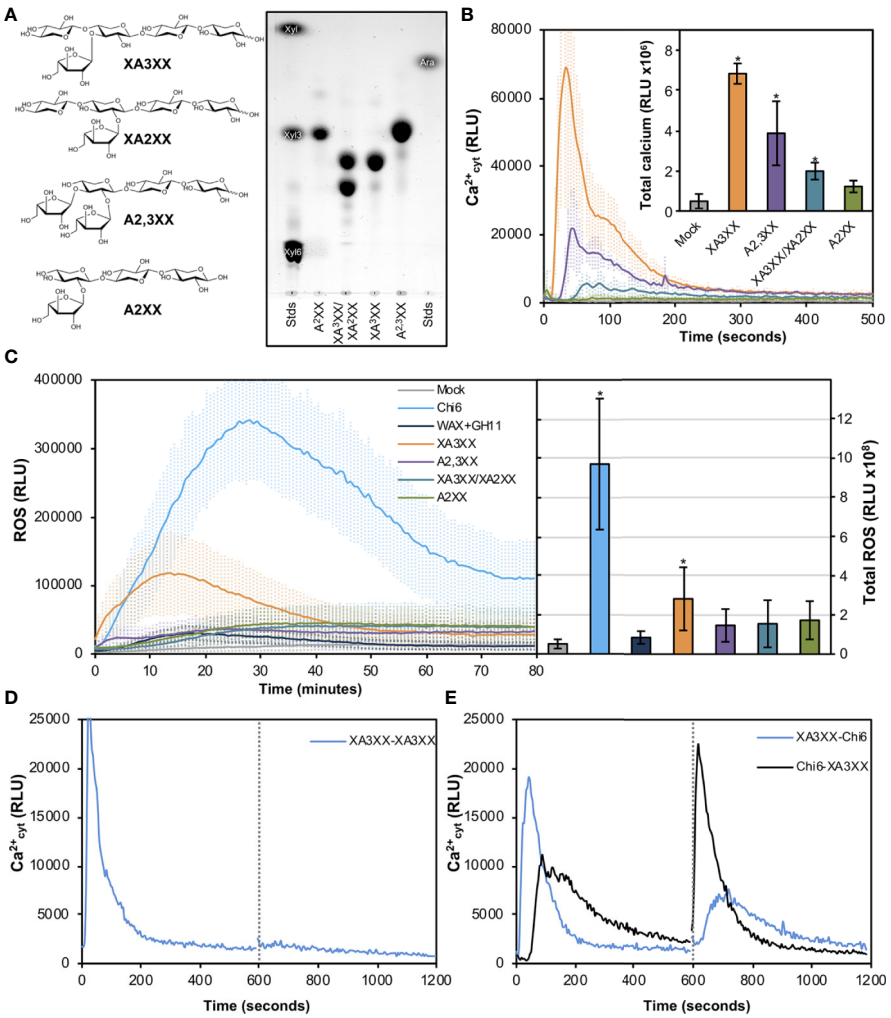

FIGURE 3 | Pure arabinoxylan (AX) oligosaccharides trigger early immune responses in Arabidopsis. **(A)** Molecular structures of the different AX oligosaccharides used in the experiments. 3^3-α-L-arabinofuranosyl-xylotetraose (XA3XX), 2^3-α-L-arabinofuranosyl-xylotetraose (XA2XX), 2^3,3^3-di-α-L-arabinofuranosyl-xylotriose (A2,3XX), and 2^3-α-L-arabinofuranosyl-xylotriose (A2XX). Thin layer chromatography profiles of the pure commercial AXs used in the experiments using 1-propanol/ethyl-acetate/water (9:7:4 by volume) as mobile phase. Left and right lanes show xylose (Xyl), xylotriose (Xyl3), xylohexaose (Xyl6) and arabinose (Ara) which were used as markers. TLC shown is from one run representative of three independent ones with similar results. **(B)** Calcium influx measured as relative luminescence units (RLU) over the time in 8-d-old Arabidopsis Col-0^{AEQ} seedlings after treatment with 500 μM of pure AX oligosaccharides. Water (mock) was used as negative control. The total areas-under-the-curves were integrated and their values are represented at the right side of the panel. Data represent mean ± SD (n=8) from one experiment representative of three independent ones with similar results. Statistically significant differences according to Student's t-test (*$p < 0.05$) compared to negative control are shown. **(C)** Reactive oxygen species (ROS) production (by Luminol reaction) after treatment with 500 μM of pure AX oligosaccharides in Arabidopsis leaf-discs measured as RLU over the time. Water (mock), GH11-digested wheat AX before chromatographic purifications (WAX+GH11) and chitohexaose (Chi6; 100 μM) were used as negative (mock and GH11+WAX) and positive (Chi6) controls. The total areas-under-the-curves were integrated and their values are represented at the right side of the panel. Data represent mean ± SD (n=8) from one experiment representative of three independent ones with similar results. Statistically significant differences according to Student's t-test (*$p < 0.05$) compared to water control are shown. **(D, E)** Cross elicitation during the refractory period of calcium signaling between XA3XX (250 μM) and chitohexaose (Chi6; 100 μM). Data show the elevation of cytoplasmic calcium concentration, measured as relative luminescence units (RLU), over the time in 8-d-old Arabidopsis Col-0^{AEQ} seedlings after treatments. Dashed line (600 s) indicates the application time of the second elicitor within the refractory period of the first elicitation. In **(E)**, blue line represents a first treatment of XA3XX followed by Chi6 after 600 s, while black line represents a first treatment with Chi6 followed by a second of XA3XX. Data represent mean (n=8) from one experiment representative of three independent ones with similar results.

(**Figures 3B, C**). Interestingly, cross-elicitation experiments, by sequential application of two compounds in 600 s interval, demonstrated that fractions #4 and #5 and commercial XA3XX had a refractory period of Ca^{2+} influx. Notably, this effect was not observed when the well characterized MAMP chitohexaose was used in the experiments (**Figures 3D, E** and **Supplementary Figure S2**). Although a refractory state does not necessarily indicate the same perception mechanism or receptor, these results indicated that these pentose oligosaccharides ranging from DP 3 to 5 have equivalent activities and, at least in

Arabidopsis, differ from chitin-based signaling. According to manufacturer XA3XX is a pure carbohydrate, but given the low required doses of peptide MAMP/DAMPs to be perceived by plants (Stegmann et al., 2017), we performed proteinase K proteolytic digestions of XA3XX solutions, and of two MAMPs solutions (chitohexaose and flg22), used as controls (**Figures 4A, B**). Of note, both carbohydrate-based elicitors remained fully active in the Ca^{2+} system after proteolytic treatment, which contrasted with the immune activity abolishment observed in case of flg22 after proteinase K treatment (**Figures 4A, B**). On the other hand, in order to confirm that XA3XX elicitor activity was linked to its oligomer structure, acid hydrolysis of the oligosaccharide was performed. Our results indicated that the hydrolyzed XA3XX lost its capacity to trigger Ca^{2+} influxes as it was the case for chitohexaose (**Figures 4C, D**). All these data, together with the observation that XA3XX was also able to trigger a ROS burst in soybean plants (**Supplementary Figure S3**), clearly indicated that this pentasaccharide deserved a more detailed investigation.

Arabinofuranosyl-Xylotetraose (XA3XX) Activates Several PTI Hallmarks Through a Novel Plant Sensing Mechanism

In order to further characterize the early immune responses triggered by XA3XX, we performed a more detailed analysis of the Ca^{2+} kinetics following the application of the pentasaccharide and the peptide MAMP flg22 (**Figures 5A, B**). Flg22 induced a double Ca^{2+} burst peak at about 90 and 180 s followed by a maintained decrease in luminescence that lasted about 600 s (**Figure 5A**). However, XA3XX kinetics was very different, with a very fast single peak at 20 s post-application and a rapid signal lost at about 200 s (**Figure 5B**). In Arabidopsis, lysin motif-(LysM)-PRR CERK1 (Chitin Elicitor Receptor Kinase 1) plays a central role as a co-receptor for several glycan MAMPs such as chitin, peptidoglycan a β-1,3-glucans (Willmann et al., 2011; Liu et al., 2012; Mélida et al., 2018). The use of *cerk1-2* mutant aequorin lines demonstrated that the perception of XA3XX, like that of flg22, was CERK1-independent, which was in line with the refractory experiments with XA3XX/chitin (**Figures 5A, B**).

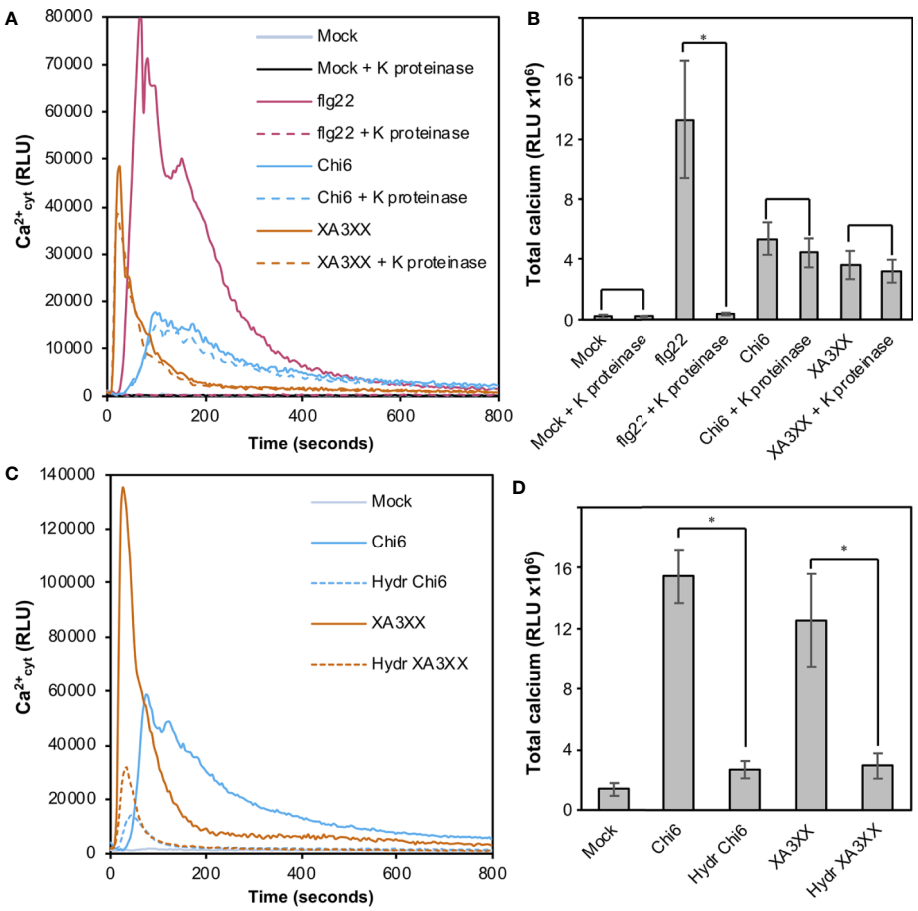

FIGURE 4 | Proteolysis and acid hydrolysis effects on XA3XX calcium signaling. Variation of intracellular Ca^{2+} concentration after 800 s of treatment of Col-0^{AEQ} seedlings with untreated flg22 (1 μM), chitohexaose (Chi6; 100 μM), or XA3XX (250 μM) **(A, B)** with or without proteinase K previous digestion and **(C, D)** with or without acid hydrolysis previous digestion. Data represent mean ± SD (n=8) from one experiment representative of two independent ones with similar results. Statistically significant differences according to Student's t-test (*$p < 0.05$).

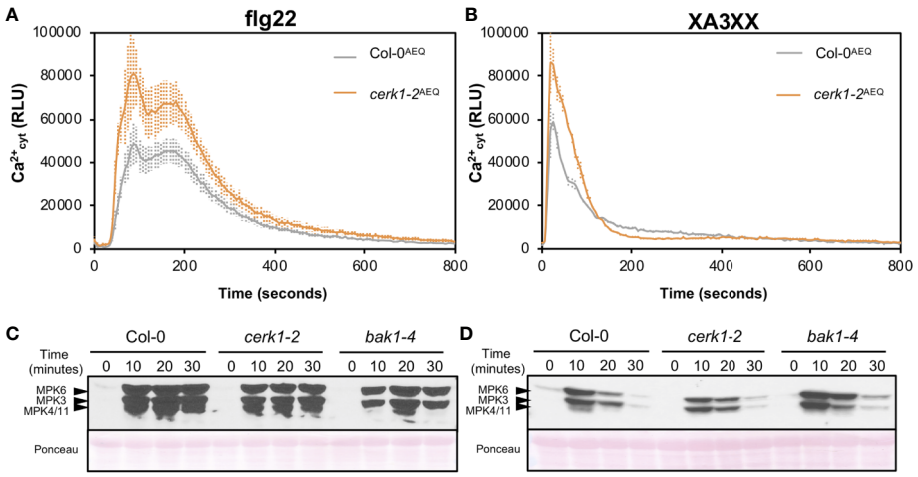

FIGURE 5 | Pattern-triggered immunity hallmarks comparison between flg22 and arabinofuranosyl-xylotetraose (XA3XX). Flg22 **(A, B)** and XA3XX **(C, D)** final concentrations were 1 and 500 μM respectively. **(A, B)** Elevations of cytoplasmic calcium concentrations over time in 8-d-old Arabidopsis Col-0^{AEQ} and *cerk1-2*^{AEQ} seedlings upon treatments. Data represent mean ± SD (n=8) from one experiment representative of three independent ones with similar results. **(C, D)** MAPK activation in 12-d-old Arabidopsis seedlings of the indicated genotypes. The phosphorylation of MPK6, MPK3, and MPK4/MPK11 was determined by Western blot, using the anti-pTEpY antibody, at the indicated time points (minutes) after treatment. Ponceau red-stained membranes show equal loading. Western-blot shown is from one experiment representative of three independent ones with similar results.

Next, we monitored phosphorylation of downstream protein kinases (MPK3/MPK6/MPK4/MPK11), a PTI hallmark, in Col-0 wild-type plants and *cerk1-2* and *bak1-4* mutants (in Col-0 background) impaired in PRR co-receptors required for the perception of chitin and flg22, respectively (**Figures 5C, D**). These analyses confirmed that XA3XX recognition by Arabidopsis plants was CERK1-independent and demonstrated that it was also BAK1-independent, while a partial BAK1-dependence for flg22 was observed, as described (Chinchilla et al., 2007). Western-blot assays confirmed MPK3- and MPK6-phosphorylation after application of a XA3XX solution to Arabidopsis seedlings, reaching the highest level of phosphorylation at 10 min post-treatment (**Figure 5D**). MPK4/11-phosphorylation was almost not-detectable in XA3XX-treated plants. MPKs phosphorylation levels of plants treated with XA3XX was weaker than phosphorylation of MPK3/MPK6/MPK4/MPK11 at all time points tested after elicitation with flg22 (**Figure 5C**).

Global gene reprogramming is the expected output of earlier PTI events such as Ca^{2+} influxes, ROS production and MAPK phosphorylation. Such alteration in the expression patterns of specific genes would determine the adaptation ability of a given plant to respond to a potential infection by pathogens. To further characterize the basis of XA3XX-mediated immunity, we performed RNA-seq analyses of Col-0 seedlings treated for 30 min with XA3XX (**Figure 6** and **Supplementary Tables S1** and **S2**). Elicitation with XA3XX changed the expression of 511 genes, most of which (460) were up-regulated (**Supplementary Table S1**). XA3XX up-regulated genes mainly grouped into gene ontology (GO) terms related to innate immune and defense response to different stimuli, kinase and signal transduction activities, and indole-containing compound metabolic processes (**Figure 6A**), further corroborating the function of XA3XX in modulating PTI. We validated RNA-seq data of five PTI-marker genes (*CYP81F2*, *WRKY53, PHI1, FRK1*, and *NHL10*) by qRT-PCR in seedlings 30 min after treatment, and we found that all these genes were up-regulated after XA3XX elicitation compared to mock-treated seedlings (**Figure 6B**) confirming a full PTI response of Arabidopsis plants treated with XA3XX. Together, these analyses suggest that XA3XX-induced responses are addressed to a global immune response.

XA3XX Crop Pre-Treatment Diminishes Pathogen Disease Symptoms Through a Non-Yet Characterized PRR Complex

Exposure of plants to active MAMP/DAMPs prior to subsequent pathogen attack may allow a more efficient plant defense activation through PTI activation (Héloir et al., 2019; Schellenberger et al., 2019). We showed that XA3XX was perceived by Arabidopsis and soybean (**Figure 3** and **Supplementary Figure S3**). Therefore, we tested the elicitor7nbsp;activity of XA3XX in three-week-old tomato plants (Moneymaker) treated by foliar spray with XA3XX 2 d before inoculation with the biotroph *Pseudomonas syringae* pv *tomato* DC3000 (10^8 cfu/ml). Notably, bacterial population, determined as colony forming units (cfu) per leaf area, was significantly reduced in tomato XA3XX-pretreated plants compared to mock-treated plants (**Figure 7A**). Bacterial growth reduction found at 5 d post-inoculation (dpi) were in the order of 0.8–0.9 log of cfu/cm^2 when 0.25 and 0.5 mg of XA3XX per tomato plant were applied as pre-treatment, respectively (**Figure 7A**). Previous studies using similar approaches have also shown protection results of carbohydrate-based DAMPs against fungal necrotrophs (Claverie et al., 2018).

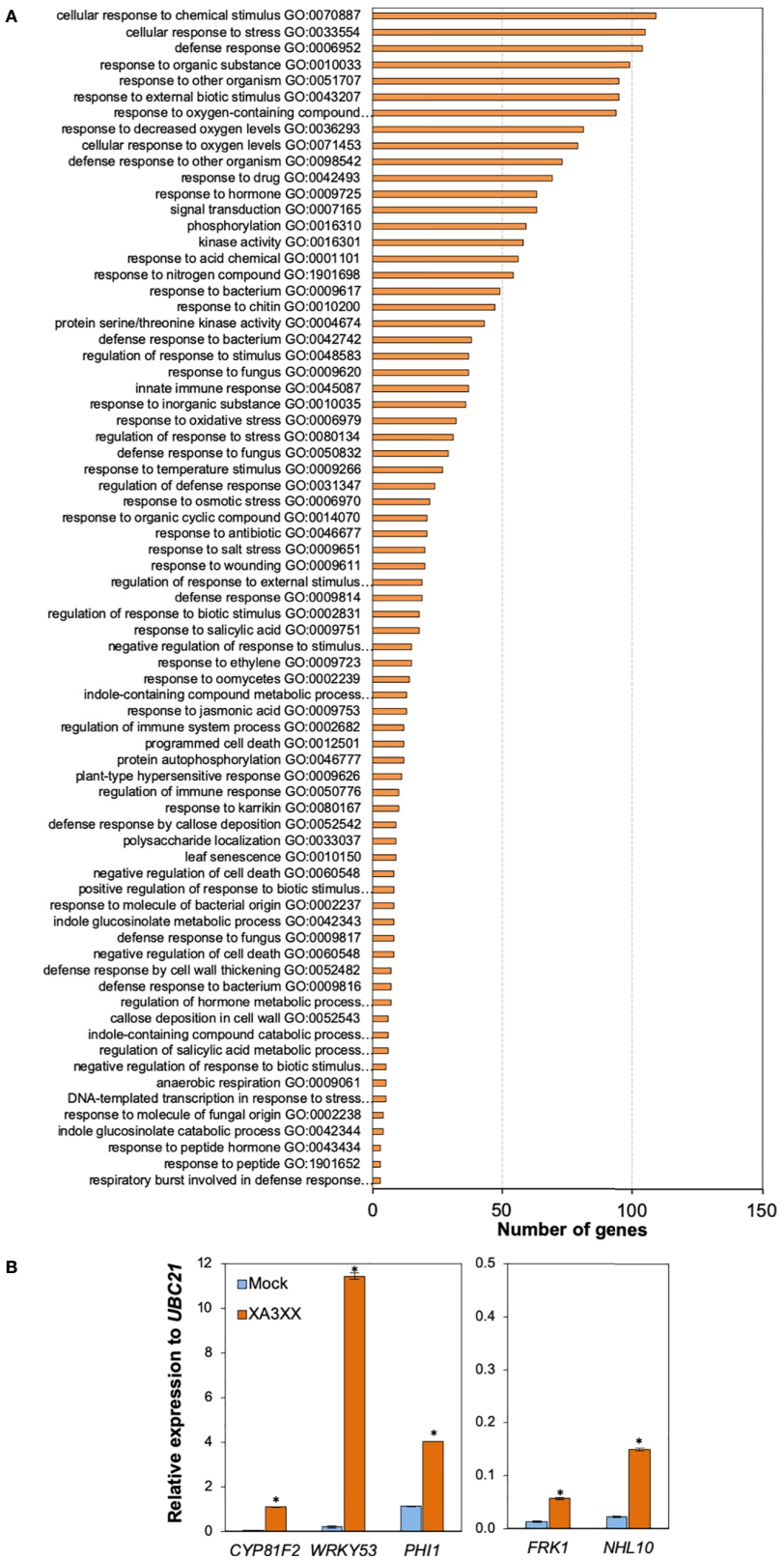

FIGURE 6 | Continued

FIGURE 6 | Functional classification of arabinofuranosyl-xylotetraose (XA3XX)-differentially expressed genes in Arabidopsis. **(A)** Biological process Gene Ontology (GO) term enrichment map of the 460 overexpressed genes in 12-d-old Arabidopsis Col-0 plants at 30 min after 250 μM XA3XX treatment. GO term enrichment is expressed as number of mapped genes. Data used to build the histogram can be retrieved from **Supplementary Table S2**. **(B)** RNA-seq data validation by quantitative RT-PCR analysis in 12-d-old Arabidopsis seedlings. Relative expression levels to *UBC21* (*At5g25769*) gene at 30 min are shown. Data represent mean ± SD (n=3) from one experiment representative of two independent ones with similar results. Statistically significant differences between XA3XX and mock according to Student's t-test (*$p < 0.005$).

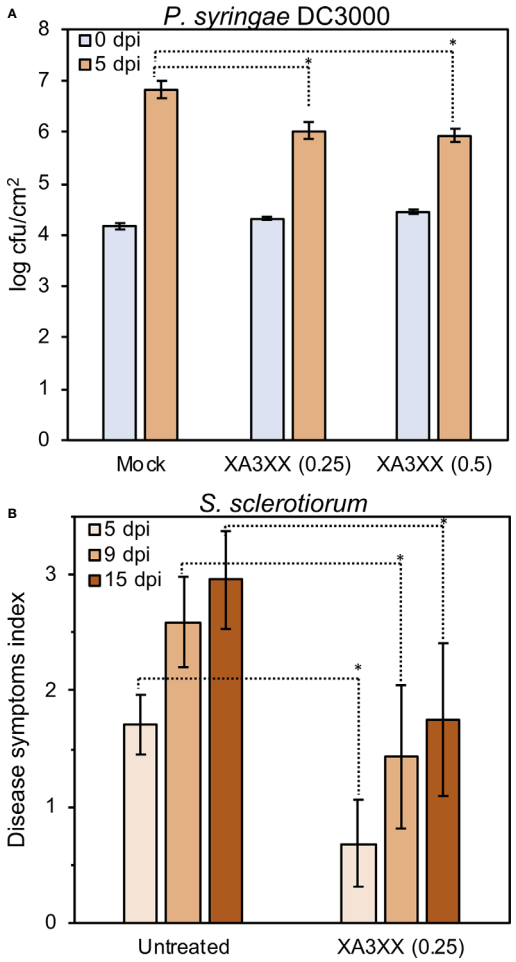

FIGURE 7 | Treatment of tomato and pepper plants with arabinofuranosyl-xylotetraose (XA3XX) confers enhanced disease resistance to pathogens. Plants were foliar-treated with XA3XX (0.25–0.5 mg/plant) 2 d prior bacterial or fungal inoculation. **(A)** Colony forming units (cfu) of *Pseudomonas syringae* pv. *tomato* DC3000 per leaf area (cm^2) at 0- and 5-d post-inoculation (dpi) in three-week-old tomato plants. Cfu/cm^2 were determined after plating serial bacterial dilutions obtained from tomato leaf discs of known area onto KB plates. Data represent mean ± SD (n=8). **(B)** Disease symptoms index produced by *Sclerotinia sclerotiorum* at 5-, 9-, and 15-dpi in leaves of pepper plants. Data represent mean ± SD (n=24). Statistically significant differences according to Student's t-test (*$p < 0.05$).

We next tested XA3XX-pre-treated pepper plants against the necrotroph fungi *Sclerotinia sclerotiorum*. Treated pepper plants showed a reduction in the disease symptoms index at 5, 9, and 15 dpi in comparison to control plants (**Figure 7B**). These data indicate that XA3XX is able to trigger immune responses in some dicot crops conferring disease resistance.

DISCUSSION

Plant pathogens and their hosts have co-evolved an arsenal of CWDE to break-down the opponent's cell wall during the interactions (Rovenich et al., 2016). Thus, plant and microbial cell walls are rich sources of carbohydrate-based defense signaling molecules (DAMPs/MAMPs), that are under-characterized. Recently, we have showed that impairment of *ARR6* gene in Arabidopsis affects cell wall composition, which may lead to the accumulation of DAMPs that would favor a "defense-ready" state, thus affecting plant-pathogen interactions (Bacete et al., 2020). Remarkably, pectin-enriched cell wall fractions extracted from *arr6* cell walls resulted to be enriched in carbohydrate-based DAMPs compared to wild-type fractions. However, the composition of such DAMPs could not be deciphered. In this work we have attempted to unveil the nature of these DAMPs. Unexpectedly, analytical data obtained from size-exclusion chromatography purifications suggested, for the first time, that pentose-based oligosaccharides co-extracted with pectins (using calcium chelators) could play a role as plant DAMPs. Next, we asked whether pentose-based oligosaccharides could be a novel group of plant DAMPs, and indeed we have demonstrated here that AXs can be perceived as molecular patterns by plants. In particular, we identified several active oligosaccharides structures, with pentasaccharide XA3XX being the most active one (**Figure 3**). In particular, XA3XX triggers Ca^{2+} influxes, ROS production, MAPK phosphorylation, and a global gene reprogramming in Arabidopsis at micromolar concentrations. Bearing in mind that the presence of glucuronoAXs has been suggested to be a component of Arabidopsis cell walls (Zablackis et al., 1995), XA3XX and related structures characterized in this work could be considered as plant DAMPs even in Arabidopsis model species.

Structurally, the most similar plant DAMPs characterized so far would be other β-1,4-linked hemicelluloses such as xyloglucan and mannan (Claverie et al., 2018; Zang et al., 2019). Xyloglucan recently proposed as DAMP consists of a β-1,4-glucan backbone associated with xylosyl, galactosyl, and fucosyl-type branching, mainly of DP 7 (Claverie et al., 2018). The purified xyloglucan triggered MAPK phosphorylation and immune-associated gene expression in Arabidopsis and *Vitis vinifera*, but no ROS production was found (Claverie et al., 2018). In another recent work, Zang et al., produced mannan oligosaccharides (DP 2–6) by enzymatic hydrolysis of locust bean gum and demonstrated their DAMP potential on *Nicotiana*

benthamiana and *Oryza sativa* (Zang et al., 2019). Mannan oligosaccharides triggered Ca^{2+} influxes, ROS production, stomata closure, and over-expression of defense-related genes such as *PR-1a* and *LOX*. Interestingly, these novel groups of hemicellulosic DAMPs, including xyloglucans, mannans, and AXs display the same type of glycosidic linkage in their backbone (β-1,4-linked) as the previously characterized plant DAMPs pectic OGs and cello-oligosaccharides (Aziz et al., 2007; Ferrari et al., 2013; Benedetti et al., 2015; Souza et al., 2017; Johnson et al., 2018). In contrast to cello-oligomers (DAMP) and chitin oligosaccharides (MAMP), which are actively triggering broad immune responses in the low micromolar range, the rest of glycan cell wall derived DAMPs characterized (including XA3XX) are only active at high micromolar concentrations, although the activities of the DAMPs/MAMPs are difficult to compare since experiments were performed in different labs, using different experimental setups and species (Aziz et al., 2007; Claverie et al., 2018; Johnson et al., 2018; Mélida et al., 2018; Zang et al., 2019; and this work). It should also be noted that cell wall polysaccharides can be very abundant in plant cells and they could yield very high concentrations of their main components, such as cellulose and xylans (over 5% of Arabidopsis fresh weight; Sakamoto and Mitsuda, 2015), and of the DAMPs derived from these polymers. Anyway, in spite of the high doses required of these novel groups of hemicellulosic DAMPs to be perceived by plants as such, they were able to enhance plant protection against different plant pathogens. Xyloglucan effectively protected grapevine and Arabidopsis against the necrotrophic fungus *Botrytis cinerea* or the oomycete *Hyaloperonospora arabidopsidis* pathogens while mannans improved rice protection against the bacteria *Xanthomonas oryzae* and the oomycete *Phytophthora nicotianae*, respectively (Claverie et al., 2018; Zang et al., 2019). In this work we have shown the protection capacity of XA3XX on tomato and pepper plants against bacterial plant pathogen *P. syringae* and the fungus *S. sclerotiorum* (**Figure 7**).

Xylans are main hemicelluloses of dicot secondary cell walls whose presence is essential for plant development, as exemplified in Arabidopsis plants with reduced xylan quantity, which show weakened cell walls and are unable to develop a vascular system (Brown et al., 2007; Wu et al., 2009). The importance of xylans in plant resistance to pathogens has been suggested previously, though the molecular bases of xylan-associated resistance phenotypes were largely unknown. For example, Arabidopsis plants with enhanced levels of xylose in their cell walls, as it occurs in Arabidopsis *de-etiolated3* (*det3*) and *irregular xylem6* (*irx6*) mutants (Brown et al., 2005; Rogers et al., 2005) or with modifications in their xyloglucan structure, as it is the case of the Arabidopsis *xyl1-2* mutant (Sampedro et al., 2010), show an enhanced resistance to the necrotrophic fungus *Plectosphaerella cucumerina* (Delgado-Cerezo et al., 2012). In contrast, Arabidopsis *er* plants, impaired in ERECTA Receptor-Like Kinase, and *agb1* and *agg1 agg2* mutants, impaired in the Gβ and Gγ subunits of heterotrimeric G proteins, that are hypersusceptible to the same necrotrophic fungus, show a reduced xylose content (Llorente et al., 2005; Sánchez-Rodríguez et al., 2009; Delgado-Cerezo et al., 2012). Also, alteration of cell wall xylan acetylation caused by Arabidopsis ESKIMO1 impairment was shown to enhance plant disease resistance to several pathogens, including *P. cucumerina* (Escudero et al., 2017). Whether these modifications in cell wall xylans are linked to an enhanced pentose-based DAMPs release from weakened walls (increased resistance) or to the alteration of pathogen capability to penetrate host tissues upon secretion of their CWDE repertories are two interesting questions to address in future works. CWDE able to hydrolyze xylan polysaccharides to DAMPs such as those described in this work, are endo-1,4-β-xylanases belonging to GH families 10 and 11 (McCleary et al., 2015). In particular, studies on GH11 β-xylanases crystal structures, such as that from *N. patriciarum* used in this work, showed that the α-L-Ara*f* can be accommodated on O-2 and O-3, thus being able to release structures such as XA3XX and XA2XX (Vardakou et al., 2008). However, Arabidopsis only displays a handful of GH10 endo-xylanases in its genome, but any GH11 (see CAZy database at www.cazy.org/e1.html). GH10 endo-xylanases would cleave arabinose-decorated non-reducing ends instead of xylose-free ones as is the case of XA3XX (Suzuki et al., 2002; McCleary et al., 2015). Therefore, our hypothesis is that the activity of GH10 and GH11 xylanases from pathogens might be associated to the release of xylan-derived DAMPs such as XA3XX and that such release might be differential in cell wall mutants displaying a modified architecture which could make some structures more or less accessible to GHs from pathogens, that could be the case of *arr6* mutants (Bacete et al., 2020). Indeed, bacterial and fungal endo-1,4-β-xylanases have been shown to be required for full virulence of plant pathogens such as *B. cinerea* and *Xanthomonas* (Brito et al., 2006; Santos et al., 2014).

Notably, we show here that XA3XX is perceived by dicot crops, like soybean, tomato, and pepper, supporting that other plant species than Arabidopsis harbor the mechanisms required to perceive xylan-derived DAMPs. This perception, at least in the case of Arabidopsis, is independent of the co-receptors CERK1 and BAK1, further indicating that the mechanism of AX perception differs from that of chitin and β-1,3-glucans (Liu et al., 2012; Cao et al., 2014; Mélida et al., 2018). Taken together that arabinofuranose substitutions are less frequent in dicot than in monocot xylans and that these hemicelluloses are quite more abundant in the monocot branch, it will be interesting to test whether these and related pentose-based DAMPs trigger stronger or lower (if any) responses in plant species at different phylogenetic positions than those included in this study (all dicots). Regarding MAMPs, only a minor fraction of them (flg22, peptidoglycan, and chitin) are recognized by PRRs that are widespread among plants and can be found in both monocot and dicot plant species (Albert et al., 2020). Sensor systems for such patterns are considered an ancient set of PRRs, however the majority of PRRs known to date exhibit genus-specific distribution patterns. On the other hand, PRR-independent perception mechanisms for carbohydrate-based DAMPs could have been evolutionary selected. Therefore, future work in the characterization of the perception mechanisms and the specific

immune pathways triggered by AXs in different species will be necessary to unveil their functions and to determine if this is part of an additional mechanism of inter plant species recognition. Our findings support the use of carbohydrate-based DAMPs/MAMPs as biological products for the regulation of crops immunity and disease resistance responses. The use of these biological products in agriculture production can contribute to reach the social demand of a more sustainable agriculture.

MATERIALS AND METHODS

Biologic Material and Growth Conditions

All Arabidopsis lines used in this study were in the Columbia-0 (Col-0) background. Arabidopsis plants used for $[Ca^{2+}]_{cyt}$, MAPKs and gene expression analyses were grown in 24-well plates (10 seedlings per well) under long day conditions (16 h of light) at 20–22°C in liquid MS medium [0.5x Murashige & Skoog basal salt medium (Duchefa), 0.25% sucrose, 1 mM MES, pH 5.7]. Soil-grown Arabidopsis plants used for cell wall isolation and ROS assays were maintained under short day conditions (10 h of light). Tomato (*Solanum lycopersicum*, Moneymaker), pepper (*Capsicum annuum*, Murano), and soybean (*Glycine max*, Annushka) plants were grown in soil under greenhouse conditions.

Statistical Methods

As a general rule, data shown are means ± standard deviation (SD) from a given number of replicates (n≥3). Data was normally retrieved from one representative independent out of three, however, given the particularity of each assay specific details are indicated in figure footnotes and in specific method subsections below. Asterisks indicate significant differences according to Student's t-test analysis, * $p \leq 0.05$ (R software).

Carbohydrates

Details about carbohydrates used in this work can be found in **Supplementary Table S3**. AX polysaccharides (from wheat and rye), oligosaccharides (XA3XX, XA2XX, XUXX, A23XX, A2XX), and chitohexaose (β-1,4-D-$(GlcNAc)_6$; Chi6) were purchased from Megazyme. Xylan (from birch, beet, and oat) and arabinan (from sugar beet) were purchased from Sigma-Aldrich.

Arabidopsis Cell Wall Fractionation

Three-week-old Arabidopsis plants (n>50) were collected and immediately frozen in liquid nitrogen. Cell walls and their fractions were prepared as previously described (Bacete et al., 2017). The pectin-I fractions were size-fractionated by size-exclusion chromatography on Sepharose CL-6B (GE Healthcare, 140 ml bed-volume in a 1.6 cm diameter column) in 0.33 M sodium acetate buffer (pH 5.0). The column was connected to a Biologic-LP instrument (Bio-Rad) and the flow rate was 1.8 ml/min. The Sepharose column was calibrated with commercial dextrans (Sigma) of known weight-average relative molecular mass. Resulting sub-fractions were dialysed (Spectra/Por MWCO 1000 Daltons, Repligen) against deionized water to remove solutes of a small molecular mass (dialysis tubings were thoroughly washed before use to eliminate any contaminants potentially associated to the membranes). The entire process was repeated three times.

Xylan Enzymatic Digestion and Oligosaccharides Purification

Five hundred mg of low viscosity wheat flour AX (P-WAXYL, Ara : Xyl 38:62) were added to 24.5 ml of deionized water at 60°C and dissolved by stirring on a magnetic stirrer until complete dissolution. Then, the solution was equilibrated to 40°C and 0.5 ml of 0.5 M sodium phosphate buffer, pH 6, were added. This solution was placed in a water bath at 40°C and 97.5 U of endo-1,4-β-D-xylanase from *Neocallimastix patriciarum* (Megazyme #E-XYLNP) were added and incubated at 40°C for 16 h. Reactions were terminated by incubating the solutions at 95°C for 5 min. Solutions were centrifuged at 9,400 *g* for 10 min to remove insoluble materials. Digestion products were freeze-dried, desalted and pre-purified using a Sephadex G-10 column (90 cm^3 bed-volume in a 1.5 cm diameter column; Merck) and size-fractionated using a Biogel P2 Extrafine column (140 cm^3 bed-volume in a 1.6 cm diameter column; BioRad). Columns were connected to a Biologic-LP instrument, distilled water was used as mobile phase and the flow rates were 0.24 ml/min. The entire process was repeated three times.

Carbohydrate Analysis

The dried purified cell wall fractions (0.5 mg) were hydrolyzed in the presence of 2 M trifluoroacetic acid (TFA) at 121°C for 3 h. *Myo*-inositol was used as an internal standard. The resulting monosaccharides were converted to alditol acetates (Albersheim et al., 1967). Derivatized monosaccharides were separated and analyzed by gas chromatography (GC) on a SP-2380 capillary column (30 m x 0.25 mm i.d.; Supelco) using a Scion 450-GC system equipped with EVOQ triple quadrupole (Bruker). The temperature programme increased from 165°C to 270°C at a rate of 2°C min^{-1}. MALDI-TOF MS analyses were performed using a 4800 Plus Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, MDS Sciex) as described (Mélida et al., 2018). Technical replicates were considered from the TFA hydrolysis step of a given cell wall fraction from the same extraction procedure.

Purified oligosaccharides were monitored by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Spotted-AXs were run twice on TLC Silicagel 60 plates (Merck) using 1-propanol/ethyl-acetate/water (9:7:4 by volume) as mobile phase. TLC plates were developed by dipping in a solution of 0.5% (w/v) thymol and 5% (v/v) H_2SO_4 in 96% (v/v) ethanol and heated at 80 °C for 5–8 min. The HPLC-ELSD analysis was performed as previously described (Senf et al., 2017). The oligosaccharides were injected into an Agilent 1200 Series HPLC equipped with an Agilent 6130 quadrupole mass spectrometer (MS) and an Agilent 1200 Evaporative Light Scattering Detector (ELSD). The purified oligosaccharides were separated on a graphitized carbon Hypercarb column (150 x 4.6 mm, Thermo Scientific) using a water (including 0.1% formic acid)-acetonitrile (ACN) gradient.

The peaks in the ELSD traces were assigned based on their retention time and the corresponding masses in the MS. For additional MS analyses, a fraction of each oligosaccharide sample was injected directly into an Agilent 1260 Infinity II Series, LC/MSD XT (Single Quadrupol mit ESI-Jetstream-source).

Aequorin Luminescence Measurements

Arabidopsis 8-d-old liquid-grown transgenic seedlings of ecotype Col-0 carrying the calcium reporter aequorin (Col-0^{AEQ}; Ranf et al., 2012) were used for cytoplasmic calcium ($[Ca^{2+}]_{cyt}$) measurements using the method previously described (Bacete et al., 2017). Negative controls (water) were included in all the experiments. Aequorin luminescence was recorded with a Varioskan Lux Reader (Thermo Scientific). Data shown represent mean ± SD (n=8 seedlings) from one experiment representative of at least three independent ones with similar results.

Reactive Oxygen Species

Five-week-old Arabidopsis or 6-week-old soybean plants were used to determine ROS production after treatments using the luminol assay (Escudero et al., 2017) and a Varioskan Lux luminescence reader (Thermo Scientific). Data shown represent mean ± SD (n=8 leaf discs from at least 4 different plants) from one experiment representative of three independent ones with similar results.

Immunoblot Analysis of MAPK Activation

Twelve-day-old seedlings (n=10) grown on liquid MS medium in 24-well plates were treated with water (mock) and oligosaccharides for 0, 10, 20, and 30 min, and then harvested in liquid nitrogen. Protein extraction and detection of activated MAPKs using the Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (Cell Signaling Technology) were performed as described (Ranf et al., 2011). Western-blot shown is from one experiment representative of three independent ones with similar results.

Gene Expression Analyses

For gene expression analysis (qRT-PCR and RNA sequencing), 12-d-old seedlings grown on liquid MS medium were treated with the oligosaccharide or water (mock) solutions for 0 and 30 min. Total RNA was purified with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. qRT-PCR analyses were performed as previously reported (Delgado-Cerezo et al., 2012). *UBC21* (*At5g25760*) expression was used to normalize the transcript level in each reaction. Oligonucleotides used for detection of gene expression are detailed on **Supplementary Table S4**. Analysis of mock-treated seedlings showed no alterations in the expression levels of the marker genes used in this study. Data shown represent mean ± SD (n=3) from one experiment representative of two independent ones with similar results.

For RNA-seq analyses, samples from three biological replicates for each treatment were sequenced using 50bp Illumina Hiseq 2500. RNA-seq read raw data can be retrieved from the NCBI Sequence Read Archive (SRA) under BioProject accession ID PRJNA639010 (http://www.ncbi.nlm.nih.gov/bioproject/639010). Transcripts obtained were aligned against Arabidopsis annotation Araport11 (Cheng et al., 2017) using Hisat2 2.10.0 release (Kim et al., 2019). Afterwards, they were processed using Stringtie v1.3.6 (Pertea et al., 2015) and Ballgown R packages (Frazee et al., 2015) as previously described (Pertea et al., 2016). Differential expression analysis was performed with FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values from the treatment against FPKM mock values in order to obtain the n-fold. For the up-regulated genes, a coverage cutoff of 50% of the dataset was applied to the treatment genes while for the down-regulated genes it was applied to the mock genes. N-fold of above or equal than 2 was used to prove up-regulation and an n-fold below or equal than 0.5 was applied to look for down-regulated genes. ClueGO 2.5.6 app for Cytoscape (Bindea et al., 2009) was used to determine which Gene Ontology (GO) categories were statistically overrepresented in the differentially expressed set of genes. Significant enrichments were determined using the Enrichment/Depletion (Two-sided hypergeometric) test and Bonferroni step down corrected *p* values are represented. Additional parameters are detailed in **Supplementary Table S2**.

Crop Protection Assays

Three-week-old tomato plants (*Solanum lycopersicum*, Moneymaker) were sprayed with 2 ml of a XA3XX solution (0.125 or 0.25 mg/ml) containing 2.5% UEP-100 (Croda) and 2.5% Tween 24 MBAL (Croda) as adjuvants. Adjuvant solution was used as mock. *Pseudomonas syringae* pv. *tomato* DC3000 infections were performed 48 h after pre-treatments according mainly to Santamaría-Hernando et al., 2019. Briefly, plants were sprayed with a suspension of the bacterium (10^8 cfu/ml) and two tomato leaf discs were collected from four different plants at 0- and 5-d post-infection (dpi). Colony forming units (cfu) per foliar area (cm^2) were determined after plating serial bacterial dilutions obtained from tomato leaf discs of known area onto KB plates with rifampicin (25 μg/ml). Data shown represent mean ± SD (n=8) from one experiment representative of three independent ones with similar results. For *Sclerotinia sclerotiorum* experiments, 5-weeks-old pepper plants (*Capsicum annuum*, Murano) were treated using 5 ml of a XA3XX solution (0.05 mg/ml) containing 0.5% UEP-100 and 0.05% Tween 24 MBAL as adjuvants. Two-days after treatment, plants were moved to a 75% humidity greenhouse chamber and spray-inoculated with 5 ml of a 250 cfu/ml suspension of *S. sclerotiorum* homogenized mycelia according to Chen and Wang (2005). Disease symptoms were determined at 5 and 9 dpi in all the leaves of each plant (n=24) using a scale from 0 to 4 where 0 = no symptoms; 1 = little necrotic spots (< 20% of leaf area); 2 = two or more notable necrotic spots (20–50% of leaf area); 3 = more than 50% of leaf area affected, 4 = leaf senescence. Data shown represent mean ± SD (n=24) from three experiments.

DATA AVAILABILITY STATEMENT

RNA-seq read raw data can be retrieved from the NCBI Sequence Read Archive (SRA) under BioProject accession ID PRJNA639010.

AUTHOR CONTRIBUTIONS

HM and AM initiated, conceived and coordinated all the experiments. HM performed most of the experiments with support of GL, LB, and DR. IH performed the RNA-seq data analysis. CR and FP performed the HPLC-ELSD and MS experiments. HM prepared figures and tables. HM and AM wrote the paper with contributions of all the authors. 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.2020.01210/full#supplementary-material

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Purinoceptor P2K1/DORN1 Enhances Plant Resistance Against a Soilborne Fungal Pathogen, *Rhizoctonia solani*

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The purinoceptor P2K1/DORN1 recognizes extracellular ATP, a damage-associated molecular pattern (DAMP) released upon cellular disruption by wounding and necrosis, which in turn, boost plant innate immunity. P2K1 is known to confer plant resistance to foliar biotrophic, hemi-biotrophic, and necrotrophic pathogens. However, until now, no information was available on its function in defense against root pathogens. In this report, we describe the contribution of P2K1 to resistance in *Arabidopsis* against *Rhizoctonia solani*, a broad host range, necrotrophic soilborne fungal pathogen. In pot assays, the *Arabidopsis* P2K1 overexpression line *OxP2K1* showed longer root length and a greater rosette surface area than wild type in the presence of the pathogen. In contrast, the knockout mutant *dorn1-3* and the double mutant *rbohd/f*, defective in two subunits of the respiratory burst complex NADPH oxidase, exhibited significant reductions in shoot and root lengths and rosette surface area compared to wild type when the pathogen was present. Expression of *PR1*, *PDF1.2*, and *JAZ5* in the roots was reduced in *dorn1-3* and *rbohd/f* and elevated in *OxP2K1* relative to wild type, indicating that the salicylate and jasmonate defense signaling pathways functioned in resistance. These results indicated that a DAMP-mediated defense system confers basal resistance against an important root necrotrophic fungal pathogen.

Keywords: extracellular ATP, damage-associated molecular patterns, purinoceptor P2K1, root disease, *Rhizoctonia solani*

INTRODUCTION

The necrotrophic soilborne pathogen *Rhizoctonia solani* infects more than 250 plant species, including corn, potato, soybean, pulses, brassicas, and small-grain cereals. *Rhizoctonia solani* primarily infects the below-ground organs of the plant (Weinhold and Sinclair, 1996; Keijer et al., 1997), resulting in damping-off, root rot and bare patch of cereals, brown patch of turfgrass, black scurf of potato, and sheath blight of rice (Sneh et al., 1991; DeShields et al., 2018). For example, *R. solani* anastomosis group 8 (AG-8), causes chronic and acute yield losses from 10-30% in dryland cereal production systems of the Pacific Northwest, USA and up to 100% in parts of the world, amounting to billions of dollars of annual losses to agriculture worldwide (Okubara et al., 2014; Okubara et al., 2019). It is a broad host range pathogen, causing disease on a variety of crop plants, including canola and other brassicas. *Rhizoctonia solani* AG2-1 is the premier causal agent of

Rhizoctonia damping-off of brassicas and pulses (Paulitz et al., 2006; Sturrock et al., 2015). It is frequently associated with wheat roots but generally is less pathogenic relative to *R. solani* AG-8 on both wheat and brassicas (Sturrock et al., 2015). Management of Rhizoctonia root diseases has been under development for decades. Genetic resistance to *R. solani* AG-8 has been reported for wheat (Okubara et al., 2009; Mahoney et al., 2016; Mahoney et al., 2017) but is not readily deployable in wheat breeding programs due to the multigenic nature of resistance and absence of molecular markers (Okubara et al., 2019). However, a broad-spectrum resistance gene from rice that confers *R. solani* resistance in *Arabidopsis* has been recently described (Maeda et al., 2019) and would be more readily deployable than multigenic resistance. Foliar plant defense activators such as probenazole are not effective for crop protection from *R. solani* infection (Kouzai et al., 2018). Crop rotation can have limited utility due to the exceptionally broad host range of *R. solani*. Biological control has not been practiced in large-scale production systems, but one study demonstrated that the use of hypovirulent *Rhizoctonia* isolates was effective in protecting against virulent isolates (Sharon et al., 2011). Current management practices include direct seed or minimum tillage, applications of nitrogen at the time of planting, and reduction of weeds and volunteers using herbicides (Mahoney et al., 2016).

Molecular genetic approaches have been successful in boosting plant defense to *R. solani* infections. For example, rice plants with increased jasmonate (JA)-mediated defense responses and ethylene (ET) production showed enhanced resistance to *R. solani* (Peng et al., 2012; Helliwell et al., 2013). Using *Arabidopsis*, researchers have demonstrated that the heterologous expression of a germin-like protein from sugar beet enhanced *R. solani* resistance (Knecht et al., 2010). Interestingly, NADPH oxidase components, such as RBOHD and RBOHF, were also suggested to play an important role in plant resistance to *R. solani* (Foley et al., 2013). Introduced defense genes provided about 50% protection against *Rhizoctonia* and *Pythium* in wheat (Okubara et al., 2014). Although the genes described above have provided plant resistance to *R. solani*, more genetic information is needed to effectively control this difficult disease.

Accumulating evidence suggests that extracellular ATP is released, triggering plant responses to various biotic and abiotic stresses (Dark et al., 2011; Ramachandran et al., 2019). Extracellular ATP is a damage-associated molecular pattern or DAMP (Tanaka et al., 2014). In response to cellular disruption by wounding, necrotizing invasion or predation, ATP is released into the apoplast and perceived by the plant cell surface purinoceptor P2K1 (also known as DORN1) (Choi et al., 2014). Upon ATP binding to the extracellular lectin domain of the purinoceptor (Nguyen et al., 2016), its intracellular kinase domain is activated (Chen et al., 2017), which subsequently results in the activation of a number of intracellular signaling pathways for the reprogramming of many plant defense-related genes (Jewell et al., 2019). Overexpression of P2K1 enhances plant resistance against various foliar pathogens, such as *Phytophthora brassicae* (biotrophic oomycete), *Pseudomonas syringae* (hemibiotrophic bacterium), and *Botrytis cinerea* (necrotrophic fungus) (Bouwmeester et al., 2011; Balagué et al., 2017; Chen et al., 2017; Tripathi et al., 2018), suggesting that extracellular ATP plays an important role in plant defense against a broad range of pathogens. However, there is no report on whether extracellular ATP enhances plant resistance to root necrotrophic pathogens such as *R. solani*.

We hypothesized that extracellular ATP acts as a DAMP to enhance root resistance to the necrotizing root pathogen *R. solani*. Here, we used the *Arabidopsis-R. solani* pathosystem to assess plant defense responses mediated by extracellular ATP signaling. The model plant *Arabidopsis* can be infected with two different *R. solani* anastomosis groups, AG2-1 and AG-8 (Foley et al., 2013). Using these fungal strains, we evaluated the susceptibility in *Arabidopsis* wild-type plants (WT), DAMP-related knockout mutants and plants overexpressing the P2K1 receptor. Our results demonstrated that P2K1 mediates a root defense response against both *R. solani* AG-8 and AG2-1. This is the first report of the role of extracellular ATP for resistance to the root pathogen *R. solani*.

MATERIALS AND METHODS

Plant Materials

All *Arabidopsis* genotypes used in the studies were in the ecotype Col-0 background. The knockout mutant *dorn1-3*, the overexpression line *OxP2K1*, and *rbohd/f* (a double knockout mutant of two NADPH oxidase subunits) were previously reported (Torres et al., 2002; Choi et al., 2014). Seeds were surface sterilized and plated onto half-strength Murashige & Skoog (MS) medium containing 2.2 g/L MS salt with vitamins (Caisson Labs Inc., Smithfield, UT), 1% (w/v) sucrose, 1% (w/v) agar, and 0.05% (w/v) MES (pH 5.7) in a square petri dish. Seeds were kept in the dark at 4°C for 3 days to synchronize germination and transferred to a growth chamber (Conviron Inc., Winnipeg, Canada) at 22°C under a 16-h light/8-h dark cycle (100 μmol m^{-2} s^{-1} light intensity). The petri dishes were oriented vertically during plant growth.

Preparation and Enumeration of *Rhizoctonia solani* AG-8 Inoculum

Rhizoctonia solani AG-8 isolate C1 was cultured on potato dextrose agar (PDA) for up to 5 days (Okubara et al., 2009). Substrate for the inoculum was prepared by autoclaving feed-quality oats for 60 min per day on two consecutive days, with a 20- to 24-h period of cooling in between (Okubara et al., 2009). Approximately 15–20 agar cubes (~5 mm^3) from the leading edge of the fungal colony were distributed among the cooled oats by gentle shaking. Inoculated oats were incubated in the dark for 3 weeks at 23°C. The oats were shaken gently every seven days for 2 weeks to redistribute the fungi. In the third week, the inoculum was air dried in a laminar hood on clean Kraft paper for 24–48 h. Dried inoculated oats were homogenized using a coffee grinder and passed through 1,000 μm and 250 μm sieves (Okubara et al., 2009). The ground inoculum was stored at -20°C for up to 3

weeks. *Rhizoctonia* colonization was quantified immediately before use from triplicate suspensions of 100 mg inoculum in 5 ml of water and 1:10 dilutions of each suspension. Two-hundred microliters of each suspension were plated on water agar containing 100 μg mL^{-1} of chloramphenicol. Colonies were counted for 3 successive days under a dissecting scope. Colony forming units (CFU) per gram of oat was the average of the cumulative colony counts for each dilution.

Comparison of *Rhizoctonia solani* AG-8 and AG2-1 Pathogenicity

Surface-sterilized seeds of *Arabidopsis* (WT of Col-0) were germinated for 11 days on 0.8% M9 agar and then transferred to 4" pots containing pasteurized Sunshine Potting Mix #4 moistened with Miracle Gro solution (0.9 g/L). Soil was infested immediately prior to sowing with 0 or 50 CFU g^{-1} soil of *R. solani* AG2-1 or AG-8 isolate C1. Plants were grown at 22°C in 16-h light/8-h dark in an environmental growth chamber, with Miracle Gro supplements. Seedling weight and root morphometric analysis (total root length) were obtained after 14 days of infection. Non-infested soil served as the control.

Rhizoctonia solani AG-8 Infection of Soil-Grown Plants

Eleven-day-old *Arabidopsis* seedlings were used for fungal infection in pots. Seedlings were transferred to a soil/sand mixture with a 1:1 (v:v) ratio containing *R. solani* AG-8 C1 inoculum in ground oats at a final population density of 150 CFU g^{-1} soil. No-pathogen controls were included for each genotype. Plants were grown for 2 weeks in a growth chamber at 22°C with 70% humidity under a 12-h light/12-h dark cycle (150 μmol m^{-2} s^{-1} light intensity), with 50 mL water per pot (1.50" × 3.38" × 2.27") added on alternate days. At the time of harvest, water was added to the pot to loosen the soil and minimize root breakage. Eight replicates of the control and pathogen-challenged samples were evaluated in each experiment. The experiment was repeated three times. To evaluate the fungal growth in roots, total DNA was extracted and subjected to PCR-based quantification using primers for the AG-8 ITS region: AG-8_F (AGTTGGTTGTAGCTGGTCCATTAAT) and AG-8_R (AGTAGACAGAGGGGTCCAATAAATGA) (Budge et al., 2009). The data were normalized to an *Arabidopsis* reference gene, *AtSAND* (At2g28390).

Morphometric Analysis of Soil-Grown Plants

Rosette areas and total root length of all the plants were measured from digital images after 14 days of growth in infested soil. Rosette area was quantified *in situ* using ImageJ (https://imagej.nih.gov/ij/). The roots were washed to remove soil and debris. Individual root systems were stored at 4°C between wet paper towels in polythene bags for about 24 h. Roots were transferred to a glass tray (25 × 25 × 2 cm), and water was added to just submerge the roots. The roots were carefully spread to minimize tangles and crossovers. Digital images were acquired using the Epson Twain Pro scanner at 600 dpi resolution. Total root length (cm) was determined for each root system using WinRHIZO Regular software (ver. 2016b, Regent Instruments, Inc., Quebec, Canada).

Total RNA Extraction and Real-Time PCR

Total RNA in roots was extracted using a Quick-RNA Microprep kit (Zymo Research, Irvine, CA). Frozen tissues were powdered using ceramic beads for 60 s in a Mini-bead beater (Biospec, Bartlesville, OK). One microgram of total RNA was used to synthesize the first strand of cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time PCR quantification of *PR1*, *PDF1.2* and *JAZ5* transcripts was performed using the SsoAdvanced SYBR Green Supermix kit on the CFX96 Touch Real-Time System (Bio-Rad, Hercules, CA). Thermal cycling conditions were composed of initial denaturation for 3 min at 95°C, followed by 40 cycles of denaturation for 10 s at 95°C and annealing and extension for 30 s at 60°C. The melting protocol was 65 to 95°C (increments 0.5°C/5 s) to ensure a unique PCR product was produced. *AtUBQ10* (At4g05320) or *AtSAND* genes were used to normalize the expression data. The Cq values of three biological replicates were used to calculate the expression of genes using the 2^-(ΔΔCq) equation (Schmittgen and Livak, 2008).

Rhizoctonia solani AG2-1 Infection of Young Seedlings in the Presence of ATP

R. solani AG2-1 was grown on PDA for 2–3 days. A single agar plug (5-mm diameter) with actively growing fungus was placed in the middle of an MS plate amended with 100 μM ATP (Sigma-Aldrich, St. Louis, MO). Control plates did not contain exogenously added ATP. Ten to twelve 10-day-old *Arabidopsis* seedlings were placed around the fungal plug with the root tips facing the plug. After incubation for 2 days, the seedlings were washed three times with 3% (v/v) H_2O_2 and sterile water and then subjected to PCR-based quantification using primers for the AG2-1 ITS region. Primer sequences used are as follows: Rs2.1/8F (GTTGTAGCTGGCCCATTCATTTG) and Rs2.1/8R (GAGCAGGTGTGAAGCTGCAAAAG) (Okubara et al., 2008). The data were normalized to an *Arabidopsis* reference gene, *AtSAND*. The experiment was repeated at least three times.

Statistical Analysis

All experiments were performed independently at least three times or more. The results were shown as the mean ± SE. The significant differences among the means were analyzed using Student's t-test or an ANOVA model and a *post hoc* analysis using a t-test (P-value < 0.05).

RESULTS

Pathogenicity of *R. solani* AG-8 and AG2-1 on *Arabidopsis*

First, a pathogenicity comparison was carried out to determine which AG caused more disease on *Arabidopsis* and would be more likely to distinguish the responses of the mutants. *Arabidopsis* Col-0 plants showed differential susceptibility to *R.*

solani AG2-1 compared to *R. solani* AG-8 in the pathogenicity assays (**Figure 1**). Reductions in seedling weight and total root length were less than twofold in the soil infested with 50 CFU g^{-1} to *R. solani* AG2-1 but approached fivefold for *R. solani* AG-8 relative to the non-infested control. The findings supported observations that the latter pathogen produced more severe disease symptoms than the former for *Arabidopsis* Col-0 plants. Therefore, subsequent experiments were focused on *R. solani* AG-8.

P2K1 Confers Plant Resistance Against *R. solani*

To measure the contribution of the purinoceptor P2K1 to plant resistance to the soilborne pathogen *R. solani* AG-8, we performed a pot-based fungal pathogenicity assay using four genotypes of *Arabidopsis* grown in soil infested with the pathogen. The NADPH oxidase double mutant *rbohd/f* was used as a positive control since it was reported to show complete loss of resistance to *R. solani* (Foley et al., 2013). After only one week of growth in soil containing 150 CFU g^{-1} of pathogen, severe stunting of aerial growth was observed. Reduction in rosette area was an indicator of underground-derived stress, usually due to root loss, in plants infected with soilborne pathogens (Mahoney et al., 2016; Julkowska et al., 2016). **Figure 2A** shows the rosette leaves of all four genotypes grown in noninfested and infested soils for 14 days. Both *dorn1-3* and *rbohd/f* showed a very high reduction in total rosette area (**Figure 2C** and **Supplementary Figure S1A**). Based on a visual observation, *dorn1-3* and *rbohd/f* also showed a yellow to tan chlorosis on their leaves (**Figure 2A**). In contrast, *OxP2K1* showed the least reduction compared to wild type (WT) plants although the difference between them are not statistically significant (**Figure 2C**). Similar results were obtained with infested field soil locally collected from the Spillman Agronomy Farm, Pullman, WA (**Supplementary Figures S2A, C**).

Total root length is an informative phenotype for assessing plant resistance against soilborne pathogens (Okubara et al., 2009). After 14 days of growth in infested soil, significant reduction (>50%) in total root length was observed in the infected plants compared to the noninfected control plants (**Figure 2B**). Similar to the data in the rosette area, the total root length values in both *dorn1-3* and *rbohd/f* were severely reduced by fungal infection. The *OxP2K1* and WT lines exhibited lesser degrees of reduction (**Figure 2D** and **Supplementary Figure S1B**). The *OxP2K1* line sustained the least reduction compared to all other genotypes. We also measured the infection level of *R. solani* in the root tissues by real-time PCR (**Figure 2E**), showing a similar trend as seen in the fungal biomass result in the infected roots. Similar results were obtained when field soil was infested (**Supplementary Figures S2B, D**). Altogether, our observations showed that *dorn1-3* and *rbohd/f* were highly susceptible, while *OxP2K1* was more resistant than the WT. The data suggested that P2K1 is involved in a basal plant resistance to *Rhizoctonia* infection, and that defects in DAMP signaling through

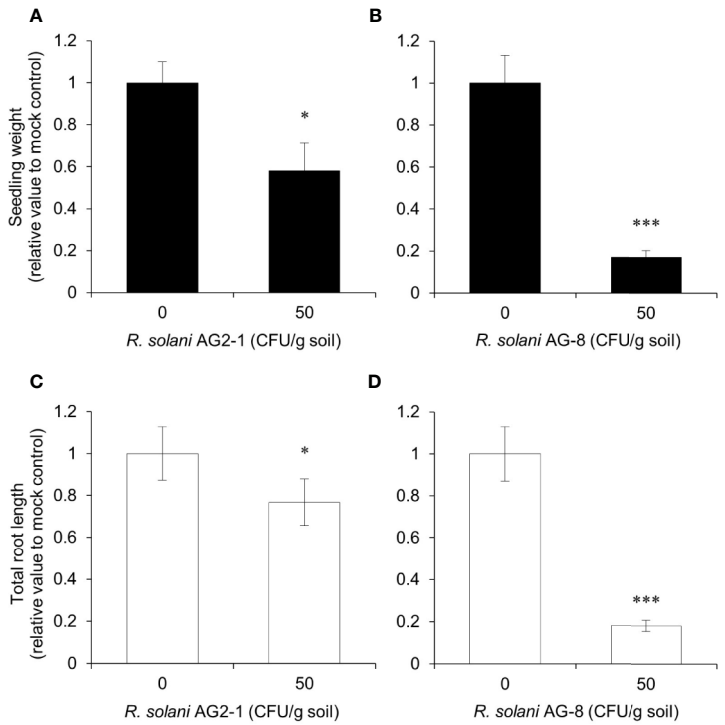

FIGURE 1 | Pathogenicity assay for *Rhizoctonia solani* AG2-1 and AG-8 against *Arabidopsis* Col-0 WT. Eleven-day-old seedlings were planted in pasteurized soil infested with 0 and 50 CFU g^{-1} of *R. solani* AG2-1 **(A, C)** or AG-8 **(B, D)**. At 14 days post-infection, total seedling weight **(A, B)** and total root length **(C, D)** were quantified. Means ± S.E. are the average of 8 to 16 plants. Data are presented as the relative value to mock control (0 CFU g^{-1} soil). Asterisks indicate statistically significant differences compared with the control: Student's t test, *P < 0.05 and ***P < 0.001.

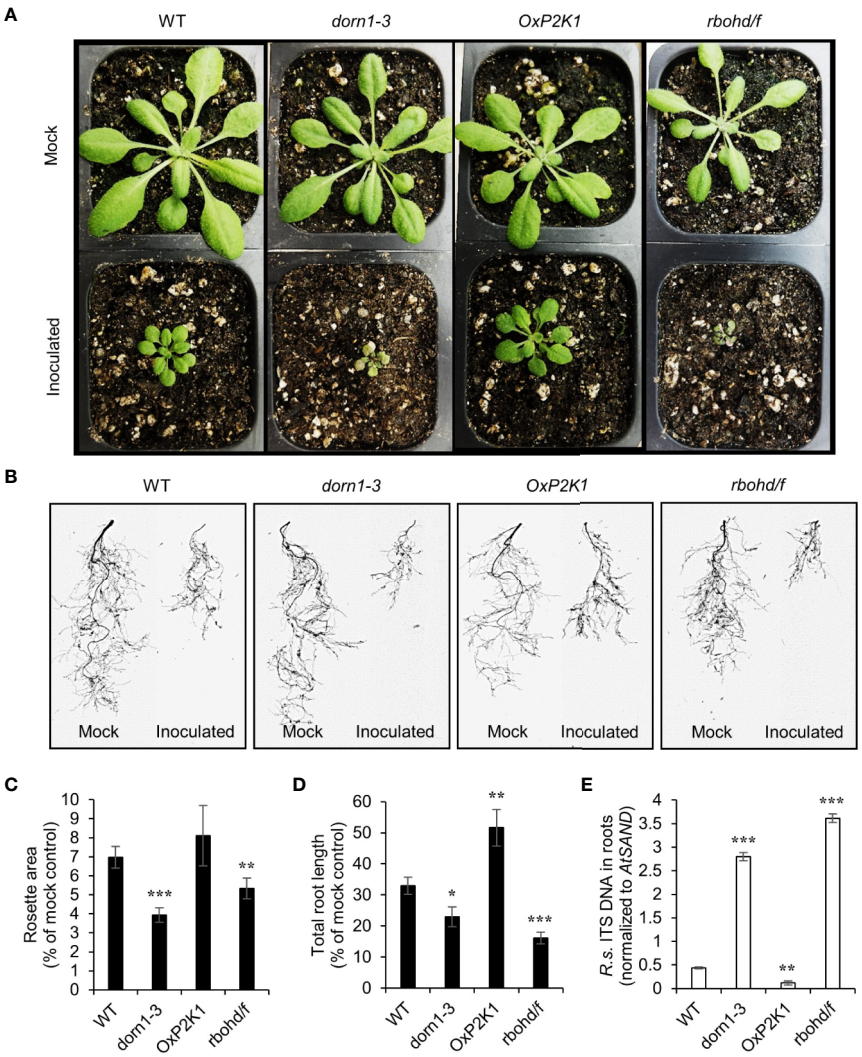

FIGURE 2 | Effect of *Rhizoctonia solani* AG-8 infection on the aboveground rosette growth and the root growth of *Arabidopsis*. Eleven-day-old *Arabidopsis* seedlings were transferred to noninfested soil (mock) or soil infested with *R. solani* AG-8 C1 (150 CFU/g soil). Pictures of aboveground **(A)** and scan images of the root systems **(B)** were taken at 14 days of growth in the soil. The rosette areas of all four genotypes were measured as the smallest circle area enclosed by the convex hull of the rosette **(C)**. The total root length was quantified using the WinRHIZO **(D)**. Data are presented as the mean percentage ± S.E. (n = 8) relative to mock control. The level of infection in the roots quantified by real-time PCR based **(E)**. Abundance is expressed as the ratios of the fungal-specific ITS region relative to the *Arabidopsis* reference gene *AtSAND*. The values are the means ± S.E. of three biological replicates. Asterisks indicate statistically significant differences compared with the WT control: Student's t test; *P < 0.05; **0.001 < P < 0.01; ***P < 0.001.

P2K1 and reactive oxygen species (ROS) generation by the NADPH oxidases resulted in enhanced susceptibility.

P2K1 Is Involved in the Upregulation of Defense-Related Genes During *R. solani* Infection

The expression of a salicylic acid (SA)-induced gene, pathogenesis-related *PR1* (Wildermuth et al., 2001), and two JA/ET-induced genes, the defensin *PDF1.2* and the JA-ZIM-domain protein *JAZ5* (Penninckx et al., 1998; Smirnova et al., 2017), were measured in the root tissues of the WT, *dorn1-3*, *OxP2K1*, and *rbohd/f* plants after growth for 14 days in noninfested and infested soil conditions. Consistent with a previous report (Foley et al., 2013), all these defense-related genes were upregulated after fungal infection in all genotypes tested, but highly upregulated in *OxP2K1* compared to the WT, and weakly upregulated in *dorn1-3 and rbohd/f* after fungal infection (**Figure 3**). Additionally, the expression level of the defense-related genes were comparable in mock-inoculated roots among all genotypes tested (**Supplementary Figure S3**). The result further demonstrated that the expression of the defense-related genes was dependent on the P2K1-mediated pathway only upon host damage. In other words, P2K1 plays an important role for the induction of defense-related genes during *R. solani* infection.

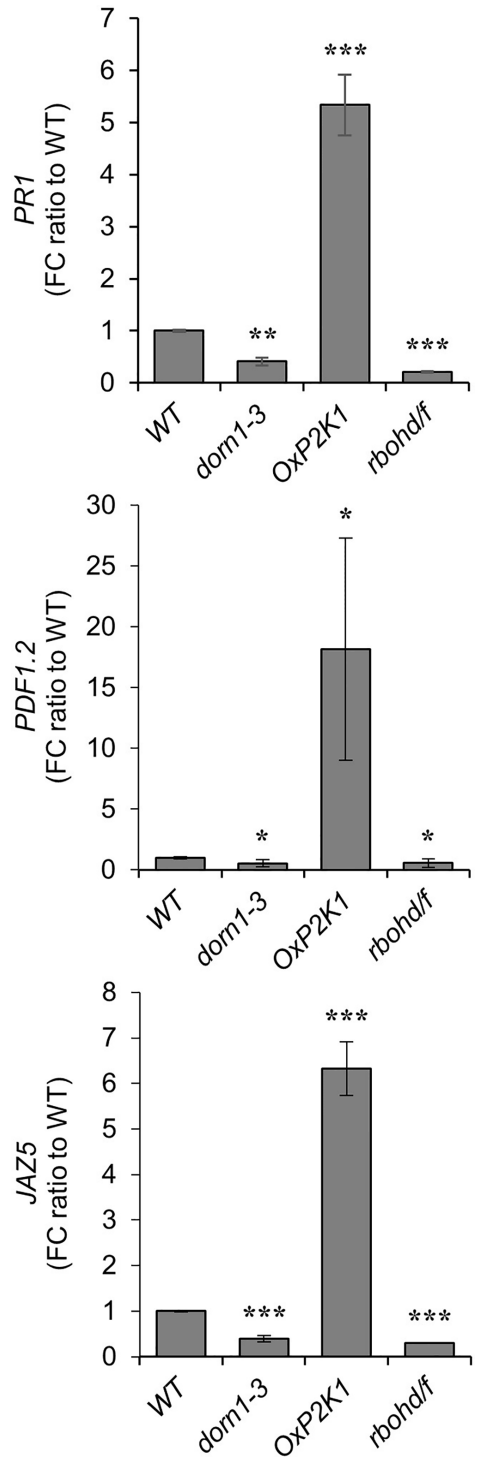

FIGURE 3 | Expression of defense-related genes in the *Rhizoctonia solani* AG-8-infected roots. The expression of defense-related genes, *PR1*, *PDF1.2*, and *JAZ5*, were measured in the root tissues of the WT, *dorn1-3*, *OxP2K1*, and *rbohd/f* plants after growth for 14 days in noninfested and infested soils. Data are presented as the mean value of fold change (FC) ± S.E. (n = 5) relative to that of WT. Asterisks indicate statistically significant differences compared with the WT control: Student's t test; *P < 0.05; **0.001 < P < 0.01; ***P < 0.001.

Application of ATP Reduces Infection of *R. solani* and Alleviates Stunting of Root Growth

It was important to do one experiment with *R. solani* AG2-1, the causal agent of the main *Rhizoctonia* disease, post-emergence damping off in the brassicas. This AG group would be useful to see the effect of exogenously applied ATP on fungal colonization (biomass) at a high resolution, even in the susceptible mutant lines. To this end, colonization of roots by *R. solani* AG2-1 was tested in a petri plate assay in the presence or absence of ATP. Fungal growth was visible 2–3 days after inoculation (**Figure 4**), and the infection level was estimated by measuring the fungal growth on the plates and also by quantifying the fungus inside or closely associated with the root tissue. The root-associated fungal biomass varied according to the genotype and ATP treatment. **Figure 4** shows that *R. solani* biomass was reduced in the *OxP2K1* line, although it was no difference in *dorn1-3*, in comparison to that for WT (**Figures 4A, B**). Additionally, exogenous ATP significantly reduced fungal biomass in roots of the WT and *OxP2K1* lines, whereas it failed to attenuate the fungal biomass in the *dorn1-3* mutant (**Figure 4B**). We also measured the infection level of *R. solani* in the root tissues by real-time PCR (**Figure 4C**), showing a similar trend as seen in the fungal biomass result on the plates. These findings suggest that extracellular ATP mediates defense against *R. solani* through P2K1.

DISCUSSION

The soilborne fungus *R. solani* is an economically important pathogen (Sneh et al., 1996) causing diseases primarily in the roots or tubers and secondarily in the stems of plants (Keijer et al., 1997; Weinhold and Sinclair, 1996). Although *R. solani* AG2-1 is typically associated with disease in the brassicas and pulses, it primarily causes post-emergence damping off, and isolates can vary in pathogenicity. In contrast, *R. solani* AG-8 causes more severe disease symptoms in the field, including pre-emergence damping off and bare patch (Khangura et al., 1999; T. Paulitz, personal communication). Our findings indicated that it was also the stronger pathogen of *Arabidopsis* in our pathogenicity assay and therefore preferable for generating disease in the *Arabidopsis* mutants under controlled conditions. *Arabidopsis* was the host of choice, given that mutants in eATP signaling were not available for other crops.

Rhizoctonia solani is a necrotroph that typically causes cellular damage to acquire nutrients from the host. Necrosis-mediated cellular disruption is a direct mechanism for releasing intracellular ATP into the extracellular matrix (i.e., apoplast). In animals, ATP is released as a DAMP signal when endogenous physiological factors and foreign entities cause cell damage and death, e.g., apoptosis, pyroptosis, and necrosis (Dosch et al., 2018). Similarly, in plants, a high concentration of cytoplasmic ATP is released into the extracellular spaces following exposure to abiotic and biotic stresses (Dark et al., 2011; Ramachandran et al., 2019), suggesting a role of extracellular ATP in stress responses.

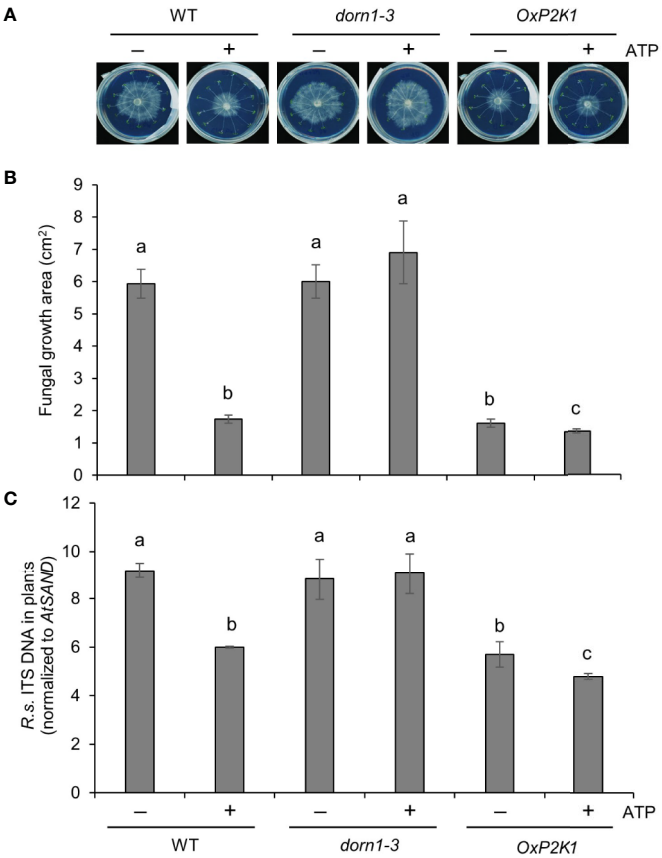

FIGURE 4 | Extracellular ATP reduces *Rhizoctonia solani* AG2-1 infection in roots. **(A)** Representative pictures of *R. solani* infection in MS plates with wild type (WT), *dorn1-3*, and *OxP2K1* seedlings at 2 days post infection. **(B)** The area of fungal growth on the MS plates in **(A)**. **(C)** The level of infection in the seedlings quantified by real-time PCR. Abundance is expressed as the ratios of the fungal-specific ITS region relative to the *Arabidopsis* reference gene *AtSAND*. The values are the means ± SE of three biological replicates. Different letters indicate significant differences at $P < 0.05$.

In the present study, we hypothesized that extracellular ATP, released upon *R. solani*-induced cellular damage (i.e., necrosis), activates the purinoceptor P2K1, which initiates a signaling cascade leading to the induction of defense genes. Our data showed that the purinoceptor P2K1 mediated defense against a necrotrophic root infection. The overexpression line of P2K1 was more resistant to both anastomosis groups (AG-8 and AG2-1) of *R. solani* than the WT. The knockout mutant *dorn1-3* exhibited a high susceptibility to AG-8, but not to AG2-1 (this is probably due to different experiment systems used). In addition, ATP addition attenuated the fungal growth in roots of the WT and overexpression lines. This ATP-induced inhibition of fungal growth was abolished in the knockout mutant. These results demonstrated that extracellular ATP is involved in plant immune response to *R. solani* in roots, where the P2K1 receptor play an essential role to mediate ATP-induced defense. Given that there is no significant difference in the P2K1 expression upon *R. solani* inoculation (GSE26206) and ATP addition (GSE52610) based on previous transcriptomics (Foley et al., 2013; Choi et al., 2014; Jewell et al., 2019), the numbers of the purinoceptor is likely maintained same no matter when the plant cells get insulted by the fungal pathogen and exposed by extracellular ATP. This evidence further supports our hypothesis that extracellular ATP released upon fungal infection, not upregulation of the receptor, is critical for induction of defense responses to R. solani in the plant roots. To our knowledge, this study is the first demonstration of the role of extracellular ATP for basal resistance against a root pathogen, i.e., *R. solani*.

Reactive oxygen species produced by NADPH oxidases play a crucial role in resistance to several pathogens, such as *Alternaria brassicicola, Magnaporthe oryzae*, *P. parasitica*, *B. cinerea, and R. solani* (Segmueller et al., 2008; Pogany et al., 2009; Larroque et al., 2013; Foley et al., 2013; Nozaki et al., 2013; Li et al., 2015). Interestingly, our data with the knockout mutant of P2K1 showed a complete loss of resistance that was comparable to the ROS-deficient double mutant *rbohd/f*, suggesting that ROS generation was as important as extracellular ATP perception in the root defense response. Given that P2K1 directly interacts with and activates RBOHD by phosphorylation (Chen et al., 2017), it is possible that extracellular ATP released upon *R. solani* infection activates P2K1-RBOHD-mediated ROS signaling, thereby enhancing plant resistance to fungal infection.

Defense against *R. solani* has been linked to the overall effect produced by the signaling pathways of SA, ET, JA, abscisic acid, and auxin (Foley et al., 2013). In general, the plant defense response is a coordinated mechanism facilitated by the major defense hormones SA and JA/ET (Glazebrook, 2005; Spoel et al., 2007; Hillmer et al., 2017). Salicylic acid is a key modulator of local and systemic resistance. This molecule accumulates in tissues along with a coordinated expression of defense genes, including *PR* genes (Malamy et al., 1990; Métraux et al., 1990; Maleck et al., 2000). Jasmonic acid and ET are induced during necrotrophic pathogen infection, which induces the expression of several other defense genes, including defensins and proteinase inhibitors (Reymond and Farmer, 1998; Feys and Parker, 2000; Schenk et al., 2000; Devadas et al., 2002). A previous study showed that *R. solani* induces multiple defense hormone-related genes in potato sprouts (Lehtonen et al., 2008) and *Arabidopsis* (Foley et al., 2013). In this study, we confirmed that SA- and JA/ET-regulated genes were proportionally higher in *OxP2K1* and lower in the *dorn1* mutant during fungal infection, suggesting that the expression of these defense-related genes was dependent on the P2K1-mediated pathway upon host damage. Indeed, extracellular ATP induces various defense responses in parallel with plant defense hormones and through an independent mechanism (Jewell et al., 2019). Some of these responses are involved in enhancing resistance against the necrotroph *B. cinerea* mediated by extracellular ATP-JA-mediated synergistic signaling that required ROS as well as other second messengers, nitrous oxide, and calcium (Tripathi et al., 2018; Tripathi and Tanaka, 2018). Particularly, ROS and calcium signalings mutually interplay in plant immune response (Marcec et al., 2019). Additionally, the expression of many defense-related genes was further exaggerated in *OxP2K1*, in which ET-regulated genes were shown to be involved additional defense-related functions (Jewell and Tanaka, 2019).

Plants have basal resistance to necrotrophic pathogens, for which extracellular ATP reinforces plant defense responses *via* defense hormone pathways that may involve secondary metabolites, including tryptophan-derived compounds such as camalexin and glucosinolates, based on previous transcriptomic data (Jewell et al., 2019). To date, many efforts have been made to identify the resistance mechanism against *R. solani* in different crops. Genetic studies in wheat indicate that both single-gene and multigenic resistance are involved and that several mechanisms of resistance are likely, but until now, the molecular basis for basal resistance has been elusive. A better understanding of extracellular ATP signaling would contribute to breeding crop varieties with increased resistance to soilborne necrotrophic pathogens. Further studies are required to understand the complete mechanism by which extracellular ATP enhances defense against this and other soilborne pathogens, which might provide leads to new management strategies for growers. Very recently, putative orthologs of P2K1/DORN1 have been reported in banana, camelina, and wheat (Li et al., 2016; Okubara et al., 2019; Shan et al., 2020). Such functional studies using crops other than *Arabidopsis* would allow us to directly use the resistance mechanism against the necrotrophic pathogens.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

PO and KT conceived and designed the study. SK, DT, PO, and KT performed research, analyzed data, and wrote the article.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Plant–Plant Communication: Is There a Role for Volatile Damage-Associated Molecular Patterns?

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Damage-associated molecular patterns (DAMPs) are an ancient form of tissue-derived danger or alarm signals that initiate cellular signaling cascades, which often initiate defined defense responses. A DAMP can be any molecule that is usually not exposed to cells such as cell wall components, peptides, nucleic acid fragments, eATP and other compounds. DAMPs might be revealed upon tissue damage or during attack. Typically, DAMPs are derived from the injured organism. Almost all eukaryotes can generate and respond to DAMPs, including plants. Besides the molecules mentioned, certain volatile organic compounds (VOCs) can be considered as DAMPs. Due to their chemical nature, VOCs are supposed to act not only locally and systemically in the same plant but also between plants. Here, we focus on damage-induced volatiles (DIVs) that might be regarded as DAMPs; we will review their origin, chemical nature, physiochemical properties, biological relevance and putative function in plant–plant communications. Moreover, we discuss the possibility to use such airborne DAMPs as eco-friendly compounds to stimulate natural defenses in agriculture in order to avoid pesticides.

Keywords: DAMP, defense, plant–plant communication, signaling, volatiles, wounding

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INTRODUCTION

As other eukaryotic organisms, plants are able to perceive typical, endogenous cell molecules or fragments thereof, when these are released at increased concentrations into the extracellular space. This occurs during cellular stress or mechanical damage upon herbivore and pathogen attack. Subsequently, the endogenous compounds contribute to activate local and systemic defense-related responses or the plant innate immunity (Howe and Jander, 2008; Boller and Felix, 2009). The whole dynamic immunity response is induced by the recognition of specific insect-derived [herbivore-associated molecular patters (HAMPs) (Mithöfer and Boland, 2008)] or pathogen-derived [pathogen-associated molecular patters (PAMPs) (Ausubel, 2005)] signals, and signals from the injured plant cells. These latter signaling molecules function as danger signals, stress signals, (endogenous) elicitors, alarmins, or damage-associated molecular patterns (DAMPs). Although various synonyms exist for the aforementioned molecules, the term DAMP is to our knowledge the most prominent example and will be further referred to in this review. With the increasing acceptance of the "damaged-self recognition" concept (Heil, 2009) for plants, the number of DAMPs, their putative reception and signaling and the corresponding literature continuously increased. Thus, here we avoid providing another collection of DAMPs and refer to recent reviews

that give comprehensive overviews (Boller and Felix, 2009; Heil and Land, 2014; Gust et al., 2017; Quintana-Rodriguez et al., 2018; Hou et al., 2019; Ferrusquía-Jiménez et al., 2020). Nevertheless, some typical examples must be mentioned such as peptides, cell wall components, nucleic acid fragments, and extracellular ATP (eATP). However, a new putative class of DAMPs that would be unique for plants (Heil and Land, 2014) will be addressed in the following: volatile DAMPs.

In recent years, plant-derived volatile organic compounds (VOCs) gained much attention as cues in plant–plant communication. However, the concept of VOCs released by attacked plants transmitting information to warn neighboring individuals is far from posing as a novelty, being described almost 40 years ago in various caterpillar-infested tree species (Baldwin and Schultz, 1983; Rhoades, 1983). Criticism regarding the lack of true replication and artificial experimental conditions (Fowler and Lawton, 1985) resulted in the rejection of this popular phenomenon known as "talking trees." It took almost 20 years to revisit and revive the concept of plant–plant communicating via volatile cues by intensely searching for evidence of VOC-induced plant protection against herbivory (Heil and Karban, 2010; Karban et al., 2014). This review focuses specifically on wounding-/damage-induced plant volatiles that fulfill the criteria of DAMPs in *stricto sensu*. We highlight their chemical nature and their ability to induce defense responses in neighboring plants and critically examine their putative role in the field.

A SHORT SURVEY OF PLANT VOLATILES

A plethora of studies is available highlighting the versatility of VOCs and in particular of herbivory-induced plant volatiles (HIPVs). Apart from activating direct and indirect plant defenses against herbivores, HIPVs are also known to mediate a diverse array of interactions between plants and insects (Turlings et al., 1990; De Moraes et al., 1998, 2001; Hoballah and Turlings, 2001). In numerous plant species HIPVs are involved in repelling herbivores, attracting their predators of a higher trophic level as well as upregulating and priming defense responses (Kessler and Baldwin, 2002; Arimura et al., 2004; Engelberth et al., 2004; Kessler et al., 2006; Arimura and Pearse, 2017). Although plants release distinct volatile bouquets with differing compositions and concentrations depending on the given stimulus, e.g., herbivory, mechanical wounding, or touch (Mithöfer et al., 2005; Bricchi et al., 2010; Meents et al., 2019), many taxa share common constituents (McCormick et al., 2012). The most well-known representatives described within the past decades are terpenoids, phenylpropanoids as well as fatty acid and amino acid derivatives (**Figure 1**) (Dudareva et al., 2004, 2006).

Among the most ubiquitous VOCs emitted after mechanical damage, herbivory, or microbial infection are green leaf volatiles (GLVs, for a full review see, Ameye et al., 2018) named after their typical odor of freshly cut green leaves. GLVs are C_6 alcohols, aldehydes, and esters such as (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, and (*Z*)-3-hexen-1-yl acetate generated via oxidation of fatty acids such as linoleic and α-linolenic acid within the oxylipin pathway (for example, see, Matsui, 2006).

Considering the largest class of plant secondary metabolites, terpenes provide a wide array of volatile compounds which are subdivided depending on the number of C_5 units (Dudareva et al., 2004; McCormick et al., 2012). The main representatives of this family are hemiterpenes (C_5; e.g., isoprene), monoterpenes [C_{10}; e.g., linalool, *(E)*-β-ocimene], sesquiterpenes [C_{15}; *(E)*-β-caryophyllene, *(E,E)*-α-farnesene, α-humulene], and homoterpenes displaying irregular structures such as *(E)*-4,8-dimethyl-1,3,7-nonatriene (DMNT; C_{11}) and (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT; C_{16}) (Boland et al., 1992; Maffei et al., 2011; McCormick et al., 2012). The formation of the abovementioned terpenes from the basic C_5 building blocks occurs via two compartmentalized pathways: the cytosol-localized mevalonate pathway (MVA) and the methylerythritol phosphate (MEP) pathway localized in the plastids (Dudareva et al., 2004). Both pathways are strictly enzymatically regulated by a large family of terpene synthases (Dudareva et al., 2013).

Another structurally diverse category of VOCs are the shikimate pathway-derived phenylpropanoids and benzenoids, originating from the amino acid phenylalanine. Sharing a single or multiple benzene rings, these two classes undergo miscellaneous modifications such as acetylation, hydroxylation or methylation, thereby creating a variety of side chains and resulting compounds (Dudareva et al., 2004, 2006). Being often specific to certain plant species and genera, methyl salicylate (MeSA), benzaldehyde, chavicol, eugenol, phenylethanol, and benzylalcohol are typical compounds of this category which can be found in numerous volatile bouquets (Dudareva et al., 2006; Arimura and Pearse, 2017). By performing radioactive labeling studies, several other amino acid-derived VOCs ranging from, e.g., isothiocyanates, sulfides, nitriles, oximes, and amines have been discovered over the years (Dudareva et al., 2006; McCormick et al., 2012). One of the key volatiles released after herbivore damage is indole, which is biosynthesized via anthranilate as an intermediate product in the tryptophan pathway (Paré and Tumlinson, 1996; Frey et al., 2000).

In the context of plant volatiles and their effects on atmospheric chemistry, short-chain oxygenated volatiles (oxVOCs) such as formic and acetic acids, formaldehyde, acetone, methanol, and ethanol, have gained increasing importance in research respective to climate change and contribution to formation of aerosol particles and ozone (Seco et al., 2007).

MECHANICAL DAMAGE-INDUCED VOCs

While studies investigating HIPVs became increasingly popular over time, volatiles solely induced by and emitted after mechanical damage [from now on *damage-induced volatiles* (DIVs)] without any contribution of other organisms, were predominantly shortly mentioned or being considered as not representative for natural processes. In more recent years, VOCs received increasing attention as a DAMP-related cue whilst

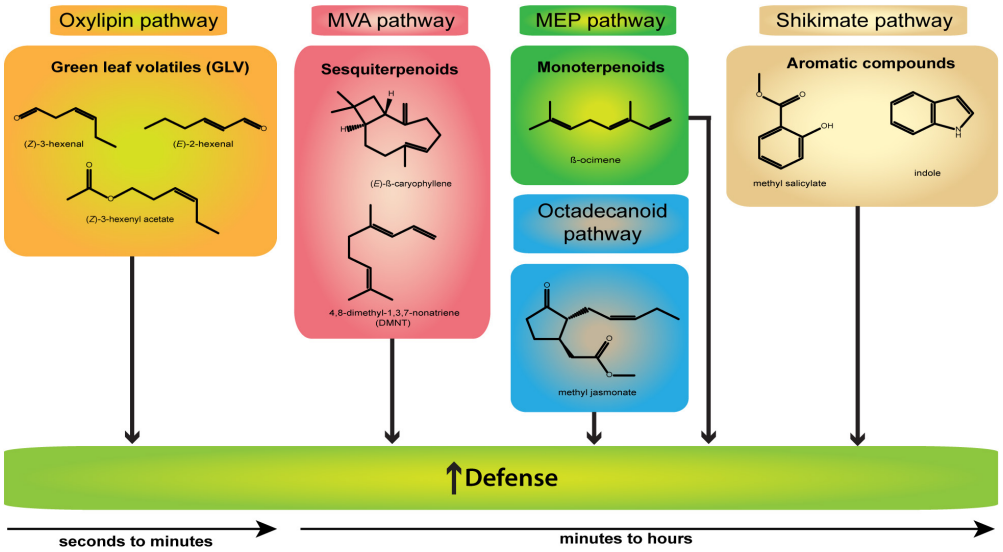

FIGURE 1 | Representative damage-induced volatiles (DIVs). Shown are structures and the biosynthetic origin (indicated by different colors) of the main DIVs involved in defense response induction (↑) and released after wounding or mechanical damage only. Compounds of the oxylipin pathway appear early within seconds to minutes, other compounds later within minutes to hours.

serving as reliable responses upon damage in various plant tissues. In **Figure 2** different key players involved in volatile induction and their relationship among each other are depicted. Particular studies by Quintana-Rodriguez et al. (2018) placed volatiles emitted upon wounding-induced tissue damage in the absence of elicitors in an entirely new context. They pointed out that such DIVs are synthesized upon cell disruption and possess the ability to trigger systemic responses and herbivore resistance, therefore functioning as a DAMP in plants (**Figure 3**) (Heil, 2009; Duran-Flores and Heil, 2016). However, it is also conceivable that DIVs are generated downstream of classical DAMPs such as oligosaccharines or peptides and therefore should be seen as second messengers rather that the initial signals. Most likely, DIVs are synthetized *de novo* after damage. However, here we must discriminate two situations. First, synthesis is initiated within seconds upon tissue damage by constitutively present enzymes as in case of GLVs. Second, synthesis is induced only upon damage perception within hours as for example in case of phenolic compounds and many terpenes. In any case the release of all these volatile compounds can be considered as early and late damage-induced responses, respectively; in contrast to classic DAMPs which are not synthesized upon damage. Only some stored terpenes are released immediately upon disruption of tissue containing pre-existing secretory structures.

Considering that damaging of a plant without the introduction of foreign molecular patterns (e.g., insects) completely omits evolutionary factors such as arms race (Heil, 2009), investigation of the underlying mechanisms will improve the understanding of "ancient" plant defense responses. Thus, we next would like to take a closer look which volatiles are actually released solely upon mechanical damage without associated herbivore feeding or other stress factors in order to identify DIVs, which might serve as potential ancient DAMPs.

One of the most well-known DIVs is the characteristic smell of freshly cut grass, mainly caused by the emission of GLVs. Karl et al. (2001) identified predominantly C_6 compounds

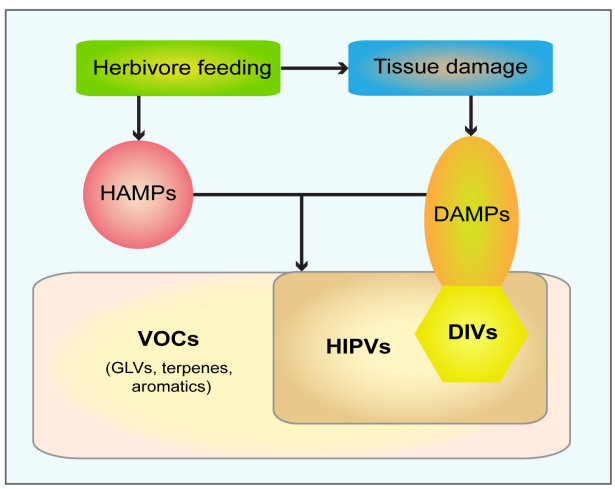

FIGURE 2 | Scheme of the relationship between groups of volatile compounds induced upon herbivore feeding and tissue damage. Herbivore feeding provides chemical signals, HAMPs (herbivory-associated molecular patters), and causes tissue damage, which in turn generates DAMPs (damage-associated molecular patters). The combination of HAMPs and DAMPs induce the emission of HIPVs (herbivory-induced plant volatiles), which all belong to the huge group of VOCs (volatile organic compounds) that includes GLV (green leaf volatiles), terpenes and aromatic compounds. DAMPs are also generated by tissue damage/wounding alone. DIVs (damage-induced volatiles) represent a sub-group of DAMPs, due to their volatile character; all DIVs belong to HIPVs. For simplicity damage-induced electrical signals are neglected.

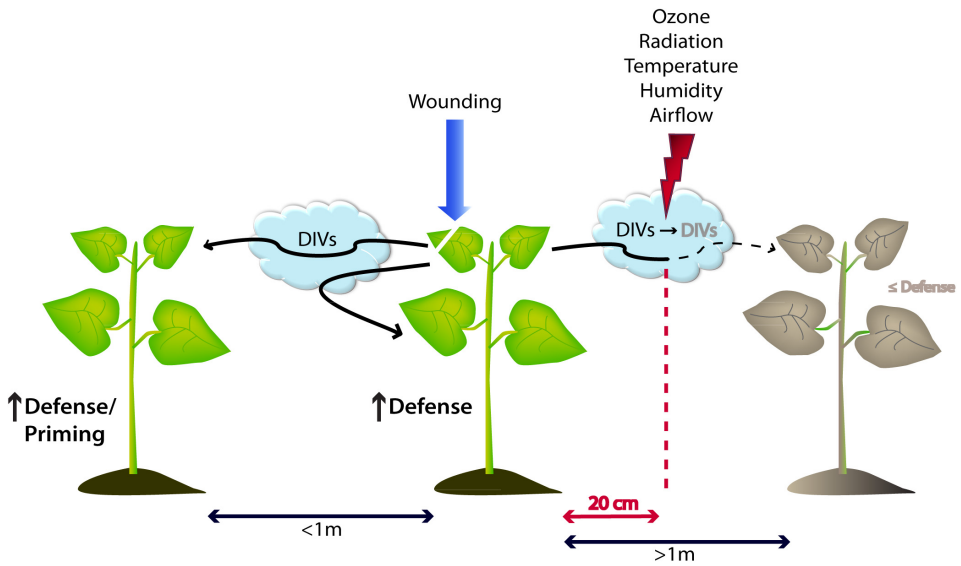

FIGURE 3 | Model of damage-induced volatile (DIV) emissions that trigger intra- and interspecific defense responses in plants. Upon wounding events without contribution from other organisms, plants release specific DIVs possessing the ability to upregulate molecular and chemical defense mechanisms within the same individual as well as in neighboring plants up to a distance of 1 m. The signal intensity and distance of DIVs is highly dependent on environmental factors such as tropospheric reagents (ozone), temperature, radiation as well as the direction of airflow. All of the aforementioned conditions can drastically reduce the effectiveness of DIV signaling by lowering it to a ∼20 cm radius.

including (*Z*)-3-hexenal, (*E*)-2-hexenal, hexenol, hexanal, and acetaldehyde to be emitted within minutes after lawn mowing and lasting for several hours in the field, therefore causing this distinct bouquet. The rapid emission reported in this field study confirmed previous findings in aspen (*Populus tremuloides*), beech (*Fagus sylvatica*), and clover (*Trifolium repens*), where cutting of leaves with scissors elicited a release of (*Z*)-3-hexenal within 1–2 s paving the way for the release of the aforementioned compounds plus hexenyl acetates (Fall et al., 1999). The sensitivity of such measurements was immensely improved by new measuring techniques, such as proton−transfer−reaction mass spectrometry (PTR-MS), enabling monitoring of the release of selected VOCs simultaneous and on-line in the laboratory or in the field.

In addition to the aforesaid rapidly emitted GLVs, mechanical wounding has been shown to generate a variety of DIVs in many different species ranging from common agricultural crops (tomato, *Solanum lycopersicum*; potato, *Solanum tuberosum*; lima bean, *Phaseolus lunatus*), model organisms (*Arabidopsis thaliana;* common liverwort, *Marchantia polymorpha*), herbs, shrubs, and grasses (sagebrush, *Artemisia tridentata*; common reed, *Phragmites australis*; *Plantago lanceolata*) to even trees (aspen, *Populus tremula*; beech, *Fagus sylvatica*; poplar, *Populus nigra*). Although the emission of such DIVs occurs in a species and/or cultivar dependent manner, similar constituents are found in the emitted bouquets.

Jackson and Campbell (1976) observed the release of the plant hormone ethylene after excision of petiole segments from tomato plants. In the following years the list of known DIVs became increasingly refined, adding β-caryophyllene, (*E*)-β-farnesene, germacrene D, and β-bisabolene discovered in potato and common broad bean (*Vicia faba*), to the mix (Agelopoulos et al., 1999). Headspace analyses in *A. thaliana* revealed, apart from GLVs, an increased emission of various terpenoids (β-ionone, β-cyclocitral), sulfides (dimethyl disulfide, dimethyl trisulfide), alcohols (3-pentanol, 1-penten-3-ol, 2-ethyl-1-hexanol), and ketones (3-pentanone, 1-penten-3-one) after rubbing of the leaf midrip with carborundum powder (Van Poecke et al., 2001). Using a more common wounding approach by punching holes into lima bean leaves, Arimura et al. (2000) paved the way for extensive VOC studies using this species by demonstrating the upregulated release of, e.g., DMNT, MeSA, α-pinene (in addition to previously mentioned compounds). From the 2000s onwards, more and more DIVs comprising methyl jasmonate (MeJA) in sagebrush (Preston et al., 2001), linalool and linalool oxide in damaged wheat (*Triticum aestivum*) (Piesik et al., 2006), acetaldehyde, methanol, isoprene, and additional C_6 compounds in common reed (Loreto et al., 2006), the essential oils pulgeone and menthone in the medicinal plant *Minthostachys mollis* (Banchio et al., 2005), as well as C_8 VOCs in the model liverwort species *Marchantia polymorpha* (Kihara et al., 2014), were identified and further investigated. In addition to DIVs found in agriculturally relevant species such as cotton (*Gossypium hirsutum*), Brussel sprouts *(Brassica oleracea*), and sweet potato (*Ipomoea batatas*) (Röse and Tumlinson, 2005; Connor et al., 2007; Meents et al., 2019), more recent studies included traditional medicinal plants and trees (Fontana et al., 2009; Martins et al., 2017; Kanagendran et al., 2018; Portillo-Estrada and Niinemets, 2018). Although the inclusion of a wider array of species highlights common DIV constituents, the potential as a functional DAMP yet remains to be verified for the majority of them. Quintana-Rodriguez and colleagues compiled

valuable information regarding VOCs triggering responses at multiple levels, identifying, e.g., GLVs, methanol, and MeJA as resistance-enhancing compounds (Duran-Flores and Heil, 2016; Quintana-Rodriguez et al., 2018). In recent studies, a combination of wounding and additional abiotic stresses (e.g., gasses, temperatures, dark treatments) revealed more volatile profiles in various species (Loreto et al., 2006; Brilli et al., 2011; Kanagendran et al., 2018). However, the focus of these investigations was mainly on the combined stress treatments, just mentioning DIVs for the sake of completeness of individual effects and not for its sole purpose. Apart from studies investigating the physiological and ecological role of DIVs, research unraveling the impact of wounding on plant volatile composition during food processing has also entered the global industry (Moretti et al., 2002; Farneti et al., 2013; Zeng et al., 2016).

WOUNDING VERSUS WOUNDING – PITFALLS IN STANDARDIZATION

A crucial aspect of all studies implementing artificial wounding is the standardization and reproducibility of such methods, especially regarding the comparability of obtained results. As discussed by Heil, mechanical damage was shown to be sufficient to induce responses in various species that are comparable to those observed after herbivore feeding – however not in all cases (Heil, 2009). The ambiguity of reports containing artificial wounding is mainly caused by the flexibility of the treatment itself. As recently highlighted by Waterman et al. (2019), the execution of mechanical damage can comprise cutting, scratching, piercing, grinding, or pinching of leaf areas differing in size whilst in- or excluding the midrip; therefore resulting in highly variable responses even within the same species (Mithöfer et al., 2005). Regarding its effect on VOC release, artificial wounding is known to produce elevated DIV levels (see above) although not as intense and diverse as during herbivory (Fontana et al., 2009; Holopainen and Gershenzon, 2010). These shortcomings were omitted by adding specific elicitors or oral secretion obtained from the respective herbivore. Furthermore, the construction of a robotic caterpillar ('MecWorm') revealed that continuous mechanical damage simulates herbivory more accurately than single wounding events, yielding DIV patterns comparable to actual herbivory (Mithöfer et al., 2005). Taken together, although the possibility of standardized wounding patterns to study DAMPs and DIVs in a comparable manner exists, the extent of reported artificial damage still varies tremendously.

WHICH DIVs CAN ELICIT DOWNSTREAM RESPONSES ON A MOLECULAR LEVEL?

While DAMPs activate defense-related signaling such as membrane depolarization, cytosolic Ca^{2+} concentration changes, generation of reactive oxygen species (ROS), MAPKinase activation, octadecanoid (jasmonate) and/or salicylic acid (SA) signaling, as well as downstream defense responses like the expression of digestion inhibitors and of defense-related genes (Duran-Flores and Heil, 2016; Li et al., 2020), our knowledge of VOC-induced defense-related responses is still fragmentary. In particular studies on early signaling events are missing. To answer the question whether any of the volatiles mentioned above could actually function as a DAMP, either systemically or between plants, it is crucial to consider whether they are (i) emitted after mechanical damage only and (ii) possess the ability to trigger detectable downstream responses on a molecular or physiological level. Although the exact mechanism of volatile perception still remains an enigma, evidence for perception of DIVs in trees, e.g., sugar maple (*Acer saccharum*) and poplar (*Populus x euroamericana*), was already found by Baldwin and Schultz (1983). This study demonstrated that airborne cues emitted from trees with artificially torn leaves triggered an enhanced accumulation of phenolic compounds and condensed tannins in nearby undamaged individuals. Over the following 20 years, an extensive amount of research was published, identifying specific DIVs and their ability to induce a plethora of responses in a broad spectrum of species ranging from trees, shrubs (sagebrush) to crops (cotton, tomato, potato), and model organisms (*A. thaliana*). The main observed responses to DIVs included accumulation of secondary metabolites, especially phenolic compounds and tannins (Baldwin and Schultz, 1983; Zeringue, 1987; Choi et al., 1994), upregulation of proteinase inhibitor gene expression and proteinase inhibitor biosynthesis (Farmer and Ryan, 1990; Reid, 1995), activation of defensive oxidative enzymes (Karban et al., 2000) by compounds such as MeJA or ethylene, which could even lead to an enhanced herbivore resistance (Karban et al., 2003).

A groundbreaking study by Arimura et al. (2000) continued to disentangle the impact of individual compounds in the upregulation of defense-related genes in lima bean. It was demonstrated that only VOCs emitted by *T. urticae*-infested leaves resulted in the upregulation of defense-related genes encoding pathogen-related (PR) proteins including PR-2 (β-1,3-glucanase), PR-3 (chitinase), as well as lipoxygenase (LOX), phenylalanine ammonia-lyase (PAL), and farnesyl pyrophosphate synthetase (FPS), whereas exposure to VOCs from artificially damaged plants only slightly triggered PR-2 gene upregulation. Although VOC emission profiles revealed the presence of (*Z*)-3-hexenol, α-pinene, (*E*)-β-ocimene, DMNT, α-copaene, junipene, β-caryophyllene, and MeSA after artificial wounding by punching holes into the detached leaves, the available concentration of the individual compounds was seemingly not sufficient to trigger defense mechanisms. Follow-up studies with whole plants revealed that GLVs such as (*Z*)-3-hexenol, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate were in fact able to induce the expression of defense genes in non-infested plants (Arimura et al., 2001; Farag et al., 2005). Findings by Bate and Rothstein (1998) corroborated the importance of C_6- GLVs (mainly (*E*)-2-hexenal) triggering plant defense response genes in *A. thaliana.* Additionally, the potential of DIVs such as DMNT or β-ocimene to activate transcript accumulations, if present in high enough amounts, was shown by their individual application resulting in upregulation of several

defense genes (Arimura et al., 2000). A similar observation was made by Meents et al. (2019) showing that VOCs released after mechanical wounding with tweezers or the application of DMNT only induced several defense genes and trypsin inhibitors in sweet potatoes in a cultivar- and concentration-specific manner. Both studies highlight the potential of single components as putative DAMPs; however the experimental setup, execution and magnitude of artificial wounding, air exchange, incubation time, and concentration of applied volatiles need to be critically taken into account.

More recent findings focused on the effect of mainly HIPVs in intra- and interspecific plant signaling, omitting artificial treatments and placing VOC signaling in a more ecological context. Matthias Erb and his team found the mainly herbivory-induced aromatic compound indole (**Figure 1**) to be a potent priming agent in maize (*Zea mays*) which increased the accumulation of defense-related phytohormones and volatiles in undamaged neighboring plants (Erb et al., 2015). Although the indole-mediated priming response was specific for maize only, exposure to synthetic indole triggered the emission of DMNT, α-pinene, and (*E*)-β-caryophyllene also in cotton and cowpea (*Vigna unguiculata*) (Erb et al., 2015). This highlights the potential of indole as a putative universal information transmitter among various species based on the fact that – although in small amounts only – it can be found in other species as well (Zeng et al., 2016; Li et al., 2019; Meents et al., 2019). Again, the mode of damage seems to play a crucial role for defense upregulation, based on studies showing the occurrence of small amounts of indole only after continuous mechanical wounding in certain species (Bricchi et al., 2010; Zeng et al., 2016; Meents et al., 2019) and not after single wounding events (Zhuang et al., 2012). These observations highlight that VOCs mainly declared as HIPVs are not necessarily limited to herbivory, but might also act as a damage-inducible priming agent and triggering DAMP mechanisms with sufficient indole released after wounding. Taken together all of these findings, there is a strong evidence for some DIVs regulating as volatile DAMPs various plant responses *via* different pathways.

How these DAMP signals act on and in neighboring plants and the receiving tissue is still not known. For sure, plants harbor the potential to perceive and transmit volatile signals. Some scientists highlighted the ability of DIVs to further induce VOC emissions in the receiver plant, e.g., via upregulating ethylene biosynthesis genes in lima bean (Arimura et al., 2002), local and systemic terpene production in tomato (Farag and Paré, 2002), or production of HIPVs-mimics in cotton, tobacco (*Nicotiana attenuata*), or maize as a response to MeJA or (*Z*)-3-hexen-1-ol (Halitschke et al., 2000; Rodriguez-Saona et al., 2001; Ruther and Kleier, 2005). Especially airborne MeJA connects different possible pathways, being taken up by the plant and consecutively converted into jasmonic acid and its active conjugates (Tamogami et al., 2008). Jasmonic acid and its conjugates are then able to regulate defense responses including VOC emission; sometimes in cooperation with peptide signaling as shown for prosystemin in tomato (Degenhardt et al., 2010). However, as shown for sweet potato, DIV-induced defense is not necessarily connected with the activation of the jasmonate pathway (Meents et al., 2019). These observations support the possibility of dual functions of certain volatile DAMPs such as DMNT, which could act with and without including known defensive pathways. Moreover, such DAMPs can either directly initiate defense as in the case of sweet potato (Meents et al., 2019) or being involved in priming (Erb et al., 2015).

It should be noticed that DIVs must also be seen in the original sense of tissue damage; i.e., this cue is not necessarily exclusively triggered in the event of an herbivore or pathogen attack but might be involved in activation in vital wounding repair mechanisms within the damaged individual, therefore serving as a shortcut. However, to our knowledge, volatile DAMPs-related to wound healing processes in plants have not been described yet.

SPECIFICITY, STABILITY, AND RANGE OF INFLUENCE

One recurring point of controversy has been the distance over which HIPV signals can be received by plants (Baldwin et al., 2002; Karban et al., 2003; Kessler et al., 2006). Recent work has shown that vascular constraints on systemic induction can be overcome with HIPVs (Karban et al., 2006; Frost et al., 2007; Heil and Bueno, 2007), as hypothesized by Farmer (2001) and Orians (2005). However, the potential of emitted VOCs to trigger a systemic response in the emitter or conspecific individuals is a complex interplay of various factors starting from released concentrations of active compound, cue specificity, stereochemistry-related configuration, field *vs* laboratory conditions, and the distance to the emitter (**Figure 3**) (Preston et al., 2004).

Among several well-studied volatiles, MeJA gained increasing attention from the 90s on after a study by Farmer and Ryan (1990) finding its emission significantly increased after excision of branches from sagebrush. Being conducted in enclosed bell jars only, Karban and colleagues transferred this knowledge to the field, performing further experiments demonstrating that wild tobacco plants growing near clipped sagebrush exhibit less herbivore damage than individuals without a wounded neighbor present (Karban et al., 2000), highlighting the defensive ability of DIVs. Upon further characterization of the emitted plume after mechanical damage in sagebrush, Preston et al. (2004) identified *cis*-MeJA as the main released epimer compared to the *trans* conformation. Subsequent experiments aiming to reproduce the emission of MeJA via application of lanolin paste or aqueous sprays revealed that neither *cis*- nor *trans*-MeJA elicited direct defenses in *N. attenuata* when applied in concentrations consistent with sagebrush emissions. This study exquisitely addressed that besides structural specificity, the application and the released amount of compounds is a crucial aspect making it tremendously difficult to treat plants in physiologically relevant quantities in order to reproduce observations made in the field.

Follow-up field studies on sagebrush conducted by Karban et al. (2006) found air contact and proximity of conspecific plants to be key to intra- and interplant communication. It was shown that adjacent conspecifics of clipped sagebrush were not

only influenced within a range of 15 cm but even up to 60 cm. Additionally, a downwind airflow toward the neighboring plant was necessary to establish volatile-mediated contact, ultimately triggering induced resistance among branches as well as within the wounded individual itself which was not observed by clipping and trapping released DIVs (Karban et al., 2000, 2006). In the case of neighboring tobacco plants, 5 days of exposure to clipped sagebrush increased the overall resistance for the whole season with up to 48% decreased herbivore damage (Karban, 2001; Karban et al., 2003). All of these findings underlined the possible longevity of volatile-based protective mechanisms even across different species, however suffering limitations based on airflow and spatial distribution of such cues. Considering the proximity of neighboring individuals, MeJA-based communication appears to be useful in sagebrush due to adjacent plants growing within a maximum distance of 60 cm apart (Karban et al., 2006) including the branches of the clipped individual itself.

Apart from warning neighboring (potentially eavesdropping) individuals, DIVs might also provide a fast and efficient mechanism of within-plant-signaling, reaching further locations of the wounded plant itself as has been demonstrated in lima bean, poplar, blueberry (*Vaccinium corymbosum*), and sagebrush (Karban et al., 2006; Frost et al., 2007; Heil and Bueno, 2007; Rodriguez-Saona et al., 2009; Heil and Adame-Álvarez, 2010). Depending on the growth form, Heil and Karban predicted that large and anatomically complex plants (especially lianas and vines) are more prone to use VOC-mediated protective mechanisms, omitting a time-consuming signaling cascade via the vascular system (Heil and Karban, 2010). Evidence for this hypothesis and the distance over which VOCs can travel was found in lima bean plants grown in the field. Heil and Adame-Álvarez (2010) demonstrated that cues from emitter plants triggered with JA or benzothiadiazole (BTH) increased secretion of extrafloral nectar as an output for resistance in independent receiver plants at a distance up to 50 cm. Interestingly, over 80% of the leaves located around a single leaf at this range still belonged to the same plant, therefore inducing resistance mainly in the same individual (**Figure 3**) (Heil and Adame-Álvarez, 2010). Additional findings were presented by Girón-Calva et al. (2012) highlighting the specificity of plant perception in lima bean, depending on the applied VOC and the dose and exposure time. Taken together, volatiles are representing a cue for within-plant-signaling as well as an alarm signal for surrounding plants of a possible threat, however in a limited range from 15 up to 60 cm, which was extended to 100 cm by work of Piesik et al. (2010) for some cereal crops and recently by Sukegawa et al. (2018) in a mint (*Mentha* × *piperita*) emitter – soybean (*Glycine max*) receiver system.

HOW ATMOSPHERIC EFFECTS CAN SHAPE VOLATILE DISTRIBUTION PATTERNS

In nature, plants are exposed to a vast number of environmental stimuli and stress factors, leading to drastic physio-chemical changes in the plant. These external factors are often omitted in studies that are performed in the laboratory. As addressed in a review by Holopainen and Gershenzon (2010), the co-occurrence of biotic and abiotic stresses such as high temperatures, nutrient availability in the soil, and increasing herbivore attacks, can significantly alter the volatile profiles in plants. These effects can be additive and result in an increased VOC emission, as observed in maize and lima bean (Gouinguené and Turlings, 2002; Vuorinen et al., 2004) under high temperature or ozone stress combined with herbivory, or prioritize a single response, e.g., anti-pathogen instead of anti-herbivore defense (Rostás et al., 2006). Strikingly, after degradation or condensation on leaf surfaces VOCs can play an entirely different biological role (Holopainen and Gershenzon, 2010).

As worked out recently, many different physico-chemical parameters can affect the occurrence and concentration of released VOCs in the close environment. Their particular vapor pressure, but also temperature, wind speed, relative humidity, and radiation are such factors (**Figure 3**) (Douma et al., 2019). In addition, an important key factor for volatile communication is the atmospheric lifetime of emitted VOCs which can range from 30 s up to several days (Atkinson and Arey, 2003). As stated again by Douma and colleagues, the chemical class of a certain compound is less important than its reactivity with atmospheric oxidants, biosynthesis rate, and volatility (Douma et al., 2019). Thus, the longevity of such a signal strongly depends on the presence of reactive radicals (OH, NO_3, O_3) and the number of C double bonds (Mofikoya et al., 2017). Especially ozone, known as the most important tropospheric air pollutant in rural areas (Ashmore, 2005), is highly reactive with a variety of VOCs (Pinto et al., 2007). As demonstrated by Blande et al. (2010) in laboratory studies, this can lead to a significantly decreased signaling distance and, hence, limited plant–plant communication. In numbers, the exposure of *T. urticae*-infested lima beans to 80 ppb ozone (representing concentrations of semi-urban areas) reduced VOC signaling distances from 70 cm (control) to 20 cm, mainly due to degradation of compounds such as (*E*)−β−ocimene, DMNT, and TMTT. Additionally, recent field studies revealed that priming of cabbage (*Brassica oleracea* var. *capitata*) after exposure to HIPVs of *Pieris brassicae*-infested neighbors was significantly disturbed (Girón-Calva et al., 2017) by elevated tropospheric ozone levels, therefore inhibiting a crucial VOC-mediated protective mechanism of plant communication. However, this adverse effect does not apply to all compounds and plant responses. Compounds such as MeSA or 2-butanone were not significantly affected and exposure to even higher ozone concentrations (160 ppb) stimulated extrafloral nectar production in lima bean, representing an increased defense mechanism (Blande et al., 2010). Apart from its influence in the plant itself, oviposition by *P. xylostella* was generally lower in plots under elevated ozone (Mofikoya et al., 2017), indicating that behavioral patterns by the herbivore are also altered in the process. The question whether this activation of defensive mechanisms might be used as a plant protection strategy or simply puts the plant under constant stress, still remains to be answered. All of these findings create a rather puzzling image regarding the benefit or drawback of air pollutants on plants and their VOCs; however representing

a major external factor that has to be considered when applying VOCs in the field.

VOLATILE DAMPs – ARE THEY USEFUL IN AGRICULTURE?

Over the last decades, numerous studies proposed the use of plant-based VOCs (DIVs as well as HIPVs) for crop protection as means for an environment-friendly pest management (**Table 1**). All having the same aim, various strategies have been suggested targeting different volatile-based mechanisms. Groundbreaking field studies by Pickett and colleagues (Cook et al., 2007; Hassanali et al., 2008; Pickett et al., 2014) introduced the push-pull-system by intercropping repellant and attractant plant species, luring pests toward attractive odors whilst protecting the important crop from damage.

Following up, various publications aimed to identify suitable crop species and cultivars based on their natural ability to release and induce VOC-mediated defenses in adjacent plants. Studies by Piesik et al. (2010) investigated the influence of mechanical damage and herbivory on the VOC emission in common cereals, e.g., wheat, barley (*Hordeum vulgare*), and oat (*Avena sativa*), revealing tremendous differences in quantities of especially GLVs emitted by injured plants. These species-specific differences in DIV quantity could even be observed in different cultivars of the same species in sweet potato (Meents et al., 2019). In both cases, herbivory resulted in the emission of higher amounts and more different VOCs compared to mechanical injury. However, low amounts of released DIVs after mechanical damage were already sufficient to induce the release of GLVs in uninjured crop plants within 1 m distance (**Figure 3**) (Piesik et al., 2010). The ability of DIVs to trigger an upregulated VOC release in adjacent plants might serve as an interesting starting point of signal amplification within an agricultural land plot. Supposing that artificial wounding of few individuals can trigger upregulation of VOCs in uninjured neighbors, which subsequently serve as relays amplifying the signal, it would be intriguing to test whether it could actually prime or induce resistance in larger areas of one plot. However, the feasibility of this concept strongly depends on the intensity and frequency of the given stimulus, stability and complexity of the signal, the ability of the receivers to perceive and respond to the given stimulus, the longevity of the response, and whether there is a trade-off between defense and yield. At this point, it might be worth to mention a very recent study demonstrating that among released VOCs – GLVs in particular – were the best candidates to indicate herbivore occurrence, suggesting their longer presence in the environment compared with other VOCs (Douma et al., 2019).

Independent of initial stimuli or wounding events, studies by Sukegawa et al. (2018) suggested mint species due to their constitutive emission of resistance-enhancing volatiles as suitable companion plants for soybean, *Brassica rapa,* and kidney bean (*Phaseolus vulgaris*). Cultivation or pre-incubation for up to 7 days in the greenhouse next to mint plants resulted in lowered herbivore damage and transcript accumulation of defense marker genes for up to 8 days. These promising findings confirmed previous studies in potato by Vucetic et al. (2014) highlighting the potential of constitutively emitted aromatic VOCs to elicit defense or priming in crop species. Another field study showed convincingly that repeated weeding-induced release of DIVs from goldenrod (*Solidago altissima*) plants reduced both leaf and seed damage in soybeans. It could be further shown that at least three different goldenrod-derived monoterpenes were involved in the induction of the respective soybean defense (Shiojiri et al., 2017). However as critically pointed out by Sukegawa et al. (2018), one has to consider whether the recipient crop species (such as soybean) is grown in large monocultures in the field, which might drastically attenuate the beneficial effect of mint as companion plants, making it more suitable for small scale house gardening and glasshouse cultivation.

Another interesting principle regarding volatile-based protection comprises the addition of a third trophic level. Various studies (Dicke et al., 1990, 2003; Turlings et al., 1990; Takabayashi and Dicke, 1996; Arimura et al., 2009; Baldwin, 2010) revealed that plants release distinct volatile blends upon herbivory in order to attract natural enemies of the attacking herbivore. Making direct use of this knowledge, researchers tested common HIPVs such as DMNT or (*Z*)-3-hexenyl acetate among many others, in field studies regarding their attractiveness toward parasitoids. In the process, MeSA as both a DIV and an HIPV, was revealed to be a promising candidate for commercial application due to its luring ability of predatory mites, bugs, and lacewings whilst repelling aphid plant pests (Dicke and Sabelis, 1987; Dicke et al., 1990; Drukker et al., 2000; Ozawa et al., 2000; James, 2003, 2005). Although being able to bait certain insect species in hop yards over a distance of 15 m away from the dispenser, studies using commercially available MeSA lures in strawberry (*Fragaria* × *ananassa*) fields did not result in decreased local pest abundance (Lee, 2010). This study just posing as an example, it nevertheless reveals the complexity of this strategy due to the predator's preferences and the potential lack of a rewarding system.

Combining aforementioned strategies, studies by von Mérey et al. (2011) constructed dispensers in maize fields releasing synthetic GLVs in order to induce and/or prime defense in neighboring plants while simultaneously monitoring predator and herbivore attractiveness. Although GLV-exposed maize plants emitted increased concentrations of sesquiterpenes, the hypothesis this would improve herbivore resistance could not be maintained but caused even higher numbers of herbivores, depending on the distance to the dispenser. Another crucial aspect is again the emitted concentration of each compound especially in complex mixtures, since repellent cues can be turned into attractants in the process or covering the desired function, especially when presented in the wrong context (D'Alessandro and Turlings, 2005; Mumm and Hilker, 2005; Snoeren et al., 2010). As addressed by Heil and Walters (2009) (VOC-mediated) induced systemic resistance seems to come with ecological costs. This effect is again highly species-specific and strongly dependent on the applied volatile, which was shown in a field study where lima bean and pepper (*Capsicum annuum*) were exposed to low doses of (*Z*)-3-hexenyl acetate for 7 days (Freundlich and Frost, 2019). Volatile treatment resulted in increased leaf

TABLE 1 | Overview of plant-derived DIVs and their application in the field.

Compound/molecule class	Classification	Emitter/source	Receiver plant	Applied VOC dosage	Distance emitter-receiver	Response	References (and ref. therein)
Methyl jasmonate (MeJA)	DIV	*Artemisia tridentata* (clipped)	*Nicotiana attenuata*	20–30 ng/g FW/h	15 cm	↑Polyphenol oxidase ↑Herbivore resistance	Karban et al., 2000
Methyl jasmonate (MeJA)	DIV	*Artemisia tridentata* (clipped)	*Artemisia tridentata*	n.a.	0–60 cm	↑Herbivore resistance	Karban et al., 2006
Methyl jasmonate (MeJA)	DIV	Dispenser (Chem-Tica sachet)	*Vitis labrusca* (var. Concord)	1 g; 7 mg/d released	0–30 m	↑Parasitoid abundance	James and Grasswitz, 2005
Methyl salicylate (MeSA)	DIV	MeSA dispenser (Predalure)	*Fragaria* × *ananassa*	2 g /lure	0–10 m	→Pest abundance	Lee, 2010
Methyl salicylate (MeSA)	DIV	Dispenser (Chem-Tica sachet)	*Vitis labrusca* (var. Concord)	5 g; 40 mg/d released	0–30 m	↑Parasitoid abundance	James and Grasswitz, 2005
Methyl salicylate (MeSA)	DIV	Dispenser (Chem-Tica sachet)	*Vitis labrusca* (var. Concord)	5 g; 60 mg/d released	0–30 m	↑Parasitoid abundance	James and Price, 2004
Methyl salicylate (MeSA)	DIV	Dispenser (Chem-Tica sachet)	*Humulus lupulus*	5 g; 60 mg/d released	0–30 m	↑Parasitoid abundance	James and Price, 2004
(*Z*)-3-Hexenyl acetate	DIV	Dispenser (Chem-Tica sachet)	*Vitis labrusca* (var. Concord)	1 g; 7 mg/d released	0–30 m	↑Parasitoid abundance	James and Grasswitz, 2005
(*Z*)-3-Hexenyl acetate	DIV	Lanolin paste	*Phaseolus lunatus*	30 ng/μl; 10 ng/h released	1 m	↑Height and biomass ↑Flower and fruit production ↓Herbivore damage ↓Cyanide production	Freundlich and Frost, 2019
(*Z*)-3-Hexenyl acetate	DIV	Lanolin paste	*Capsicum annuum* (var. Cayenne)	30 ng/μl; 10 ng/h released	1 m	↓Height and biomass ↓Flower and fruit production →Herbivore damage	Freundlich and Frost, 2019
(*E*)-β-Caryophyllene	DIV/ HIPV*	*Zea mays* ssp. *parviglumis*	n.a.	n.a.	1 m	↑Parasitoid abundance	Rasmann et al., 2005
n.a.	DIV/ HIPV°	*Mangifera indica* (var. Criollo)	n.a.	n.a.	20 cm	↑Parasitoid abundance	Carrasco et al., 2005
VOC mixture monoterpenes, GLVs, terpenes, N- and S-containing VOCs, DMNT, (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene	DIV/HIPV	*Brassica oleracea* (var. Capitata)	*Brassica oleracea* (var. Capitata)	n.a.	30 cm	↑VOC emission (priming)	Girón-Calva et al., 2017
VOC mixture (*E*)-2-hexenal, (*Z*)-3-hexen-1-yl acetate, (*E*)-β-ocimene	DIV	*Solidago altissima* (cut)	*Glycine max* (cv. Hyokei Kuro-3)	500 mg cut *S. altissima* pieces	0–15 m	↓Leaf damage ↓*Spodoptera litura* damage	Shiojiri et al., 2017
GLV mixture (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate	DIV	Dispenser	*Zea mays* (var. Tuxpeño Sequía)	0.2 ml	<0.1–1 m	↑Sesquiterpene emission ↑Herbivore damage ↑Herbivore abundance →Parasitism rate	von Mérey et al., 2011
(*E*)-β-farnesene	HIPV/cVOC (GMO)	*Triticum aestivum* (cv. Cadenza)	n.a.	Maximum 30.7 μg/plant/h released	0.5 m	→Grain yield →Aphid abundance →Parasitoid abundance	Bruce et al., 2015
VOC mixture 1,8-cineole, menthone, menthol	cVOC	*Mentha* × *piperita* (cv. Candy)	*Glycine max* (cv. Tanba—Kuro) *Brassica rapa* *Phaseolus vulgaris* (cv. Nagauzuramame)	n.a.	50–100 cm	↓Herbivore damage ↑Defense genes	Sukegawa et al., 2018
Push-pull-intercropping systems	DIV/cVOC	For a full review see					Pickett et al., 2014
Plant extracts	DAMP	For a full review see					Quintana-Rodriguez et al., 2018

*DIV, damage-induced volatiles; HIPV, herbivore-induced volatiles; cVOC, constitutive volatiles; GLV, green leaf volatiles; GMO, genetically modified organism; n.a., not applicable. ↓ decrease; ↑ increase; → no change. *VOC emission induced by herbivore feeding. °Beneficial effect observed after herbivory only.*

and flower formation, overall taller growth and decreased herbivory in lima bean plants, however coming at the cost of a reduced cyanide induction (trade-off). An entirely different output was observed in pepper, producing fewer flowers and fruits conjoined with reduced above- and belowground biomass and unaltered herbivore damage. These observations illustrate the effect of a single VOC on traits such as reproductive fitness and growth in a species-specific manner which very carefully needs to be considered while choosing a suitable VOC-plant pairing in agriculture. Having a large scale application of volatile treatments in agriculture in mind, in addition to the compounds' environmental compatibility and efficacy also their production costs must be considered, which may become a limiting factor.

CONCLUSION

Within the past decades, plant-based signaling compounds became increasingly popular as eco-friendly priming compounds or resistance boosters in the fields of biotechnology and agriculture. Unfortunately, up to now most of the proposed concepts have not yet proven to be successful enough to pose as viable alternatives for conventional crop protection strategies. This observation is mainly based on the variety of drawbacks addressed by Brilli et al. (2019) which still need to be further discussed and overcome in the future. However, new concepts exploring the potential of DAMPs as plant protective compounds found especially eDNA (Ferrusquía-Jiménez et al., 2020) to be a new candidate for application in the field. In addition to such treatments directly spraying compounds produced by wounded plant tissues on unwounded crops, we would like to focus onto damage-induced volatile compounds (DIVs). These DIVs are (i) specifically synthesized and emitted upon tissue disruption and (ii) can serve as intra- and interplant signals initiating immune responses as well. Due to their generation upon injuries or damage, these compounds can also be classified as DAMPs. Mainly GLVs but also DMNT and indole fulfill the criteria to be classified as volatile DAMPs in *stricto sensu*. Their airborne nature opens new possibilities for applications but also reveals new challenges. A general issue is the volatile-based communication itself, involving the plant as an emitter as well as a receiver. On the one hand, even in conspecific plants a high genetic identity does not guarantee a functioning communication between varieties as shown for sweet potato (Meents et al., 2019). On the other, VOC-emitting plants do not necessarily release "private messages" and may attract unwanted organisms as well as advantage eavesdropping adjacent plants competing for nutrients (Gershenzon, 2007). The intensity and longevity of the volatile "messages" itself is highly fluctuating as well since environmental conditions can strongly reduce the efficiency of the particular volatile compound not only on a physico-chemical level but simply by fast dilution due to strong winds. On a physiological scale, the cost-benefit ratio for the emitting plant and the effect on conspecific individuals need to be further investigated to prove an actual profit and not simply a trade-off. Taken together, up to this point DIVs pose as a promising approach for DAMP-based crop protection – however, mainly restricted to a controlled and space-limited area such as phytochambers and greenhouses.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Breaking Bad News: Dynamic Molecular Mechanisms of Wound Response in Plants

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Recognition and repair of damaged tissue are an integral part of life. The failure of cells and tissues to appropriately respond to damage can lead to severe dysfunction and disease. Therefore, it is essential that we understand the molecular pathways of wound recognition and response. In this review, we aim to provide a broad overview of the molecular mechanisms underlying the fate of damaged cells and damage recognition in plants. Damaged cells release the so-called damage associated molecular patterns to warn the surrounding tissue. Local signaling through calcium (Ca^{2+}), reactive oxygen species (ROS), and hormones, such as jasmonic acid, activates defense gene expression and local reinforcement of cell walls to seal off the wound and prevent evaporation and pathogen colonization. Depending on the severity of damage, Ca^{2+}, ROS, and electrical signals can also spread throughout the plant to elicit a systemic defense response. Special emphasis is placed on the spatiotemporal dimension in order to obtain a mechanistic understanding of wound signaling in plants.

Keywords: wound response, damage, damage-associated molecular pattern, systemic signaling, herbivory, jasmonic acid, regeneration

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INTRODUCTION

Plants are especially susceptible to damage as they are unable to run away when facing danger. Wounds can originate from harsh weather conditions (e.g., strong wind, hail, fire, and frost), physical damage (e.g., trampling), exposure to chemicals (e.g., DNA damage and toxic substances), or biotic attack (e.g., microbes and herbivores). Damage can range in severity from single cell death to complete removal of organs and in duration from single events to repeated injury, for example, from chewing insects. In the lab, mechanical damage can be rather "clean" as in cutting with a sharp razor blade, application of pin pricks, and laser-mediated wounding, or "messy" by bruising tissue with pinches of a forceps or hemostat. We define here "wound" (wounding, wound-induced, etc.) as a general term, while the type of damage that produced the wound can be further specified, such as mechanical- or herbivore-induced damage.

In contrast to metazoans, plants do not rely on a dedicated nerve system or mobile immune cells to sense or respond to wounds. Nevertheless, plants have evolved efficient mechanisms to

perceive wounds and mount an appropriate defense response. Each plant cell has the ability to transduce a signal to its neighboring cells *via* damage-associated molecular patterns (DAMPs; previously reviewed in Heil and Land, 2014). Depending on the severity of the damage in size or location (e.g., leaf midvein; Kiep et al., 2015; Toyota et al., 2018), the complete plant can be alerted through a systemic signal, spreading from local to distal tissues that comprises waves of hydraulic, electrical, calcium (Ca^{2+}), and reactive oxygen species (ROS) signals, and the perception of wound-related hormones, such as jasmonic acid (JA), ethylene, or abscisic acid (ABA). Once activated, chemical defenses, such as the production of phytoalexins and other secondary metabolites, or structural defenses, such as increased production of trichomes and strengthening of cell walls, can protect the plant from reoccurring damage (Agrawal, 1998; Maffei et al., 2007b). Several aspects of the wound response are conserved with metazoans, including the release of certain DAMPs, Ca^{2+}, and ROS signaling. Other traits are plant-specific, such as the production of wound hormones and release of wound-induced volatiles. Some responses share similarities, such as the production of oxylipins (JA in plants and prostaglandins or leukotrienes in metazoans) and activation of membrane localized receptors by DAMPs and downstream phosphorylation cascades to activate defense gene expression (previously reviewed in León et al., 2001; Maffei et al., 2007a; Heil and Land, 2014; Savatin et al., 2014).

The ability to sense and appropriately respond to wounds is crucial for survival. On the one hand, a defective or overwhelmed defense response leads to increased plant mortality (Agrawal, 1998), especially what concerns the replenishment of stem cells and regeneration of organs in the root and shoot apical meristems and cambium (Sena et al., 2009; Heyman et al., 2013; Efroni et al., 2016). On the other hand, mechanisms are in place to prevent plants from overreacting to wounds and, when compromised, can lead to uncontrolled spread of cell death (Cui et al., 2013) or hypersensitivity to wounding (Zhang et al., 2019). Wound healing and defense responses can prevent excessive water loss (Consales et al., 2012; Cui et al., 2013; Becerra-Moreno et al., 2015), attenuate pathogen infection (Tarr, 1972; Lulai and Corsini, 1998; Zhou et al., 2020), and deter herbivores (previously reviewed in Erb and Reymond, 2019).

In nature, wounds are likely pervasive even when not visible to the naked eye and provide easy access sites for some pathogens, especially wound parasites such as wood rot and canker fungi (previously reviewed in Tarr, 1972). Pathogen colonization is prevented by wound healing processes, such as production of cork, callus, resin, or gum, and relies on rapid sealing of wounds (Lulai and Corsini, 1998). Furthermore, the immune system is activated in response to wounding (Savatin et al., 2014; Zhou et al., 2020). Therefore, wound-induced resistance can inhibit pathogen growth, for example, in the local resistance to *Botrytis cinerea* (Chassot et al., 2008; García et al., 2015), although it likely depends on environmental circumstances, such as high humidity (L'Haridon et al., 2011) and the natural genetic variation of the host plant (Coolen et al., 2019). Furthermore, effective colonization of the wound depends on the timing of contact with the pathogen (present before wounding or only after) and degree of wounding (Lulai and Corsini, 1998; Chassot et al., 2008). Therefore, pathogen entry *via* wounds merits further investigation and should be evaluated in a case-by-case scenario. Both microbes and invertebrate herbivores will attempt to subvert wound-induced defense responses. Interaction with chewing or sucking insects is further complicated as both insects and insect-borne microbes produce elicitors and suppressors of plant defense, in which JA signaling is often the target (previously reviewed in Basu et al., 2018). Due to the co-evolution of plants and pests, it is to be expected that every wound response is a potential target for suppression by pathogens and herbivores. Therefore, interactions of wounds with biotic challenges pose interesting cases, where wound responses can be enhanced or subverted, and some examples will be highlighted throughout this review.

Studies of wound response in plants present a long tradition of research. Whereas the first studies were mainly descriptive (Bloch, 1941; Lipetz, 1970), in the last decades, molecular mechanisms are increasingly becoming clear (León et al., 2001; Maffei et al., 2007a; Savatin et al., 2014). For information on wound healing and mitigation of damage in post-harvest processes in vegetables and fruit, we refer to specific literature (Cisneros-Zevallos et al., 2014; Lulai et al., 2016; Saltveit, 2016; Iakimova and Woltering, 2018; Hussein et al., 2020). This review provides a broad overview of the recent developments in molecular mechanisms with a focus on spatiotemporal dynamics in order to gain mechanistic understanding and to address open questions in the field of wound response in plants.

LOCAL VS. SYSTEMIC WOUND SIGNALING

Wound signaling can be divided in a local and systemic response. Cells at the site of injury can be completely destroyed or bruised (Iakimova and Woltering, 2018) and, at least in leaves, cell death ensues at the timescale of hours to days in 2–3 cell layers away from the site of injury (Cui et al., 2013; Iakimova and Woltering, 2018). Together with the local deposition of lignin, callose, and phenolics, cell death likely functions as a physical barrier to seal-off the injury and protects the adjacent intact tissue (Savatin et al., 2014; Iakimova and Woltering, 2018). DAMPs released from wounds signal the surrounding intact cells *via* Ca^{2+}, ROS, phosphorylation, and electrical signaling to mount defense gene expression. Most likely, direct physical responses, such as changes in mechanical forces and cell pressure surrounding the wound, play a pronounced signaling role, although these are largely unknown (Routier-Kierzkowska et al., 2012; Hoermayer et al., 2020). In parallel and depending on the severity of damage, systemic signals are propagated from the wound site to the rest of the plant, comprising leaf-to-leaf, root-to-root, leaf-to-root, and root-to-leaf signaling. Local and systemic responses are inherently linked at least through Ca^{2+}, ROS, and electrical signaling, and, where information is available, links will be highlighted throughout the review.

The Ins and Outs of DAMPs Generation and Recognition

Plants have evolved mechanisms that allow them to respond quickly to wounding and to distinguish the self from the non-self (Heil and Land, 2014; Savatin et al., 2014). Plant innate immunity relies on cell surface receptors that allows activation of defense responses *via* the recognition of conserved exogenous pathogen-derived (non-self) or endogenous (self) danger signals by transmembrane pattern-recognition receptors (PRRs). These conserved danger signals are also termed as pathogen-associated molecular patterns [PAMPs; also named microbe-associated molecular patterns (MAMPS) in the literature] for the non-self-signals and DAMPs for the self-signals (Choi and Klessig, 2016). In this review, we will discuss recent progress on several prominent DAMPs and their links to wound response, while for an extensive overview of DAMPs, we refer to recent excellent reviews (Choi and Klessig, 2016; Duran-Flores and Heil, 2016; Gust et al., 2017; Hou et al., 2019).

Primary/Constitutive and Secondary/Inducible DAMPs

Wounding either by mechanical damage, herbivores, or microbial infections results in disruption of plant tissue and subsequent release of intracellular molecules and cell wall-associated molecules into the apoplastic space (Mithöfer and Boland, 2012; Choi and Klessig, 2016; Duran-Flores and Heil, 2016; **Figure 1A**). Herbivores destroy plant tissues during feeding and/or by chemical modification while microbial infection-induced plant damage is often caused by deleterious activities of microbial hydrolytic enzymes or toxins (D'Ovidio et al., 2004; Horbach et al., 2011). Molecules released passively upon host damage conform to the definition of "classical" or primary DAMPs (Matzinger, 1994), which are molecules that have a physiological role during homeostasis but indicate damage when they appear outside the cell. Examples are ATP, cell wall fragments occasioned by wounding or pathogen derived cell wall degrading enzymes, or fragmented DNA caused by pathogen DNases (Claverie et al., 2018; Hadwiger and Tanaka, 2018; Huang et al., 2019; Jewell and Tanaka, 2019). Location is important, as these DAMPs are invisible to the immune system during homeostasis and are passively exposed to the extracellular environment, thereby acting as early and general activators of the plant immune system (Vénéreau et al., 2015; Choi and Klessig, 2016). Thus, primary DAMPs are not linked to biosynthesis or secretion from undamaged cells. The secondary or inducible DAMPs are endogenous molecules actively produced or modified during cell death and function exclusively as signals. They can be secreted passively or actively upon wounding or microbial infection by either damaged or undamaged cells and include, for example, small signaling peptides (Gust et al., 2017; Li et al., 2020). Details about the temporal activation of the signaling molecules and hormones upon perception of DAMPs mentioned in the text can be retrieved in **Table 1**.

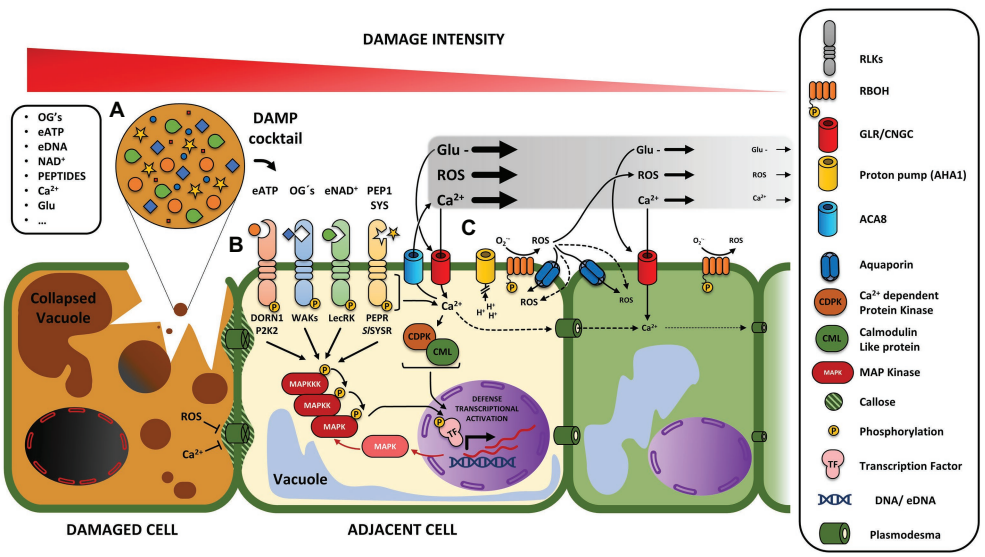

FIGURE 1 | Overview of local damage at early time-points following signaling processes. **(A)** Damaged cells suffer fragmentation of their cellular components, releasing a mixture of different damage-associated molecular patterms [DAMPs (DAMP cocktail)] to the surrounding environment. Reactive oxygen species (ROS) and Ca^{2+} contribute to plasmodesmata blockage and later accumulation of callose. **(B)** DAMPs are perceived by specific receptors, generally receptors like kinases in the plasma membrane. DAMP sensing is normally accompanied by hallmark signal transduction events, such as MAPK phosphorylation cascades, that result in transcription factor phosphorylation and modulation of defense gene expression. **(C)** Parallel to local perception by receptors, certain signals such as Ca^{2+}, ROS, and glutamate can travel from the wound site in a distance-dependent gradient along the apoplast. GLR and CNGC channels can be activated by DAMPS such as glutamate and Pep1. Intracellular Ca^{2+} serves as a component to activate calcium-dependent protein kinases (CDPK) and calmodulin-like proteins (CML), which contribute to transcriptional responses. Together, oxygen radicals are generated locally in the extracellular space and transformed to more stable ROS species by RBOHs, thus adjusting the ROS wave signal.

TABLE 1 | Timing and localization of DAMP release/generation and wound responses in plants.

DAMP	Receptor	Release/generation	Localization	Response	Source	Response time	Plant species	References
Ogs	WAK1/2	< 4 h (Polygalacturonase; Bergey et al., 1999)	-	Ca^{2+}	Ex	2 min	Tobacco	Chandra and Low, 1997
				ROS	Ex	2 h	Tobacco	Bellincampi et al., 2000
				ROS	Ex	15 min	*Arabidopsis*	Galletti et al., 2008
				MAPK	Ex	3 min	*Arabidopsis*	Denoux et al., 2008
				NO	Ex/*in vivo*	30 min	*Arabidopsis*	Rasul et al., 2012
				Callose	Ex	18 h	*Arabidopsis*	Denoux et al., 2008
eATP	P2K1	< 1 min (Song et al., 2006)	Extracellular	Ca^{2+}	Ex	30–40 s	*Arabidopsis*	Tanaka et al., 2010
				Ca^{2+}	Ex/*in vivo*	1–2 min	*Arabidopsis*	Demidchik et al., 2009
				ROS	Ex/*in vivo*	15 s	*Arabidopsis*	Demidchik et al., 2009
				ROS	Ex/*in vivo*	5 min	Medicago	Kim et al., 2006
				JA	Ex/*in vivo*	24 h	Tomato	Wu et al., 2012
				Et	Ex/*in vivo*	24 h	Tomato	Wu et al., 2012
NAD(P)+	LecRK-1.8/VI.2	< 20 min (Zhang and Mou, 2009)	Extracellular	SA	Ex	4 h	*Arabidopsis*	Wang et al., 2017
				PR genes	Ex/*in vivo*	24 h	*Arabidopsis*	Zhang and Mou, 2009
				SA	Ex	-	*Arabidopsis*	Zhang and Mou, 2009
HMGB3	-	24 h (Choi et al., 2016)	Apoplast	MAPK	Ex/*in vivo*	15 min	*Arabidopsis*	Choi et al., 2016
				Callose	Ex/*in vivo*	15 h	*Arabidopsis*	Choi et al., 2016
DNA	-	-	-	Ca^{2+}	Ex	30 min	Maize	Barbero et al., 2016
				Ca^{2+}	Ex	30 min	Lima Bean	Barbero et al., 2016
				ROS	Ex	2 h	Common Bean	Duran-Flores and Heil, 2018
				MAPK	Ex	30 min	Common Bean	Duran-Flores and Heil, 2018
Systemin	SYR1/2	3–4 h (mRNA; McGurl et al., 1992)	Intracellular	Ca^{2+}	Ex	2 min	Tomato	Moyen et al., 1998
				ROS	Ex/*in vivo*	4 h	Tomato	Orozco-Cardenas and Ryan, 1999
				MAPK	Ex/*in vivo*	2 min	Tomato	Stratmann and Ryan, 1997
		18 h (prosystemin; Narváez-Vásquez and Ryan, 2004)	Phloem	Et	Ex/*in vivo*	30 min	Tomato	O'Donnell et al., 1996
				JA	Ex/*in vivo*	15 min	Tomato	Narváez-Vásquez et al., 1999
				PI	*in vivo*	1 h	Tomato	Howe et al., 2000
Inceptin	INR	-	-	JA	Ex/*in vivo*	30 min	Cowpea	Schmelz et al., 2007
				Et	Ex/*in vivo*	120 min	Cowpea	Schmelz et al., 2007
				SA	*Ex/in vivo*	240 min	Cowpea	Schmelz et al., 2007
Glutamate	GLR	< 1 min (Toyota et al., 2018)	Vasculature	Ca^{2+}	*in vivo*	1 min	*Arabidopsis*	Toyota et al., 2018
				Ca^{2+}	Ex	1 min	*Arabidopsis*	Shao et al., 2020
				SA	Ex	6 h	*Arabidopsis*	Goto et al., 2020
				JA	Ex	7 h	*Arabidopsis*	Goto et al., 2020
AtPep1	AtPEPR1/2	0.5–5 min (Hander et al., 2019)	Intracellular	Ca^{2+}	Ex	40 s	*Arabidopsis*	Ranf et al., 2011
				MAPK	Ex	2 min	*Arabidopsis*	Ranf et al., 2011
				ROS	Ex	4 min	*Arabidopsis*	Flury et al., 2013
AtPep3	AtPEPR1	24 h (Yamada et al., 2016; Engelsdorf et al., 2018)	Extracellular	ROS	Ex	2 h	Rice cells	Shinya et al., 2018
				MAPK	Ex	15 min	Rice cells	Shinya et al., 2018
				JA	Ex	3 h	Rice cells	Shinya et al., 2018
				JA	*in vivo*	4 h	*Arabidopsis*	Klauser et al., 2015

Ex, exogenous application; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase; NO, nitric oxide; JA, jasmonic acid; SA, salicylic acid; Et, ethylene, PR, pathogenesis related; and PI, protease inhibitors.

Oligogalacturonides

Cell wall integrity is crucial for plant growth and development as well as in preventing wounding and pathogen attack (Bellincampi et al., 2014). Perception of an altered cell wall integrity is proposed to be a key event during wounding (Nühse, 2012; Wolf et al., 2012; Wolf, 2017), although experimental evidence is lacking so far. Oligogalacturonides (OGs) are released from the plant cell walls from the fragmentation of homogalacturonan, the main component of pectin, either by endogenous wound-induced polygalacturonases or during infection by microbial polygalacturonases (Savatin et al., 2014). OGs are relatively immobile in the plant vascular system and may act as a local signal; however, because polygalacturonase activity is induced systemically in response to wounding, OGs may amplify responses in undamaged leaves (**Table 1**; Bergey et al., 1999). The size of OG fragments is a major factor dictating their elicitor activity, being OGs with a degree of polymerization between 10 and 15 the most active while shorter oligomers are inactive. OG-induced defense responses include production of ROS (Bellincampi et al., 2000), mitogen-activated protein kinase (MAPK) activation (Denoux et al., 2008), nitric oxide (NO; Rasul et al., 2012), and upregulation of phytoalexins and glucanase (Davis and Hahlbrock, 1987), chitinase (Broekaert and Peumans, 1988), and callose (Denoux et al., 2008; Galletti et al., 2008). In tomato, OGs induce the accumulation of a protease inhibitor, which is effective against insect herbivores (Moloshok et al., 1992; Ryan and Jagendorf, 1995). The *Arabidopsis* wall-associated kinase 1 (WAK1) has been described as an OG receptor. *In vitro* studies have demonstrated that WAK1 binds to polygalacturonic acid, pectins, and specifically to OGs with a degree of polymerization over nine moieties (Decreux and Messiaen, 2005; Cabrera et al., 2008; Brutus et al., 2010). Furthermore, gene expression studies indicate that WAK1 is upregulated by wounding and exogenous application of OGs (Wagner and Kohorn, 2001; Denoux et al., 2008; Ferrari et al., 2013). Alterations in the expression of WAK1 and of its interactors disturb the local response to wounding (Gramegna et al., 2016; De Lorenzo et al., 2018). Hyperaccumulation of OGs may affect growth of the whole plant, eventually leading to cell death (Benedetti et al., 2015), suggesting that OGs play a role in the growth-defense trade-off (Huot et al., 2014). Hence, plants limit the hyperaccumulation of OGs by a battery of at least four *Arabidopsis* enzymes belonging to the family of the so-called berberine-bridge enzyme (BBE-like) proteins (Daniel et al., 2017). BBE-like proteins specifically oxidize OGs and produce oligosaccharides that reduce the ability to induce expression of defense genes, ROS burst, and deposition of callose (Benedetti et al., 2018). Similarly, cellodextrines, degradation products of cellulose, trigger a signaling cascade during immunity, and oxidation by other BBE-like proteins impairs elicitor activity (Locci et al., 2019). Recently, an application of OGs accelerated mechanical wound healing in tomato fruit *via* elicitation of callose deposition, defense gene expression, lignin biosynthesis, and phenylalanine ammonia-lyase activity around the wound in a Ca^{2+} signaling-dependent manner (Lu et al., 2021).

Extracellular ATP, NAD^+, and $NADP^+$

Adenosine-5-triphosphate (ATP) represents the universal energy source for metabolic processes. During wounding, ATP is released immediately from the cytoplasm to the outside of the cell (**Table 1**). This extracellular ATP (eATP) is recognized as a DAMP and has been reported to activate defense responses in fungi, mammals, and plants (Medina-Castellanos et al., 2014; Tripathi and Tanaka, 2018; Roux and Clark, 2019). Concentrations of approximately 40 uM eATP have been measured in the extracellular fluid present at wound sites within 3 min following damage to *Arabidopsis* leaves, which are sufficient to initiate an immune response (Song et al., 2006). In mammals, eATP is recognized by plasma membrane-localized P2-type purinergic receptors. In *Arabidopsis*, eATP, as a DAMP, is sensed by the L-type lectin receptor kinases P2K1 (also known as does not respond to nucleotides 1 or DORN1) and P2K2 at concentrations well below 40 uM (Choi J. et al., 2014; Pham et al., 2020). Transcriptional studies of a *p2k1* mutant in the absence of stimuli revealed only 21 differentially expressed genes compared to the wild type. Such a small number could indicate that P2K1-mediated eATP signaling does not play a major role in growth and development under homeostasis (Jewell and Tanaka, 2019). Approximately 60% of the genes induced by eATP are also induced by wounding, indicating that eATP plays an important role in response to wounding (Choi J. et al., 2014). Furthermore, physical damage in plants that overexpress *P2K1* enhanced upregulation of wound-induced gene expression, while this expression is notably reduced in the *p2k1-3* mutant (Choi J. et al., 2014). Early eATP induced responses include membrane depolarization, Ca^{2+} influx, ROS formation, malondialdehyde production, enzymatic activity (catalase and polyphenol oxidase), JA, and ethylene biosynthesis (Kim et al., 2006; Tanaka et al., 2014; Tripathi et al., 2018; Wang Q.-W. et al., 2019). eATP treatment of wounded tissue resembles a JA-dependent defense response, resulting in the secretion of extrafloral nectar in lima bean to attract predators of herbivores (Heil et al., 2012). Induced immunity by eATP has been reported at the phenotypic level in response to bacteria (Chivasa et al., 2009; Chen et al., 2017), necrotrophic fungi (Tripathi et al., 2018), and herbivores (Heil et al., 2012). ATP receptors, *p2k1-3*, *p2k2* single mutant, and *p2k1p2k2* double mutants, are more susceptible to bacterial infection compared to the wild type, whereas *P2K2* complemented lines showed no difference to the wild type and ectopically expressed *P2K2* showed elevated resistance to bacterial infection (Pham et al., 2020). Saliva from *Helicoverpa zea* larvae degrades eATP from tomato leaves *via* multiple ATPases. The ATPases also suppress wound-induced expression of glandular trichomes in newly forming leaves, thus acting as a herbivore effector suppresses eATP induced wound response (Wu et al., 2012). Similarly, mechanical stress can be coupled to the release of extracellular ATP. In fact, it plays an important role in the root avoidance response, where sensing mechanical stimulation elicited by contacting an object triggers root growth, allowing it to avoid and overcome physical obstacles. Exogenously applied ATP changes the sensitivity of the root tip to the growth-regulating plant hormone auxin and reduces shootward auxin transport (Tanaka et al., 2010). Plants respond to eATP in a

dose-dependent manner. Constitutive levels of eATP appear to be essential, as depletion can trigger cell death (Chivasa et al., 2005), while low (30 μM) or moderate (150 μM) doses of eATP can stimulate or suppress cell elongation, respectively (Clark et al., 2010). High eATP doses (>500 μM) reduce cell viability and can trigger programed cell death (Sun et al., 2012; Deng et al., 2015). While there is no direct evidence that eATP alone affects plant growth/regeneration after wounding, data suggest that a combination of several cues like DAMPS, PAMPS, ion/osmolyte concentrations, or mechanical stresses trigger a defense and regeneration response (Marhavý et al., 2019; Shanmukhan et al., 2020; Zhou et al., 2020).

NAD^+ and $NADP^+$, as di-nucleotides and similarly to ATP acting as a classical cofactor, can be released to the environment after wounding, through membrane leakage or active processes such as exocytosis in animal model species (Haag et al., 2007). In *Arabidopsis*, an application of exogenous NAD^+ ($eNAD^+$) and $eNADP^+$ is sufficient to induce salicylic acid (SA) accumulation, expression of pathogenesis-related (PR) genes, and resistance to pathogens (Zhang and Mou, 2009; Wang et al., 2017). A lectin receptor kinase, LecRK-I.8, was found to be partially responsible for $eNAD^+$ perception (Wang et al., 2017), while LecRK-VI.2 has been proposed as a receptor of both $eNAD^+$ and $eNADP^+$ (Wang C. et al., 2019). Transcriptome analyses suggest that eNAD+ signaling upregulates genes involved in PAMP triggered immunity and SA pathways but suppresses genes of the JA and ethylene pathways, which are more related to wounding (Wang et al., 2017). However, $eNAD^+$ and $eNADP^+$ leak into the extracellular space during mechanical wounding and pathogen-induced hypersensitive response in concentrations high enough to induce the latter responses (**Table 1**), raising the possibility that they act as DAMPs (Zhang and Mou, 2009; Wang C. et al., 2019).

High Mobility Group Box Proteins

High mobility group box (HMGB) proteins are highly conserved chromatin-architecture regulators found in all eukaryotes, including plants. Mammalian HMGB1 was one of the first DAMPs to be identified and is extensively characterized and considered a primary DAMP (Choi and Klessig, 2016). Briefly, human HMGB1 binds in the nucleus to DNA, facilitating nucleosome formation and transcription factor binding (Thomas and Travers, 2001; Lotze and Tracey, 2005). Upon its release outside the cell, it can be recognized by various cell surface receptors (Heil and Vega-Muñoz, 2019). In metazoans, HMGB1 facilitates tissue repair and healing by promoting the switch of macrophages to a tissue-healing phenotype (Bianchi et al., 2017). Based on their nuclear location and domain structure, plant HMGB-type proteins might function in a similar way to mammalian HMGB1. The presence of extracellular AtHMGB3 raised the possibility that, similar to the classical role of HMGB1 as mammalian DAMP, it serves in a similar way in plants (Choi et al., 2016). Notably, AtHMGB2/3/4 are present in the cytoplasm as well as in the nucleus. Cytoplasmic functions for these proteins have not yet been reported; however, it is theorized that the cytosolic subpopulation might have easy access to the apoplast after wounding in comparison to the ones found in the nucleus (Pedersen et al., 2010; Choi and Klessig, 2016). To our knowledge, there is no evidence that AtHMGB3 is secreted into the apoplast, so extracellular AtHMGB3 is most likely the result of cell membrane rupture. In fact, tissue damage during *Botrytis cinerea* infection causes the release of AtHMGB3 to the apoplast after 24 h of inoculation, whereas a control protein, histone H3, only appears in the total leaf and nuclear extracts at that timepoint, suggesting that AtHMGB3 is released early during necrosis (**Table 1**; Choi et al., 2016). Exogenous application of AtHMGB3 induces innate immune responses like MAPK activation, defense gene expression, callose deposition, and enhanced resistance to pathogen infection (Choi et al., 2016).

DNA

Plant immunity can be activated upon the sensing of DNA. Cell death during pathogen infection or abiotic stresses leads to DNA fragmentation (Ryerson and Heath, 1996; Kuthanova et al., 2008). Fragmented DNA can be exposed to the apoplast and function as a DAMP. Several recent studies have found evidence that the host-derived fragmented DNA (<700 bp) triggers early plant defense responses, such as membrane depolarization, Ca^{2+} influx, ROS production, and MAPK activation, and eventually induces changes in CpG methylation, and increases plant resistance to pathogen infections (Wen et al., 2009; Barbero et al., 2016; Duran-Flores and Heil, 2018; Vega-Muñoz et al., 2018). Intriguingly, the ability of non-self-derived DNA to trigger an immune response is lower or undetectable than the ones induced by self-derived DNA (Duran-Flores and Heil, 2018), suggesting a species-specific perception mechanism that discriminates self-derived DNA from non-self DNA. To date, no DNA receptor has been identified in plant cells, and none of the receptors that are known from mammals discriminate between self and non-self DNA (Heil and Vega-Muñoz, 2019). Extracellular DNA present on plant root tips is required for defense against a necrotrophic fungus (Wen et al., 2009), and it was recently reported that secreted DNases by a fungal pathogen (*Cochliobolus heterostrophus*) and a herbivore (*Laodelphax striatellus*) serve as effectors that suppress DNA-dependent plant immunity, reinforcing the biological relevance of DNA as a DAMP in plants (Huang et al., 2019). Importantly, to the best of our knowledge, there is no evidence for wound-induced DNA release to the apoplast in plants. However, based on evidence of DNA release in mammalian studies (Marichal et al., 2011; Pottecher et al., 2019; Gong et al., 2020), it is anticipated to be similarly present in plants, but requires further investigation.

Links between the DNA damage response (DDR), cell cycle, programed cell death, and immunity have emerged in recent years (Song et al., 2011; Yan et al., 2013; Hu et al., 2016; Johnson et al., 2018). Depending on the cell type and the severity of the DNA damage, different cellular responses are triggered. In mammals, mild DNA damage leads to cell-cycle arrest, whereas severe and irreparable damage leads to senescence or cell death programs (Surova and Zhivotovsky, 2013). In plants, the presence of damage-inducing agents or defective DNA repair leads to aberrant organogenesis and development,

as well as loss of biomass (Hu et al., 2016). In addition, other reports link DDR to the activation of the plant immune system. Pathogen infection triggers the production of SA, which in turn induces DNA damage that can be sensed by DNA repair mechanisms to the site of DNA damage for repair or activation of defense gene expression (Yan et al., 2013). Suppressor of gamma response 1 (SOG1) is a transcription factor of the NAC family and is a central regulator of the plant DDR (Yoshiyama et al., 2009). DDR has been reported to play an essential role for plants to cope with various environmental stresses (Yan et al., 2013; Hong et al., 2017; Ogita et al., 2018). *sog1-1* mutants are deficient in DDR and immune response, while *SOG1* overexpression in the presence of zeocin, a double-strand DNA break agent, enhances DDR, the expression of genes involved in chitin response, and fungal resistance (Yoshiyama et al., 2020). Ethylene response factor 115 (ERF115) is a transcription factor that is upregulated in meristematic cells that are positioned adjacent to dead ones in the root tip. Severe stress conditions may cause irreparable DNA damage resulting in cell death, followed by the induction of regeneration in an ERF115-dependent manner (Heyman et al., 2016, 2018). Besides SA, specific agents that cause DNA alterations (e.g., DNA helical distortion, intercalation, base substitutions, methylation, etc.) enhance defense gene expression. DNA damage and resulting chromatin structural changes may be a central mechanism in initiating defense gene transcription during nonhost resistance (Hadwiger and Tanaka, 2018). Links between DNA damage, immunity, and regeneration have been emerging in the last years, yet, it remains unclear how DNA is sensed as no formal DNA receptors have been reported.

Systemin and Other Small Signaling Peptides

Small signaling peptides can be generated as the product of two activities: by transcriptional responses inducing small open reading frames coding for small peptides or by proteolytic processing of precursor proteins (Tavormina et al., 2015; Hou et al., 2019). Proteolytic cleavage generates peptides that are able to alarm surrounding tissues about the imminent stress when perceived *via* plasma membrane associated receptor-like kinases (Wang and Irving, 2011; Stührwohldt and Schaller, 2019). Although experimental evidence has accumulated over the last years, the functions, receptors, mode of actions, and proteases that liberate the peptides from their precursors are still largely unexplored (Tavormina et al., 2015; Schardon et al., 2016; Hander et al., 2019; Chen et al., 2020).

Systemin was the first reported extracellular peptide that induces defense signaling in plants (Pearce et al., 1991). From its precursor, prosystemin, mature systemin (18 amino acids in length) is partially processed by the cysteine protease phytaspase and released into the apoplast during mechanical damage (Beloshistov et al., 2018). Phytaspase might get access to intracellular prosystemin *via* cellular disruption or *via* active delocalization upon programed cell death (Chichkova et al., 2010; Beloshistov et al., 2018). *Prosystemin* expression is low in unwounded leaves and increases several fold, peaking around 4 h after wounding (McGurl et al., 1992). Prosystemin accumulates mainly in the cytosol and nucleus of phloem parenchyma cells (Narváez-Vásquez and Ryan, 2004). Systemin specifically binds its receptors Systemin receptor 1 and 2 (SYR1 and SYR2), which is sufficient to induce the typical response including a ROS burst, ethylene production, and the expression of two wound induced proteinase inhibitors in tomato (Wang et al., 2018). Functionally related peptides are the hydroxyproline-rich glycopeptide systemins. Repetition of these peptides found in the polypeptide precursor proHypSys is thought to magnify the intensity of the wound response once processed (Pearce, 2011). These genes encode different peptides for tobacco, petunia, tomato, and sweet potato but have in common that they are transcriptionally responsive to wounding and/or JA, and above all, they induce similar responses as systemin (Pearce et al., 2001, 2007; Ryan and Pearce, 2003; Ren and Lu, 2006; Chen et al., 2008). Systemin has only been identified in Solanaceae species (Pearce et al., 1991). However, peptides similar to systemin have been identified in other plant species, such as HypSys, Peps, GmSubPep, GmPep914, GmPep690, and PIPs, that act as DAMPs, eliciting high levels of proteinase inhibitors, JA, and release of volatiles within minutes of exogenous peptide application (Albert, 2013; Huffaker et al., 2013; Hou et al., 2019).

Protein elicitor peptide 1 (Pep1) was extracted from *Arabidopsis thaliana* lysates (Huffaker et al., 2006) and is the founding member of a gene family in *Arabidopsis* of eight with various expression patterns under normal and biotic or abiotic stress conditions (Huffaker and Ryan, 2007; Bartels et al., 2013; Bartels and Boller, 2015). Peps are encoded in the C-terminus of their precursors, PROPEPs, which are found in both monocots and dicots (Huffaker et al., 2013; Lori et al., 2015) and play multiple roles in defenses to pathogens, herbivores, and abiotic stresses (Ross et al., 2014; Klauser et al., 2015; Yamada et al., 2016; Engelsdorf et al., 2018; Lee et al., 2018; Nakaminami et al., 2018; Zheng et al., 2018; Jing et al., 2020; Zhang and Gleason, 2020). Ca^{2+} release in mechanically damaged cells activates the cysteine protease metacaspase4 (MC4) to cleave Pep1 from its precursor PROPEP1 within 5 min after wounding (Hander et al., 2019; Zhu et al., 2020). Metacaspases are evolutionary conserved proteases with nine members in the *Arabidopsis* gene family (Klemenčič and Funk, 2018; Minina et al., 2020) of which various metacaspases can cleave different PROPEPs (Hander et al., 2019; Shen et al., 2019). Cleavage of PROPEP1 seems to be essential for release of Pep1 from the tonoplast (Bartels et al., 2013; Hander et al., 2019). However, cleavage might not be required for others as unprocessed PROPEP3 was found to accumulate in the apoplast within 24 h after Pep treatment, pathogen challenge, and in response to cell wall damage (Yamada et al., 2016; Engelsdorf et al., 2018; **Table 1**). Downstream, Peps are perceived by the receptor-like kinases PEP receptor 1 and 2 (PEPR1 and PEPR2; Yamaguchi et al., 2006, 2010; Krol et al., 2010; Tang et al., 2015). Fluorescently labeled Pep1 travels locally in root tissue within a minute after external application and undergoes endocytosis when bound to PEPR1/2 (Ortiz-Morea et al., 2016). Recently, the Ca^{2+}-permeable channel cyclic nucleotide gated channel 19 (CNGC19) was proposed to act downstream of Pep perception in generating Ca^{2+} fluxes during herbivory (Meena et al., 2019).

Peptidome approaches to identify native peptides directly from protein extracts allowed the identification of novel peptide DAMPs. A tomato pathogenesis related 1b (PR-1b) derived peptide identified from wounded and JA-treated plants forms the basis of a conserved family of CAPE peptides named after PR1b, which belongs to the cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 proteins (CAP) superfamily (Chen et al., 2014; Chien et al., 2015). CAPE peptides operate during herbivore attack by activation of stress responsive genes, including proteinase inhibitors, and treatment with exogenous CAPE retards the growth of herbivores and confers resistance to *Pseudomonas syringae* pv. tomato DC3000 in tomato (Chen et al., 2014). In a recent peptidome approach, two interesting peptides were identified from developing *Arabidopsis* tracheary element cells (Escamez et al., 2019). Kratos and Bia (named after the children of the Styx river separating the worlds of the living and the dead in Greek mythology) decrease and enhance cell death during the incubation of leaf discs on the peptides, respectively (Escamez et al., 2019). While this hints at a novel role for Kratos in reducing wound-induced cell death, further investigation is needed.

Interactions Between DAMPs, HAMPs, and PAMPs

Herbivore associated molecular patterns (HAMPs) and pathogen associated molecular patterns (PAMPs) allow plants to perceive an attack from herbivores and pathogens, respectively, and interactions with responses to DAMPs have been described in the literature. Herbivory, for example, feeding by *Spodoptera* sp. caterpillars on Lima bean (*Phaseolus lunatus*) or *Medicago truncatula* or the application of HAMPs into mechanically inflicted wounds elicits conserved downstream signal amplification cascades (Duran-Flores and Heil, 2016). These cascades involve membrane depolarization, Ca^{2+} influxes, ROS formation, and the release of green leaf-volatiles (GLVs) within minutes, followed by MAPK phosphorylation and octadecanoid signaling cascades in the first hour following stress perception (Maffei et al., 2004, 2006; Arimura et al., 2008; Fürstenberg-Hägg et al., 2013; Schmelz, 2015). None of these responses are specific for a single type of herbivore or HAMP. Furthermore, in all cases of HAMP application, the leaves are mechanically damaged; hence the presence of DAMPs is unavoidable and the specific effects of DAMPs and HAMPs are difficult to be distinguished (Huffaker et al., 2013). Albeit a more artificial system, application of elicitors to suspension cell culture circumvents the unintended consequences of wounding and to disconnect the application of elicitors from the wound response (Shinya et al., 2018). Simultaneous application of *Oryza sativa* Pep3 and oral secretions from *Mythimna loreyi* has an additive effect on the production of ROS and MAPK activity and a synergistic effect on defense metabolite accumulation in comparison to separate application. This suggests that while DAMPs and HAMPs alone can trigger a defense response, perceiving both is critical for the strength of the induced plant defenses (Shinya et al., 2018).

A recent study provides a strong evidence for the positive interaction between wounding and PAMP recognition. Whereas applications of PAMPs do not or only weakly trigger immune-related gene expression in the *Arabidopsis* root, the co-incidence of accidental- or laser-induced damage highly amplifies this response as early as 4 h after wounding (Zhou et al., 2020). A localized and specific response is produced, as mostly close cells from underlying tissues, opposed to surrounding cells of the same tissue, respond strongly to the combination of PAMPs and damage. Wounding locally gates the expression of PAMP receptor kinases, and, thereby, immune responses to both beneficial or detrimental bacteria in roots. Co-application of the typical PAMP flg22 with DAMPS, including Pep1, eATP, cellobiose, OGs, or a cocktail thereof, however, does not induce immune-related gene expression to the extent as mechanical damage, suggesting that damage perception is more complex and likely involves other cues such as mechanical stress (Zhou et al., 2020).

Inceptin peptide is generated when cowpea (*Vigna unguiculata*) leaves are consumed by armyworm (*Spodoptera frugiperda*) larvae. Inceptin is produced by proteolysis of the cowpea chloroplastic ATP synthase γ-subunit (cATPC protein) in the insect gut and is then regurgitated back to the wound site (Schmelz et al., 2006). Inceptin stands in an intermediate position between HAMP and DAMP as conceptually speaking it is very similar, for example, to systemin, as it originates from a plant protein yet is different in the way that wounding alone does not trigger processing, and it requires a biotic attacker to process the peptide in order to trigger wound response (Duran-Flores and Heil, 2016). Inceptin is a disulfide-bridged peptide containing 11 amino acids. Exogenous treatment of cowpea with inceptin promotes the production of ethylene, SA and JA, and defense metabolite cinnamic acid, upregulates transcription of cowpea protease inhibitor, and enhances cowpea resistance to herbivory. Sequence alignments of cATPC proteins from multiple plant species demonstrate a high degree of conservation in the amino acid sequence related to the predicted inceptin peptides. However, inceptins are active elicitors of defense responses only in some Fabaceae (Schmelz et al., 2007; Li et al., 2020), suggesting that inceptin perception is a recent evolutionary event in plants. Recently, a leucine-rich repeat receptor-like kinase was found for inceptin in cowpea, being the first HAMP receptor to be reported and expanding the current knowledge of surface immune recognition to include herbivory (Steinbrenner et al., 2019).

Keeping Your Friends Close: Local Damage Signaling by Ca^{2+}, ROS, and Phosphorylation

Local wound signaling is defined as occurring typically a few cell layers away but, in terms of electrical signaling, can also relate to the whole wounded leaf (but not systemic leaves; see next section) and will depend on the severity of the wound. Receptor kinases, as mentioned in the previous sections, likely play an important role in perceiving a cocktail of DAMPs that is released in the immediate surrounding of wounds (**Figure 1B**). Ca^{2+} is a conserved second messenger involved in the initial signaling cascades of multiple physiological actions

and in response to biotic and abiotic stresses (Kudla et al., 2010). Across scales of wounding, from single cell laser-mediated damage in roots to pin pricks and herbivory in leaves, cytosolic Ca^{2+} levels are the highest and remain elevated longer closest to the wound site (Beneloujaephajri et al., 2013; Costa et al., 2017; Behera et al., 2018; Nguyen et al., 2018; Toyota et al., 2018; Hander et al., 2019; Li T. et al., 2019; Marhavý et al., 2019). This observation also applies to other model species, for example, fruit fly (*Drosophila melanogaster*; Razzell et al., 2013; Shannon et al., 2017). Mechanical damaged cells themselves experience immediate and highest spikes in Ca^{2+} levels, likely because of passive influx of Ca^{2+} through perforated plasma membranes or coming from internal stores (Hander et al., 2019). Cytosolic Ca^{2+} peaks are associated with corresponding drops in cytosolic pH (Behera et al., 2018).

Calcium signaling relies on a set of channels, pumps, and effector Ca^{2+}-binding proteins (De Vriese et al., 2018) for generation and readout of information in so-called Ca^{2+} signatures – cell-to-cell differences in calcium peak duration, intensity, and repetition – as observed during wounding (**Figure 1C**). Ca^{2+} signals can be inhibited by the application of typical extracellular chelators (e.g., EGTA and BAPTA) and inhibitors of Ca^{2+} channels (e.g., verapamil and GdCl3) at least in the cells neighboring the damaged cells (Beneloujaephajri et al., 2013; Hander et al., 2019; Marhavý et al., 2019). CNGC19 is the first known Ca^{2+}-permeable channel that mediates propagation of cytosolic Ca^{2+} elevations in the vasculature of the local leaf (within a minute) during mechanical and herbivore damage (Meena et al., 2019). Loss-of-function *cncg19* mutants have a decreased production of JA, glucosinolates, and are more susceptible to herbivores (Meena et al., 2019). Free Ca^{2+} can bind to EF-hand motifs present in calmodulins, calcineurin B-like protein (CBL) and CBL-interacting protein kinase (CIPK), calcium-dependent protein kinases (CDPKs, also referred to as CPKs), and calmodulin-like proteins (CML). So far, autoinhibited Ca^{2+}-ATPase isoform 8 (ACA8) is the only known Ca^{2+} pump involved in calcium signaling in the local wound response and is regulated by phosphorylation of a CBL1-CIPK9 complex (Costa et al., 2017). The Ca^{2+}-binding protein, *CML42* is transcriptionally induced by *Spodoptera littoralis* feeding and application of insect oral secretions on *Arabidopsis* leaves but not by mechanical damage simulated by MecWorm (Mithöfer et al., 2005; Vadassery et al., 2012). Glucosinolate production is impaired in *cml42* mutants in the presence of herbivores. CML42 is responsible in part for the trichome branching formation, a structural defense against herbivores (Dobney et al., 2009), and in the negative modulation of JA-induced cytosolic Ca^{2+} elevations and JA signaling (Vadassery et al., 2012). On the contrary, *CML37* is induced both by insect herbivory and mechanical damage (MecWorm) and is a positive regulator of the defense response against herbivores, as JA accumulation and JA marker gene expression is impaired in *cml37* mutants upon herbivory (Scholz et al., 2014). The calmodulin binding protein IQD1 is induced by wounding and affects glucosinolate biosynthesis (Levy et al., 2005). From a collection of CPK mutants, *cpk3* and *cpk13* show lower levels of defense gene induction, independent of JA signaling, after wounding (Kanchiswamy et al., 2010). Interestingly, 30 min after mechanical or herbivore-induced damage, accumulation of intracellular Ca^{2+} at wound sites was significantly higher in *cpk3* than *cpk13* or wild type (Kanchiswamy et al., 2010).

Traditionally perceived as by-products of cellular metabolism, ROS have later been recognized to play active roles in stress signaling and to be essential for wound responses in plants and animals (Suzuki and Mittler, 2012). Hydrogen peroxide (H_2O_2) increases both at the injury site and systemically to reach a peak after 4–6 h, while superoxide (O_2^-) is believed to be transiently and locally generated within minutes after injury (Doke et al., 1991; Minibayeva et al., 2001; Orozco-Cárdenas et al., 2001). Next to providing structural roles in cell wall strengthening in response to mechanical damage (Bradley et al., 1992), ROS and especially the relatively more stable H_2O_2 can act as second messengers (Mignolet-Spruyt et al., 2016). Ca^{2+} and ROS accumulate locally following mechanical damage in the same cells, where Ca^{2+} accumulates in a few seconds and is required to initiate a subsequent longer-lasting increase of ROS (maximum at 10–12 min; Beneloujaephajri et al., 2013). Ca^{2+} and ROS intersect at the plasma membrane localized respiratory burst oxidase homolog (RBOH), which are plant homologs of NADPH oxidase (NOX) enzymes that contain Ca^{2+}-binding EF-hand motifs. RBOHs function in propagation of systemic ROS waves (see next section), as well as local response, at least in *Arabidopsis* roots, leading to ethylene production (Marhavý et al., 2019). Similar to ROS, lesser-studied reactive nitrogen species (RNS), such as NO, accumulate locally between 15 min and 2 h and aid in wound healing by lignin and callose deposition (Huang et al., 2004; Corpas et al., 2008; Arasimowicz et al., 2009). ROS and RNS can affect the redox status of proteins, for example, through cysteine modifications, in biotic or abiotic stresses (Mhamdi, 2019). Cysteine oxidations are found in the enzymes 1-aminocyclopropane-1-carboxylic acid oxidase (ACO; ethylene) and 12-oxophytodienoic acid reductase 3 (OPR3; JA; McConnell et al., 2019; Pattyn et al., 2020), but the importance for wound response needs further investigation.

Classically, MAPK phosphorylation cascades, notably WIPK and SIPK in tobacco and homologs MPK3 and MPK6 in *Arabidopsis*, are activated at timescales between accumulation of Ca^{2+} (faster) and ROS (slower) with a maximum at 15 min after wounding (Seo et al., 1995, 1999; Usami et al., 1995; Bögre et al., 1997; Ichimura et al., 2000). Activation of upstream kinases include MEKK1 and MEK1 phosphorylating MKK2 and MPK4 in *Arabidopsis* (Matsuoka et al., 2002; Hadiarto et al., 2006), which can be reverted by the action of PP2C-type phosphatases (Schweighofer et al., 2007). Wound-induced MPK8 activity is detected within 10 min and is peculiar in the sense that both MKK3 phosphorylation and Ca^{2+}-dependent calmodulin binding is required for full activation (Takahashi et al., 2011). Once activated, MPK8 controls the redox balance by negative regulation of *RBOHD* gene expression. Downstream of the wound-activated MKK4/MKK5-MPK3/MPK6 cascade and CPK5/CKP6 phosphorylation is the upregulation of ethylene biosynthesis genes and ethylene accumulation (Li et al., 2018). Intriguingly, next to the classical fast activation of MAPK

cascades, a later activation controlled by JA-induced MAP3Ks expression and a cascade involving MKK3 phosphorylation of MPK1/2/7 can be observed with a maximum at 1 h after mechanical and herbivore-induced damage (Ortiz-Masia et al., 2007; Sözen et al., 2020).

Plasmodesmata are plasma membrane-lined pores that connect the cytoplasm of neighboring cells that allow cell-to-cell exchange of molecules, and the regulation thereof plays important roles in signaling of stresses, including pathogen defense and wounding (Jacobs et al., 2003; Cheval and Faulkner, 2018). Locally elevated levels of Ca^{2+} and ROS will lead to rapid closure of the plasmodesmata within seconds to minutes (Holdaway-Clarke et al., 2000; Cui and Lee, 2016; Xu et al., 2017). Deposition of callose, which is mostly Ca^{2+}-dependent (Kauss et al., 1983; Leijon et al., 2018), will further "seal the deal" in prolonged closing of plasmodesmata and restricting access from the wound to intact tissues (Jacobs et al., 2003; Wu et al., 2018). In systemic signaling, plasmodesmata could be important for cell-to-cell movement of molecules or continuity of membranes and coupling of electrical signals (Cheval and Faulkner, 2018). Similarly, sieve plates of the phloem can be rapidly closed within minutes to prevent leakage of nutrients and assimilates by the deposition of callose (Mullendore et al., 2010). In Fabaceae, specialized proteinaceous structures called forisomes expand upon the binding of Ca^{2+} was released during wounding to block the sieve plate pores (Knoblauch and Van Bel, 1998). Interestingly, unidentified Ca^{2+}-binding proteins in aphid (*Megoura viciae*) watery saliva, which they inject in the phloem, can chelate Ca^{2+} and leave sieve elements unblocked for uninterrupted aphid feeding in Fabaceae (Will et al., 2007). Cytosolic Ca^{2+} elevations during aphid feeding can be observed in species that lack forisomes, such as *Arabidopsis*, so Ca^{2+} chelation by aphid saliva is likely a more general phenomenon (Vincent et al., 2017).

Systemic Wound Tides: Hydraulic Waves, Electric Torrents, and Ca^{2+} Fluxes

More than a century ago, the existence of long-distance signals of unknown nature that is able to propagate signals throughout the plant and travel through the vascular bundle was already hypothesized (Burdon-Sanderson, 1873; Ricca, 1916; Stahlberg, 2006). In recent years, significant strides have been made in understanding these systemic signals (Davies, 2006; Stahlberg et al., 2006; Vodeneev et al., 2015; Farmer et al., 2020), which can be attributed mainly to (1) very rapid changes in hydraulic pressure and (2) slower propagation of electric, ROS, and Ca^{2+} signals, and enigmatic xylem-born chemical elicitor-dubbed Ricca's factor (Ricca, 1916; **Figure 2A**). In parallel to vascular signaling, signals can be released from plants in volatile forms that may activate defense in the same plant's distal parts or in other plants in the neighborhood (Kessler and Baldwin, 2001). Volatile signals are addressed in these recent reviews (Bouwmeester et al., 2019; Ninkovic et al., 2019).

Wounding can cause a direct loss of the water content of plants and in many occasions can disrupt the plant vasculature, which has a direct effect on the turgor pressure of plant epidermal cells (Malone and Stanković, 1991). Changes in the hydraulic components were proposed to be part of the systemic damage signal that takes advantage of the organ interconnectivity of the vasculature (Malone and Stanković, 1991; Boari and Malone, 1993). Another measure of hydraulic signals, found in common in different species including wheat, tomato, soybean, faba bean, and others, is a change in leaf thickness in neighboring leaves of a damaged leaf (Boari and Malone, 1993). Changes in turgor pressure and leaf thickness are likely caused by the retraction of water through the vascular system in a pressure wave that travels the rigid xylem vessels (Malone and Stanković, 1991; Stahlberg and Cosgrove, 1992, 1995). Although the results showed differences on the magnitude of the reaction across species and capacity of responsiveness, the data obtained for leaf thickness starts within seconds and peaks around 1–4 min, lasting about 1 h or longer. Hydraulic signals propagate at an estimated speed of 10–20 $cm{\cdot}s^{-1}$, meaning that rupture of the water continuity by wounding can have relatively direct repercussions on distant locations (Malone, 1992; Boari and Malone, 1993). At present, the study of hydraulic changes during wounding is rather unexplored, likely due to the absence of tools that allow efficient detection of changes on pressure over short periods of time at distant locations. A recent study detailed the use of a non-invasive method using optical methods that measures the changes of the diffraction patterns associated to stem displacement after flaming injury (Nožková et al., 2018).

Relatively better studied are the electrical signals, which are based on changes in the membrane potentials (depolarization or hyperpolarization followed by repolarization) and were recently reviewed in Farmer et al. (2020). At least four types of electrical signals elicited by damage are reported in the literature: wound potential, action potential, slow wave potential (also named variation potential), and systems potential, each displaying different characteristics (Davies, 2006; Stahlberg et al., 2006; Zimmermann et al., 2009, 2016; Farmer et al., 2020). Wound potential depolarization spreads locally around the damaged area (<40 mm or about the length of 200 epidermal cells in cucumber hypocotyls; Stahlberg et al., 2006). While probably sharing molecular mechanisms with systemic electrical signals, such as inhibition of P-type H+ pumps (Stahlberg et al., 2006), wound potentials are technically not considered as systemic signals. Action potentials, slow wave potentials, and systems potentials spread to distal parts of the plant with the main difference that slow wave potentials are driven by hydraulic or chemical changes, as they can travel across killed or poisoned areas (Stahlberg and Cosgrove, 1992). The slow part in slow wave potential reflects a delayed repolarization, and slow wave potentials are dampened in amplitude in more distal parts of the plant. On the other hand, action potentials are characterized by their all-or-none depolarization traveling without attenuation (Favre and Agosti, 2007; Cuin et al., 2018). Systems potentials are mainly different to the aforementioned signals in that they are hyperpolarized instead of depolarized (Zimmermann et al., 2009).

Earlier studies of electrical signals, similar to hydraulic signals, were mainly performed using harsh damaging treatments, such as flaming. More recently, subtle mechanical or herbivore induced wounds were also found to induce electrical signals, likely containing mixed forms of wound

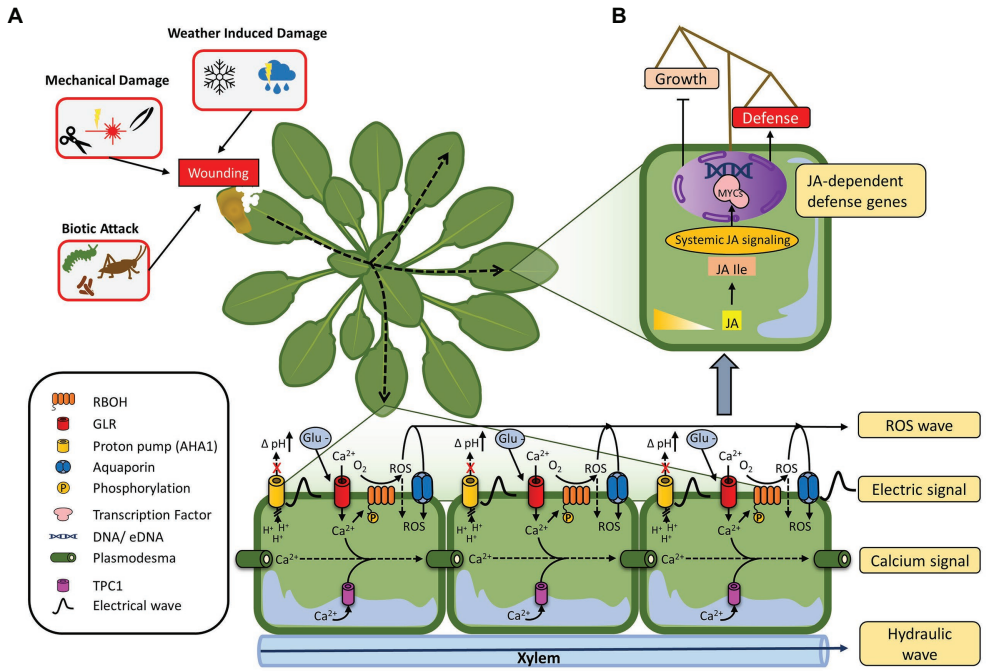

FIGURE 2 | Schematic representation of systemic response to wounding. **(A)** Different origins of wounding, including biotic attack (herbivore and pathogens), mechanical damage (cutting and laser induced), and weather induced damage (freezing and hail). Depending on severity of the wound, propagation of systemic signals ensues. Local changes in membrane potentials, increases in cytosolic Ca^{2+}, and ROS accumulation generate a wave that quickly spreads throughout the plant, in order to reach distant tissues. **(B)** Systemic-induced jasmonic acid (JA) continues to promote JA-dependent defense genes in distant tissues, leading to a systemic growth/defense trade-off to promote plant fitness.

potentials, action potentials, slow wave potentials, and systems potentials (Salvador-Recatalà et al., 2014; Zimmermann et al., 2016). Observations of different electrical signals can be very heterogenous and depend on several factors including (1) severity of the damage, for example, flaming triggering strong hydraulic waves and slow wave potentials and herbivores triggering action potentials and systems potentials, (2) the readout method of choice, for example, stomata impaled-pierced, agar-pierced, or blindly pierced electrodes or aphids as living bioelectrodes, and (3) place of recording, which mainly relates to abundance and interplay of signals from multiple vascular strands (Salvador-Recatalà et al., 2014; Zimmermann et al., 2016).

Identification of clade 3 glutamate receptor-like (GLR) genes, *Arabidopsis* H + -ATPases (AHAs), and RBOHs that shape or propagate the systemic signals illustrate the intertwining of electrical signals with Ca^{2+} and ROS waves and their impact on the downstream activation of JA synthesis (Koo and Howe, 2009; Mousavi et al., 2013; Gilroy et al., 2016; Nguyen et al., 2018; Toyota et al., 2018; Farmer et al., 2020). Mousavi et al. (2013) identified two GLRs, homologs of mammalian ionotropic glutamate receptors (iGluRs), for which double homozygous mutants have reduced wound-induced systemic membrane depolarization, and changes in JA marker gene expression. While electric signals do not propagate to neighboring leaves in the *glr3.3 glr3.6* mutant, signals in the (local) wounded leaf are unaffected (Mousavi et al., 2013; Salvador-Recatalà et al., 2014; Salvador-Recatalà, 2016), leading to the conclusion that GLR3.3 and GLR3.6 are "gatekeepers" of systemic electric signals. Interestingly, loss-of-function of a third GLR, *glr3.5*, leads to systemic electric signals in non-neighboring leaves, where usually no signals are detected, indicating that GLR3.5 acts as an off-switch (Salvador-Recatalà, 2016). Whereas GLRs are involved in propagation of slow wave potentials, *in vitro* activation of CNGC19 by hyperpolarization hints at its involvement in systems potential propagation (Meena et al., 2019).

Wounds generated by mechanical damage results in the increase of apoplastic glutamate concentration ([Glu]apo) of ~50 mM within minutes at the damage site, suggesting that [Glu]apo can act as a DAMP (Toyota et al., 2018). Glutamate, among other amino acids, are specifically perceived in plants through GLRs (Qi et al., 2006; Toyota et al., 2018; Alfieri et al., 2020; Shao et al., 2020). GLRs are calcium-permeable channels and thereby mediate the influx of cytosolic Ca^{2+} within seconds after the damage (Vincill et al., 2012; Mousavi et al., 2013; Nguyen et al., 2018; Toyota et al., 2018). Similar to slow wave potentials, systemic Ca^{2+} waves are observed following wounding (Kiep et al., 2015) and did not spread to neighboring intact leaves in the *glr3.3 glr3.6* mutant (Toyota et al., 2018), showing that electrical and Ca^{2+} signals are closely interacting through GLRs (Nguyen et al., 2018). In *Arabidopsis* systemic leaves, slow wave potentials seem to precede peak Ca^{2+} signals (Nguyen et al., 2018).

Slow wave potentials travel through the vasculature toward the center of the rosette and then disperse away from the

apex into a restricted number of parastichy leaves to initiate distal JA accumulation and signaling (Mousavi et al., 2013; Nguyen et al., 2018; Farmer et al., 2020; **Figure 2B**). The measured speed of leaf-to-leaf electrical signal was observed in the ~2 cm/min range, which is concordant with estimates of signal speeds of JA accumulation measured in leaf-to-leaf wounding studies (Chauvin et al., 2013; Mousavi et al., 2013). The signal spreads across tissues by GLRs through the phloem and xylem vascular tissues, especially when major veins are damaged (Salvador-Recatalà et al., 2014; Nguyen et al., 2018; Toyota et al., 2018). Minutes following slow wave potentials, JA is locally and systemically synthesized leading to the activation of the transcriptional JA responses (Mousavi et al., 2013). Proton pumps were long expected to take part in the return of membrane potential back to its initial status (repolarization), but the genetic evidence was lacking (Stahlberg and Cosgrove, 1996; Fleurat-Lessard et al., 1997). Kumari et al. (2019) recently found that repolarization in the *Arabidopsis* proton pump H + -ATPASE 1 (AHA1) deficient plants took longer compared to wild type, indicating a role for AHA1 in resetting the plant for sensing new stimuli. Additionally, *aha1* mutants have higher total JA accumulation and JAZ10 expression and reduced levels of herbivory (Kumari et al., 2019), which is the opposite in *glr3.3 glr3.6* plants (Mousavi et al., 2013; Nguyen et al., 2018). Recently as well, Shao et al. (2020) provided evidence that higher pH, such as during wound-induced apoplast alkalization, greatly enhances the binding of glutamate to GLR3.3 and GRL3.6. They further confirmed the effect of AHA1 on slow wave potentials. Taking in consideration theoretical models and experimental work that predict chemical agents transported by xylem mass flow or sheer-enhanced dispersion propagate slow wave potentials, as opposed to pressure waves (too fast) or chemical diffusion (too slow; Vodeneev et al., 2012; Evans and Morris, 2017; Blyth and Morris, 2019), [Glu]apo might well be (part of) the ideal candidate(s) for Ricca's long-sought chemical factor(s) that propagate the slow wave potential (Ricca, 1916). Sudden changes in the negative and positive pressure of xylem and phloem, respectively, followed by osmotic re-equilibration, might help to pull in [Glu]apo or other chemical elicitors in the vasculature (Farmer et al., 2020).

In parallel with systemic electrical signals, Ca^{2+} and ROS waves are induced by wounding, among other stresses, and depend on RBOHs (Miller et al., 2009; Choi W.-G. et al., 2014; Kiep et al., 2015; Evans et al., 2016; Choi et al., 2017; Toyota et al., 2018). In systemic tissues, mechanical damage and H_2O_2 inducible gene expression overlap considerably more than any other purportedly ROS-induced transcripts, including O_2^- and singlet oxygen (Miller et al., 2009). One of these H_2O_2-inducible genes is zinc finger of *Arabidopsis thaliana*12 (ZAT12). *ZAT12* expression, using luciferase reporter lines, is induced strongly within 10 min after wounding in the local leaf, while it spreads systemically at 8.4 cm/min to full expression within the hour and both are impaired in an *rbohd* mutant (Miller et al., 2009). New ways of visualizing ROS will improve the further study of systemic signaling in species other than *Arabidopsis*, including crops (Fichman et al., 2019; Lew et al., 2020). ROS waves can be inhibited by the Ca^{2+} channel blocker lanthanum ($La3^+$; Miller et al., 2009). Next to the N-terminal Ca^{2+}-binding EF-hand motif (Suzuki et al., 2011), RBOHD activity is regulated through phosphorylation at its N-terminus by the calcium dependent kinase CPK5 upon elicitation with flg22, a bacterial flagellin peptide and elicitor of innate immunity (Suzuki et al., 2011; Dubiella et al., 2013). Wound-induced Ca^{2+} waves are suppressed in loss-of-function mutants of the vacuolar cation channel two pore channel 1 (TPC1), whereas local Ca^{2+} elevation was largely unaffected (Kiep et al., 2015). RBOHD can interact with different partners involved in immune response such as the receptor kinases EFR and FLS2, and botrytis-induced kinase1 (BIK1; Laluk et al., 2011; Kadota et al., 2014). Furthermore, cysteine rich receptor-like kinase 2 (CRK2) controls flg22-induced H_2O_2 production through direct interaction with RBOHD and phosphorylation of its cytosolic C-terminus (Kimura et al., 2020). Whether these interactions are important for systemic wound signaling warrants investigation. A unifying concept of molecular mechanisms underpinning wound-induced systemic signals is within reach (Gilroy et al., 2016; Farmer et al., 2020) but will require the discovery of additional genetic players.

WOUND-INDUCED HORMONE SIGNALING

Upon wounding, several hormones, including JA, ethylene, ABA, auxin, and their respective cross-talks, are indispensable for damage perception and eliciting key downstream responses.

First on the Scene: Jasmonic Acid Signaling

Jasmonic acid is a phytohormone involved in many aspects of plant stress responses and development. Probably the most renowned is the regulation of mechanical wounding and immune responses against herbivores or necrotrophic pathogens, which trigger the biosynthesis of JA and of its bioactive form jasmonoyl-L-isoleucine (JA-Ile) not only at the damage site but also systemically in unharmed tissues (Glauser et al., 2008; Koo and Howe, 2009; Goossens et al., 2016). JA biosynthesis begins with release of α-linolenic acid from chloroplast membrane phospholipids, which is then converted into cis-(+)12-oxo-phytodienoic acid (OPDA) through the sequential action of chloroplast-located enzymes, such as the 13-lipoxygenases (13-LOX; Wasternack and Feussner, 2018). OPDA is then exported from the chloroplast by JASSY, a protein localized to the outer chloroplast envelope (Guan et al., 2019), and transported into the peroxisomes, presumably by the ABC transporter Comatose (AtABCD1/CTS) and acyl-CoA-binding protein 6 (ACBP6; Theodoulou et al., 2005; Ye et al., 2016). Once in the peroxisome, OPDA is reduced by OPDA reductases 2 and 3 (OPR2 and OPR3) and subsequently oxidized through two distinct pathways to form JA (Schaller and Stintzi, 2009; Chini et al., 2018). The bioactive molecule JA-Ile is synthetized by the JA resistant 1 (JAR1) enzyme

and transported to the nucleus within minutes after plant damage (Suza and Staswick, 2008). Nuclear transport of JA-Ile is mediated by the jasmonate transporter 1 (JAT1), a member of the ABC transporter family known to transport small molecules such as auxins, abscisic acid, or strigolactones (Li et al., 2017). In the nucleus, JA-Ile is perceived by a co-receptor complex composed of the JA ZIM-domain (JAZ) repressor proteins and the coronatine insensitive 1 (COI1) F-box protein that associates with CUL1, Rbx1, and the Skp1-like proteins ASK1 and ASK2 to assemble the SCF-COI1 ubiquitin-ligase complex (Thines et al., 2007; Fonseca et al., 2009; Sheard et al., 2010; Williams et al., 2019). Hormone perception requires a JAZ degron that bridges COI1 to JA-Ile (Sheard et al., 2010). In addition, inositol pentakisphosphate ($InsP_5$) was identified as a critical structural component of the receptor complex (Sheard et al., 2010). Plants with increased InsP5 showed accentuated wounding responses, suggesting that InsP5 contributes to the assembly and function of the SCF-COI1 complex (Mosblech et al., 2011). Following JA-Ile binding, the SCF-COI1 complex ubiquitinates the JAZs, which targets them for proteasomal degradation. Thereby, several transcription factors (TFs), such as the MYCs that are otherwise bound by the JAZ proteins, are released and can activate the JA response (Chini et al., 2007; Fonseca et al., 2009; Goossens et al., 2016). JA-Ile perception and signaling leads to the systemic alteration of a growth-defense balance to promote plant fitness (Wasternack and Feussner, 2018). One of the most characteristic features of JA is the transcriptional reprograming of a wide array of enzymes leading to production of specialized metabolites, including terpenes, glucosinolates, phenolics, or alkaloids (Pauwels et al., 2008; Hickman et al., 2017; Zander et al., 2020).

On the contrary, JA represses signaling pathways that lead to plant growth to reallocate resources toward defense (Hou et al., 2010; Major et al., 2017; Guo et al., 2018). It was shown that a growth penalty is restored to different extents in moderate (*jazQ*) or in severe (*jazD*) JAZ depleted mutants by the introgression of a *phytochrome B* (*phyB*) mutation, which was explained by the fact that JA and phyB transcriptional networks are uncoupled (Campos et al., 2016; Major et al., 2020). Interestingly, these findings show that the JA regulated growth-defense trade-off is not merely directed by the need of relocating metabolic resources, which opens interesting leads for plant improvement for agricultural or industrial purposes. Because of the importance in tuning the growth-defense balance, JA and growth promoting pathways are cross-regulated through different pathways in response to changing environments. DELLA proteins are plant growth repressors whose degradation is promoted by gibberellins (Davière and Achard, 2016). DELLAs have been reported to interact with JAZs to thereby compete with MYC2 and, thus, modulate JA responses (Hou et al., 2010; Wild et al., 2012; Leone et al., 2014). However, the importance of DELLAs in the cross-regulation of the JA pathway has recently been challenged by a study that shows no major role of DELLAs in restricting shoot growth of jaz mutants (Major et al., 2020). Wounding dramatically modifies the growth-to-defense balance, resulting in stunted vegetative growth effects being directly linked to the activation of JA synthesis (Yan et al., 2007).

A key function of JAs produced in damaged organs is to travel systemically across tissues in order to reprogram future growth and optimize plant defense strategies (Huot et al., 2014; Guo et al., 2018; Ballaré and Austin, 2019). Upon damage, plants tightly regulate biosynthesis, transport, and catabolism of JAs (Browse, 2009; Chini et al., 2016; Howe et al., 2018; Fernández-Milmanda et al., 2020; Yang et al., 2020). JA biosynthesis in *Arabidopsis* depends on LOX2, LOX3, LOX4, and LOX6. Each of these LOXs contribute in a different way to regulate JA biosynthesis and transport upon wounding (Chauvin et al., 2013, 2016; Grebner et al., 2013; Yang et al., 2020). *LOX2* is expressed throughout soft aerial tissues, whereas *LOX3, LOX4*, and *LOX6* are expressed in the phloem and xylem of leaves (Chauvin et al., 2013, 2016). *LOX2* is highly induced in the close vicinity of wounds in cotyledons and is necessary to ensure leaf to root axial JA transport (Gasperini et al., 2015). Upon wounding, LOX6 participates in the radial export of JAs from the leaf vasculature to the blade (Gasperini et al., 2015). It was recently suggested that LOX3 and LOX4 repress leaf growth upon wounding by acting on stem cell populations that determine the rate of leaf primordia development (Yang et al., 2020). Furthermore, the activity of LOX3 and LOX4 in leaf growth is related to the vacuolar cation channel TPC1 through a mechanism that remains unclear (Bonaventure et al., 2007; Yang et al., 2020).

The aforementioned studies together with the discovery of GLR-aided electrical signaling reveal that wounded leaves rely on at least two kinds of JA-dependent mechanisms to alert distal organs, being different whether the signal translates from leaf-to-leaf or from leaf-to-root (Mousavi et al., 2013; Gasperini et al., 2015; Schulze et al., 2019). Shoot wounding not only activates electrical signals but also triggers relocation of JA-Ile precursors, tentatively OPDA, OPC-4, OPC-6, OPC-8, and JA from wounded shoots toward undamaged roots (Schulze et al., 2019). Mobile OPDA and its derivatives activate JA signaling through their conversion into JA-Ile at the distal sites, and while leaf-to-leaf signaling relies on electrical and hormone translocation mechanisms, and leaf-to-root signaling seems to exclusively rely on hormone (precursor) translocation (Schulze et al., 2019). In complement to these studies, the development of the fluorescent biosensor Jas9-VENUS allowed visualization of the dynamic distribution of JA-Ile in wounded plants (Larrieu et al., 2015). Cotyledon wounding generated a distal increase of JA-Ile through vascular tissues of the root following two distinct temporal dynamics. The first phase started with a rapid increase of distant JA-Ile propagating at a speed <1 cm/min, few minutes after wounding, then a second slower phase that started 30 min and lasted for at least 90 min (Larrieu et al., 2015). The nature behind these phases needs further investigation to be conciliated with latter results, suggesting that leaf-to-root signaling exclusively relies on hormone translocation, which is likely a slower process than the initial observed phase.

Although glutamate was characterized as triggering rapid slow wave potentials resulting in the activation of the JA pathway, little is known about additional DAMPs triggering

distant and/or local JA signaling. A large set of cell wall-related DAMPs have been characterized for triggering wound responses; however, despite the fact that JA is one of the most well-characterized phytohormonal pathways activating wound responses, mechanisms clearly connecting cell wall perception to JA are missing (Mielke and Gasperini, 2019; Bacete and Hamann, 2020). Exogenous application of cell wall degrading enzymes or the cell wall fragments OGs (DP10–DP15) or xyloglucans (Xh) results in the activation of JA signaling (de Azevedo Souza et al., 2017; Claverie et al., 2018; Engelsdorf et al., 2018). Xh elicited resistance against the necrotrophic pathogen *Botrytis cinerea* was abolished in JA biosynthesis (*dde2*) and signaling (*coi1-40*) mutants, suggesting the specific activation of the JA pathway by Xh (Claverie et al., 2018). In *Nicotiana attenuata*, the combination of wounding with the fatty acid conjugates N-linolenoyl-l-Gln, N-linolenoyl-l-Glu, N-linoleoyl-l-Gln, and N-linoleoyl-l-Glu strongly activated JA biosynthesis and subsequent herbivore defense responses (Wu et al., 2007). Future studies should address how cell wall disruption leads to local JA signals and if they connect to systemic responses. In this respect, *Arabidopsis* mutants of the xylem-specific cellulose synthases, irregular xylem 3 and 5 (*irx3* and *irx5*), severely affect the shape and speed of slow wave potentials; however, *JAZ10* expression in systemic leaves, as a measure of JA signaling, is only slightly affected (Kurenda et al., 2019).

Likewise, the molecular events operating downstream of the Ca^{2+} influx, preceding the rapid biosynthesis and redistribution of JAs are hardly understood. Phosphorylation is postulated to be one of the major cellular modes of action for translating defined Ca^{2+} signatures into specific downstream reactions (Dodd et al., 2010; Yip Delormel and Boudsocq, 2019). Several existing lines of evidence point to the importance of Ca^{2+}/phosphorylation for JA signaling in the context of wound responses. Furthermore, Ca^{2+} signaling has been repeatedly hypothesized as a mechanism preceding JA signaling, which suggests that Ca^{2+} signals may not only relate to GLRs but also to other alternative pathways activating JA signaling (Kenton et al., 1999; Bonaventure et al., 2007; Scholz et al., 2014; Lenglet et al., 2017). The JA-associated VQ motif 1 (JAV1) protein associates in a complex with JAZ8-WRKY51 to represses the expression of JA biosynthesis genes. Wounding or insect attack activate a Ca^{2+}Calmodulin dependent pathway that phosphorylates JAV1, leading to its degradation to thereby activating transcription of JA biosynthesis genes (Yan et al., 2018). Beyond the potential importance of phosphorylation for Ca^{2+} induced JA biosynthesis, it was recently shown that wounding triggers JA signaling in the stomata through the activity of the Ca^{2+} receptor kinase complex CBL1-CIPK5 (Förster et al., 2019). Furthermore, a recent study showed that the rice homolog of brassinosteroid insensitive 2 (BIN2), OsGSK2 kinase, phosphorylates OsJAZ4 to promote its degradation in a COI1-dependent manner, thereby posing a new mechanism of growth-defense regulation (He et al., 2020). Additionally, wound-activated MAPK signaling mechanisms have been reported to regulate the JA pathway (Wu et al., 2007; De Boer et al., 2011). WIPK and SIPK regulate wound responses including JA biosynthesis in Solanaceae species. In *N. attenuata*, leaf wounding together with the herbivore oral secretion treatment elicits strong SIPK and WIPK activities resulting in the biosynthesis of JA, SA, and JA-Ile/JA-Leu conjugates and ethylene biosynthesis. The SIPK and WIPK activate the transcription of defense related genes in both wounded and unwounded regions of the local leaf but not in systemic adjacent leaves (Wu et al., 2007). In tobacco, the JA-factor stimulating MAPKK1 (JAM1) protein regulates transactivating activities of the ORC1 and MYC transcription factors in a JA dependent manner (De Boer et al., 2011). Altogether, this evidence underscores that phosphorylation is an important post-translational modification in the regulation of plant wound responses and JA signaling; however, to date, these mechanisms have only been explored to a limited extent.

Rather Late Than Never: Accumulation of Ethylene, ABA, and Auxin During Wound Response

Ethylene has many roles in plant development and stress response (Pattyn et al., 2020), including fruit ripening, where inhibition is a critical target for improved storage (shelf-life) of fruit and vegetables post-harvest (Barry and Giovannoni, 2007; Saltveit, 2016). Wound-induced ethylene accumulation is thought to proceed *via* transcriptional upregulation of its rate-limiting biosynthetic enzyme 1-aminocyclopropane-l-carboxylate (ACC) synthase (ACS) resulting in a lag-time of 20–30 min before the first accumulation of ethylene and a peak within hours after wounding (Boller and Kende, 1980; Kato et al., 2000; Marhavý et al., 2019). Ethylene production depends on both ROS and Ca^{2+} increases (Marhavý et al., 2019) and is transduced by MAPK and CDPK-dependent phosphorylation for activation of *ACS* gene expression locally at wound sites (Wu et al., 2007; Li et al., 2018; Sözen et al., 2020). Possible involvement of DAMPs cannot be ruled out, as Peps induce the accumulation of ethylene within 5 h after exogenous peptide application (Bartels et al., 2013). Furthermore, electrical signaling might lead to systemic increases of ethylene production in distal leaves (Dziubinska et al., 2003; Tran et al., 2018; Farmer et al., 2020). In the young root meristem, JA has been shown to be involved in transmitting the single cell damage signal (Zhou et al., 2019), whereas in older non-dividing root cells, a predominant role for ethylene has been demonstrated (Marhavý et al., 2019). Here, death of a single cell, through laser ablation or during the early stages of nematode infection, causes a distinct ethylene-dominated response (Marhavý et al., 2019).

Abscisic acid (ABA) accumulation is doubled within 24 h after wounding and induces, among other cues, the expression of the *proteinase inhibitor II* gene in potato and tomato (Pēna-Cortes et al., 1989; Peña-Cortés et al., 1995; Dammann et al., 1997). Arguably, ABA is best known for its role in drought-induced stomatal closure (Cardoso and McAdam, 2019). Therefore, it should come as no surprise that ABA accumulation likely depends on the level of humidity during wounding. As

a case in point, *Arabidopsis* plants accumulate ROS normally and develop wound induced resistance (WIR) to the fungus *Botrytis cinerea* in high humidity (L'Haridon et al., 2011). However, keeping plants 1.5 h in dry conditions after wounding, reduces ROS, WIR, and callose accumulation, which is linked to enhanced accumulation of ABA and is reversed in ABA biosynthetic enzyme deficient mutants *aba2* and *aba3* (L'Haridon et al., 2011). In this study, ABA biosynthesis genes are induced 15 min after wounding only in the dry condition. Probably, differences in experimental set-ups, therefore, fail to detect changes in wound-induced ABA accumulation and gene transcription (Ikeuchi et al., 2017). Interestingly, an application of exogenous ABA leads to enhanced local cell death surrounding wound sites in *Arabidopsis*, and the transcription factor botrytis sensitive1/MYB108 (BOS1/MYB108) is a negative regulator of this ABA-dependent cell death (Cui et al., 2013). Mutant *bos1* plants display runaway cell death after wounding, which interacts with ABA, cuticle permeability, and resistance to *B. cinerea* (Cui et al., 2013, 2016, 2019).

Accumulation of auxin at wound sites mainly has a role in the subsequent repair process that bridges or protects wounds and regeneration of lost tissue. Tissue reunion following incision or upon grafting requires reactivation of cell division, not so much to generate callus, but rather to bridge the cut and allow reconnection of the vascular tissue. Upon incision of the inflorescence stem, the NAC-type transcription factor NAC071 and ERF113 are activated in order to assist in the reunion process (Asahina et al., 2011). On the one hand, ERF113, an AP2/ERF-type transcription factor, is rapidly induced within 1 day following incision at the bottom part of the cut site in a JA-dependent manner. On the other hand, NAC071 is induced in the top part of the incision between 1 and 3 days as a result of auxin accumulation, and both TFs execute different functions in the reunion process (Asahina et al., 2011). Auxin response during grafting is symmetric between top and bottom of the adjoined graft junction and occurs within 12 h, consistent with earlier reports of auxin-induced transcription at 1–3 days after cutting (Yin et al., 2012; Melnyk et al., 2015, 2018; Matsuoka et al., 2016). Upon full excision of the leaf between the blade and petiole, callus is generated very locally at the cut site and an adventitious root can sprout within 8 days following excision (Liu et al., 2014). Auxin accumulates within a day at the wound site and directly activates expression of the *WUSCHEL related homeobox 11* (*WOX11*) transcription factor, which works redundantly with WOX12 to enable the transition of the local cambium cells to root founder cells within 4 days following the cut (Liu et al., 2014; Hu and Xu, 2016). Accumulating evidence from recent publications on root regeneration emphasizes the importance of auxin during the replenishment of a single cell, a cluster of damaged root cells, or even regeneration of a complete *de novo* root tip (Canher et al., 2020; Hoermayer et al., 2020; Matosevich et al., 2020). Depending on the severity and type of damage, the mode of action that allows for sufficient auxin accumulation in order to facilitate the regeneration process varies. Upon death of a single cell, for example, following laser ablation, a strictly localized auxin signaling, independent of biosynthesis or active transport, coordinates the wounding response (Hoermayer et al., 2020). Upon death of a group of vascular stem cells, for example, by bleomycin-induced DNA damage, the natural auxin flow is disrupted through downregulation of auxin transporters, resulting in an auxin redistribution, much alike rocks in a stream. However, similar to single cell death, no auxin biosynthesis could be observed during the recovery from vascular stem cell death (Canher et al., 2020). However, following full root tip excision, YUCCA9-dependent auxin biosynthesis was found to be indispensable to allow regeneration of a *de novo* tip (Matosevich et al., 2020). Among the key regeneration-related and wound-responsive transcription factors, several members of the AP2/ERF-type of transcription factors can be found, including ERF115, wound-induced dedifferentiation 1 (WIND1) and several plethora (PLT) members (Delessert et al., 2004; Iwase et al., 2011; Ikeuchi et al., 2013; Heyman et al., 2016). Although originally identified as a rate-limiting factor controlling stress-induced quiescent cell divisions, *ERF115* represents an important wound-responsive gene (Heyman et al., 2013, 2016). Being the death of a single cell, stem cell damage or even removal of the entire root tip, *ERF115* expression is instantly activated within 1–2 h in the adjacent cells and plays a key role in stimulating these cells to activate the cell division program (Heyman et al., 2016; Zhang et al., 2016). Although not being the initial trigger, auxin is required to maintain *ERF115* expression following tissue damage (Canher et al., 2020; Hoermayer et al., 2020), leaving the question open about the initial trigger activating this key regeneration granting factor following wounding.

FUTURE PERSPECTIVES

The field has come a long way since the first observations and descriptions of plant wound response more than a century ago (Burdon-Sanderson, 1873; Ricca, 1916; Bloch, 1941; Lipetz, 1970). Notwithstanding detailed molecular knowledge gathered in the last decades on several aspects, major areas of study are still largely unexplored. Keeping in the spatiotemporal spirit of the review, some of these areas can be defined from local to systemic and fast to slow.

What is the fate of damaged cells in the wound and are they actively involved in determining the outcome of the wound response? This is exemplified by the activation of metacaspases and maturation of Peps in damaged cells (Hander et al., 2019), which shows that "post-mortem" cells can still be active (Bollhöner et al., 2013). Furthermore, what are the chain of events that proceed in the dying cells bordering the damaged cells as observed in leafs (Cui et al., 2013; Iakimova and Woltering, 2018), is there a point of no return and how does it change the wound response in the neighboring tissue? While more DAMPs are being discovered, possible mechanisms that are in place to avoid unwanted or exaggerated wound response by maturation, possible controlled release, and turnover of DAMPs become important. Furthermore, are there different

dynamics of DAMP release, for example, fast elevation of eATP and [Glu]apo (Song et al., 2006; Toyota et al., 2018), compared to potential slow release of OGs due to upregulation of polygalacturonases after wounding (Bergey et al., 1999)? Some studies have detailed the release of DAMPs after wounding (**Table 1**) or extrapolate from studies in animal model species. However, most DAMPs in plants have not been directly measured in the apoplast or vasculature during wounding, while there is an abundance of indirect measurements (e.g., exogenous application). To fully understand the dynamics of DAMP release and its impact on wound response, direct measurements are needed in the future.

In this review, we had to limit ourselves to reports dealing with wounding. Certainly, molecular components that are increasingly found in other abiotic or biotic stresses for local and systemic signaling likely play roles as well in wounding. As an illustration, ROS-mediated activation of Ca^{2+} channels by the receptor kinase HPCA1 (Wu et al., 2020) or mechanisms that have been described for systemic signaling by stresses other than wounding (Gilroy et al., 2016; Szechyńska-Hebda et al., 2017; Farmer et al., 2020). Local implications and responses of cells to wounding change in different tissues. For example, mesophyll cells are differently connected as xylem or phloem cells that form conduits. Disruption of tissue integrity will therefore have different repercussions, which is obvious in the slow-down of electrical, Ca^{2+}, and ROS waves when they move from vasculature to inner tissues (Salvador-Recatalà et al., 2014; Evans et al., 2016; Toyota et al., 2018). Similarly, non-dividing full-grown cells and tissues will have different needs than expanding tissues and meristem cells, which are more plastic and essential to replace. The differences in these tissue-specific wound responses are only starting to be addressed (Hoermayer and Friml, 2019; Li T. et al., 2019; Marhava et al., 2019; Marhavý et al., 2019; Zhou et al., 2019).

Although such complex problems are difficult to predict (Lehmann et al., 2020), detailed knowledge on plant wound response will become even more needed as weather- and herbivore-induced damage is projected to increase with climate change (Deutsch et al., 2018). Development of new techniques for investigating wound response, such as MecWorm (Mithöfer et al., 2005) and SpitWorm that adds oral secretion to simulated herbivore-induced damage (Li G. et al., 2019), or laser-mediated wounding (Hoermayer and Friml, 2019; Marhavý et al., 2019) will help advance the field. Application of this newfound knowledge has the ability to improve grafting, regeneration, and crop production (Santamaria et al., 2013; Si et al., 2018; Coppola et al., 2019; Notaguchi et al., 2020; Zhang and Gleason, 2020).

AUTHOR CONTRIBUTIONS

IV-M, DD-F, and AF-F made the table and figures. IV-M and DD-F wrote the part on DAMPS. AF-F and SS wrote the part on local and systemic wound signaling. AR, JH, and SS wrote the part on hormones. All authors made comments on the manuscript, which were integrated by SS. All authors contributed to the article and approved the submitted version.

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frontiers in Plant Science

REVIEW
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The Role of DNA in the Extracellular Environment: A Focus on NETs, RETs and Biofilms

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The capacity to actively release genetic material into the extracellular environment has been reported for bacteria, archaea, fungi, and in general, for microbial communities, but it is also described in the context of multicellular organisms, animals and plants. This material is often present in matrices that locate outside the cells. Extracellular matrices have important roles in defense response and disease in microbes, animal and plants cells, appearing as barrier against pathogen invasion or for their recognition. Specifically, neutrophils extracellular traps (NETs) in animals and root extracellular traps (RETs) in plants, are recognized to be important players in immunity. A growing amount of evidence revealed that the extracellular DNA, in these contexts, plays an active role in the defense action. Moreover, the protective role of extracellular DNA against antimicrobials and mechanical stress also appears to be confirmed in bacterial biofilms. In parallel, recent efforts highlighted different roles of self (homologous) and non-self (heterologous) extracellular DNA, paving the way to discussions on its role as a "Damage-associated molecular pattern" (DAMP). We here provide an evolutionary overview on extracellular DNA in extracellular matrices like RETs, NETs, and microbial biofilms, discussing on its roles and inferring on possible novel functionalities.

Keywords: self-DNA, exDNA, extracellular matrix, self-DNA inhibitory effect, exDNA as a DAMP

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INTRODUCTION

The presence of extracellular materials, organized as extracellular matrix (ECM), glycocalyx, or mucus layers, has been described in both vertebrates (Huxley-Jones et al., 2007; Möckl, 2020) and invertebrates (Har-el and Tanzer, 1993; Schröder and Bosch, 2016) as well as in plants (Driouich et al., 2013) and microorganisms (Flemming and Wingender, 2010). Despite the specific components may vary between clades or species, also in dependence of the specific cell types, the extracellular organization mostly shares gel-like structures mainly composed of glycoproteins, proteoglycans, and glycolipids (Theocharis et al., 2016).

The extracellular structures may fulfill relevant roles in terms of structure and functional organization, contributing to fundamental processes like cell adhesion, migration, proliferation, differentiation, and apoptosis. They can act as protective barriers in preventing pathogen invasion, or represent advantageous habitats to facilitate symbiotic interactions, for example favoring adhesion of microbial communities (Yue, 2014; Schröder and Bosch, 2016; Möckl, 2020).

In vertebrates, among interesting examples of specialized ECM production, there are those from cell types like fibroblasts, contributing to the organization of the connective tissue, chondrocytes, producing cartilage, and osteoblasts, producing the bone matrix (Alberts et al., 2002). Noticeable, also neutrophils, which are terminally differentiated killer cells in vertebrates (Dale et al., 2008) and invertebrates (Jenne et al., 2018), essential for both the innate and acquired immune systems (Mantovani et al., 2011; Rosales et al., 2017), are known to form specialized extracellular organization in terms of web-like structures, that are called neutrophil extracellular traps (NETs), and appear to have a relevant protective role against pathogens.

Similar structure organizations have been also described in plants. For example, the root extracellular traps (RETs), by analogy with the NETs, are identified as high molecular weight compounds surrounding the plant root cap. They are mostly composed by carbohydrates, and are produced by the root border cells, playing a crucial role in plant defense (Hawes et al., 2011, 2016; Driouich et al., 2013).

The presence of extracellular structures has been extensively described also in the microbial world, in microalgae, fungi, bacteria, and archaea. These structures are generally associated with biofilm formations. Indeed, biofilms are defined as an agglomerate of microorganisms hold in a self-produced ECM. Biofilm formation allows single cell microorganisms to acquire a temporary multicellular lifestyle, facilitating survival in specific conditions, or under specific environmental changes (e.g., levels of oxygen and/or carbon; Monds and O'Toole, 2009; Kostakioti et al., 2013), with possible roles in the increase of microbial fitness and protection, as examples, from predation, desiccation, starvation, and exposure to antimicrobials (O'Toole, 2003).

We here propose an overview of the current knowledge on the role of extracellular DNA (exDNA) in extracellular matrices like NETs, RETs, and biofilms, highlighting specificity and conserved traits among different clades. The role of exDNA in these matrices is also discussed in relationship with the evidence of the inhibitory role of conspecific exDNA on cell growth (Mazzoleni et al., 2015a,b), thus suggesting possible additional functions for DNA in extracellular matrices.

Extracellular DNA

One of the current definitions of exDNA is "... located outside the cell and originating from intracellular DNA by active or passive extrusion mechanisms or by cell lysis" (Ceccherini et al., 2009).

Extracellular DNA is abundant in many habitats, including soil, sediments, oceans, and freshwater as well as the intercellular milieu of metazoan (Nagler et al., 2018). In all these contexts, the exDNA results from either cell lysis or active release, and can be found in both the double and single stranded, as well as more or less fragmented forms (Levy-Booth et al., 2007; Ceccherini et al., 2009; Thierry et al., 2016; Nagler et al., 2018). The fate of exDNA may include biotic degradation (mainly due to ubiquitous extracellular and cell-associated DNases) and abiotic (physical and chemical) decay, as well as environmental long-term preservation and possible incorporation by microbial cells or other living beings via horizontal gene transfer (HGT; Levy-Booth et al., 2007; Nielsen et al., 2007; Torti et al., 2015). Interestingly, the released DNA may also become part of extracellular structures, such as NETs (Brinkmann et al., 2004), RETs (Driouich et al., 2013), and biofilms (Whitchurch et al., 2002).

The presence of exDNA in the *Pseudomonas aeruginosa* biofilm was demonstrated in 2002, by Whitchurch et al. (2002), whose experiments highlighted the structural role of DNA in the establishment and development of the bacterial biofilm. In 2004 the presence of both chromatin and DNA (Brinkmann et al., 2004) was confirmed also in the context of NETs. Finally, in plant slime surrounding roots, later called RET (Driouich et al., 2013), the presence of the histone H4 was revealed in 2007 (Wen et al., 2007b) and, 2 years later, the co-presence of DNA macromolecules was demonstrated too. Thus, DNA resulted to be an essential structural component of the ECM in plants (Wen et al., 2009a).

ExDNA as a DAMP

DNA in extracellular environment has often been discussed for its contribution to "Damage-associated molecular patterns" (DAMPs), also known as "danger-associated molecular patterns," i.e., as a molecule of endogenous origin that, if present in the inappropriate compartment, is recognized as a self-damage and can initiate and perpetuate a non-infectious inflammatory response (Seong and Matzinger, 2004; Roh and Sohn, 2018). Indeed, after being released from damaged or dying cells, DAMPs may activate the innate immune system by interacting with pattern recognition receptors (PRRs; Roh and Sohn, 2018).

In animals, self-DNA of nuclear or mitochondrial origin is frequently reported to act as a DAMP and to determine various types of diseases. For instance, extracellular self-DNA is associated to several diseases and/or to their severity, like in cancers (Hawes et al., 2015), hypertension (McCarthy et al., 2015), and Parkinson and Alzheimer diseases (Lowes et al., 2020). Self-DNA is also considered to be involved in autoimmune diseases such as in rheumatoid arthritis (Rykova et al., 2017), in systemic lupus erythematosus (Barrat et al., 2005), and in other autoimmune diseases (Vakrakou et al., 2018).

In plants, it is well established that non-self-DNA (heterologous, i.e., DNA from phylogenetically unrelated species, or, more in general, distant in sequence similarity terms) of bacterial origin, triggers immunological responses with the formation of reactive oxygen species (ROS) and callose deposition (Yakushiji et al., 2009). Moreover, recent studies clearly indicated that the self-exDNA has specific effects in plants. In 2015, Mazzoleni et al. (2015a) reported evidence that fragmented exDNA accumulating in litter during the decomposition process, produces a concentration dependent, species-specific inhibitory effect, reducing root growth and seed germination of conspecifics. They highlighted for the first time that the exposure to fragmented self-DNA inhibits root growth in plants, while non-self-DNA does not trigger similar effects (Mazzoleni et al., 2015a). The authors suggested that the inhibitory effect could depend on the sequence similarity of the plant DNA with the one representing the fragmented molecules, since the toxic effect was also evident, although to

a lower extent, when exposing plants to decomposing litters of phylogenetically similar plants. These studies paved the way to further investigations on possible novel roles of exDNA in ecology, plant physiology, and in translational research. Indeed, in 2016, Barbero and colleagues demonstrated that the treatment with fragmented self-DNA triggers specific early immune signaling responses in plants. Indeed, the authors showed that fragments of self-DNA, and not of non-self-DNA, induced intracellular calcium signaling and plasma membrane depolarization in *Phaseolus lunatus* and *Zea mays* (Barbero et al., 2016). Furthermore, in 2018, Duran-Flores and Heil demonstrated that in *Phaseolus vulgaris*, the exposure to self-DNA inhibits seed germination and triggers H_2O_2 production, mitogen-activated protein kinase (MAPK) activation, extrafloral nectar release (typical of the defensive response to herbivores) in combination with a decreased susceptibility to infection by the bacterium *Pseudomonas syringae* (Duran-Flores and Heil, 2018). In 2018, Vega-Muñoz et al. (2018) suggested that the response to self- and non-self-DNA could depend on the degree of self damage detected by the plant, confirming that, in line with previous findings (Mazzoleni et al., 2015a,b; Barbero et al., 2016; Duran-Flores and Heil, 2018), this could depend on the concentration of either self-DNA or non-self-DNA and on the phylogenetically distance of non-self-DNA. Vega-Muñoz et al. (2018) also suggested that the exDNA methylation patterns could explain the mechanism for self-DNA recognition in plants.

However, despite these evidences, the mechanisms behind the differential response of plants to self- and non-self-DNA remains still unclear, to our knowledge.

Bacteria can detect foreign DNA and thus activate specific responses, as it will be discussed later, however, the role of exDNA as a DAMP has never been proposed in bacteria.

The discovery of Mazzoleni et al. (2015a) was also extended by the same authors to different organisms other than plants, including microbes, fungi, protozoa, and insects (Mazzoleni et al., 2015b). Noticeable, these studies demonstrated that the toxic effect due to exposure to self-DNA (conspecific or similar/homologous) in plants is a general phenomenon, that appear to be a typical response in all species in all kingdoms, paving the way to further studies that could address the role and the molecular mechanisms involved in self-exDNA sensing.

ExDNA Sensing

The exDNA has been demonstrated to be sensed in animals by receptors located in various cellular compartments, such as the nucleus (Brázda et al., 2012; Wang et al., 2019), the cytoplasm (Hornung et al., 2009; Herzner et al., 2015; Szczesny et al., 2018), and the endosomes (Hemmi et al., 2000).

The distinction between self and non-self DNA is also a relevant aspect to carefully consider when describing crucial processes related to the detection of exogenous DNA components. For example, the specific recognition of unmethylated CpG-rich DNA in the endosomal compartment is ascribed to the TLR9 receptor (Barton et al., 2006). CpG methylation patterns are typically underrepresented in bacteria genomes and this allows their fragments to be detected by the host. Interestingly, the underrepresentation of CpG methylation is also typical in the mitochondrial DNA (mtDNA), and its erroneous recognition as a foreign molecule can give rise to inflammatory and autoimmune pathological responses in animals (Barrat et al., 2005; Zhang et al., 2010). In the cytoplasm, the receptor cGAS is able to bind DNA in a sequence-independent manner, and preferentially binding long dsDNA or short dsDNA with unpaired open ends containing guanosines (Y-form DNA), that are primarily found in viral genomes (Herzner et al., 2015), thus favoring the recognition of non-self DNA sequences when present.

In plants, no specific DNA receptor has been reported yet. Nevertheless, it is suggested that the exposure to both self- and non-self-DNA induces an immunological response (Duran-Flores and Heil, 2015; Heil and Vega-Muñoz, 2019). It has been suggested that the recognition of exDNA in plants could involve a membrane-bound exDNA receptor that, upon recognition, triggers a downstream signaling cascade, or a membrane-bound exDNA transporter or channel, and/or a vesicle-mediated internalization that, after the exDNA internalization, could favor the detection via an intracellular sensor (Bhat and Ryu, 2016). The sensing of exDNA molecules has also been ascribed to mechanisms similar to the "well-known processes of interference, based on sequence-specific recognition of small-sized nucleotide molecules" (Mazzoleni et al., 2015a), that could justify the specific inhibitory roles of extracellular self-DNA. Some plant membrane proteins are considered good candidates as exDNA receptors (Bhat and Ryu, 2016). During infection, plants release defense proteins [pathogen-related (PR) proteins] in the extracellular environment. Certain PR proteins, such as Vpr10.1 and GaPR10, show RNase and DNase activities *in vitro*, and have a putative adenosine triphosphate (ATP)-binding domain (Xu et al., 2014). For their activities, they are considered potential candidates intercepting exDNA and/or extracellular RNA outside the cell. Interestingly, the treatment of Arabidopsis with dsRNA leads to impairment in a pathogen associated molecular pattern (PAMP)-triggered immunity response (Niehl et al., 2016). The membrane-bound SERK1 was suggested to be the potential dsRNA receptor in this process. Moreover, a transcriptome analysis of plants treated with bacterial RNA revealed over expression of ribonuclease (RNS)-1 (Lee et al., 2016). RNS1 is a member of the T(2) family of RNS proteins that are typically expressed in response to wounding in *Arabidopsis thaliana*, and this process is independent from the activation of the jasmonic acid and abscisic acid pathways, which are typical elicited by wound response. In addition, plant PRRs recognize danger signals both from self-damage and/or non-self-organisms (Medzhitov and Janeway, 2000; Seong and Matzinger, 2004). Among PRRs, surface-localized proteins, characterized by leucine-rich repeats (LLRs) motifs, have been proposed as putative exDNA receptors (Heil and Land, 2014). Nevertheless, all these evidences need additional investigations to further elucidate candidates exDNA receptors, their structure, and roles (Gallucci and Maffei, 2017).

In bacteria, the perception and recognition of exogenous DNA also occurs. In order to recognize foreign DNA, such as the viral genomes, bacteria may recognize differential patterns in DNA structure. Usually unmethylated or differently methylated DNA

of exogenous DNA are recognized through the DNA restriction-modification system (Wilson and Murray, 1991) and/or by the CRISPR–Cas systems (Dupuis et al., 2013; Shah et al., 2013; van der Oost et al., 2014). *Cis* elements as the *Chi* sequences may be recognized by the RecBCD recombination system, and may characterize the bacterial DNA because of their higher frequency and their absence in phages (Vasu and Nagaraja, 2013). In addition to the above-mentioned defense systems, bacteria can also keep track of invasive elements by specific transcriptional silencing of horizontally acquired genes or prophages recombined with their own genome through the recognition of different compositional patterns, such as the higher A-T contents in foreign molecules, and thus silencing them by the binding of repressor proteins (i.e., the heat-stable nucleoid-structuring protein (Navarre et al., 2007) or through the action of transcription termination factor (i.e., Rho protein; Cardinale et al., 2008). Interestingly, during the transformation process, the DNA uptake in most systems appears not to be sequence-specific. However, in some Gram-negative bacteria, such as *Haemophilus influenzae* (Sisco and Smith, 1979; Elkins et al., 1991) and *Neisseria species* (Danner et al., 1980; Fitzmaurice et al., 1984; Goodman and Scocca, 1988), the DNA uptake is more efficient if specific sequences called DUS (DNA uptake sequences), are present. As the genomes of these bacteria are enriched in their respective DUS (Smith et al., 1995; Parkhill et al., 2000; Tettelin et al., 2000), the uptake of self DNA is favored. Nevertheless, specific DUS receptors on the bacterial surface have not yet been identified (Chen and Dubnau, 2004).

DNA receptors have also been identified in bacteria. In gram-positive bacteria, such as *Bacillus subtilis* and *Streptococcus pneumoniae*, the DNA-binding protein ComEA is considered a DNA receptor (Inamine and Dubnau, 1995; Bergé et al., 2002). In gram-negative bacteria, such as in *Neisseria gonorrhoeae*, orthologs of the protein ComEA contain the DNA-binding domain (Chen and Gotschlich, 2001) and, presumably, may have the same role. Recently, the protein ComH has been identified in the gram negative bacteria *Helicobacter pylori* as a periplasmic DNA-binding protein, that interacts with the periplasmic domain of the inner membrane translocator ComEC to transfer the DNA into the cytoplasm (Damke et al., 2019).

NETs

Extracellular traps produced by eosinophils, mast cells, macrophages, and neutrophils are extracellular components in animals that have been demonstrated to contain DNA in their structure organization (Goldmann and Medina, 2012). Among these, NETs that are produced by neutrophils, are the most studied extracellular traps (**Figure 1**). Neutrophil cells, designed as heterophils in birds, reptiles, and some mammals, are the most abundant granulocytes (Montali, 1988), representing from 40%, up to 70%, of all white blood cells in humans. They are also present in higher invertebrates in the form of primordial neutrophils (Jenne et al., 2018), where they play active roles in the process of phagocytosis, but also share the ability to form clotting of haemolymph with platelets.

Mature neutrophils are released from bone marrow into the bloodstream (Mayadas et al., 2014), and represent the first line of defense against the invading microbes (de Bont et al., 2019). Indeed, they kill microbes by releasing proteases that favor their engulfment by macrophages, through phagocytosis, activating the immune system (Sofoluwe et al., 2019).

Neutrophils extracellular trapsosis is the process by which the NET formation occurs. This process was described for the first time in 2004 by Brinkmann et al. (2004). It consists in the expulsion of DNA, proteases, and antimicrobial peptides into the extracellular space (Sofoluwe et al., 2019). In particular, the induction of NETosis activates the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex that produces superoxide anions. Superoxide anions are converted into hydrogen peroxide, which is a substrate of the myeloperoxidase (MPO) that induces the release of the neutrophil elastase (NE) from neutrophil granules. NE and MPO migrate to the nucleus, where they induce histone degradation decondensing chromatin structure. Moreover, NE degrades actin filaments inhibiting neutrophils movement (Fuchs et al., 2007; Papayannopoulos et al., 2010; Metzler et al., 2011, 2014; Palmer et al., 2012; de Bont et al., 2019). Therefore NETs composition consists in proteins from primary, secondary, and tertiary neutrophils granules, MPO, NE, DNA, and histones (H1, H2A, H2B, H3, and H4), in addition with cathepsin G, lactoferrin, gelatinase, as initially revealed by Brinkmann et al. (2004).

DNA in NETs can be composed either by nuclear or mitochondrial DNA. In 2009, Yousefi et al. (2009) showed that, in specific conditions, NETs could be formed from pure mtDNA, and this was not accompanied by NETosis since neutrophils remained viable. In 2014, McIlroy et al. (2014) demonstrated that NETs could be released also after injury and orthopedic trauma surgery.

NETs influence the humoral innate immunity by producing part of the complement factors cascade (de Bont et al., 2019), which consists in more than 30 proteins mostly produced by the liver. They are activated by a sequence of proteolytic cleavages ending with the formation of a pore on the pathogen cell membrane that, losing its integrity, determines pathogen death (Sarma and Ward, 2011; Janeway et al., 2017). Interestingly, complement activation by NET formation is strongly decreased by DNase I (de Bont et al., 2019). Furthermore, NETs can also act as a scaffold for clot formation, highlighting novel insights on the role of neutrophils and NETosis in coagulation-mediated diseases (de Bont et al., 2019).

Neutrophils extracellular traps are structures including active molecules with strong intermolecular bindings, preventing their diffusion into neighboring tissues. *In vivo*, NETs are degraded by DNases and removed by macrophages (Hakkim et al., 2010; Farrera and Fadeel, 2013; Jiménez-Alcázar et al., 2017; de Bont et al., 2019). These are essential steps that follow NETs formation and are required for NETs clearance. Remarkably, in 2017, Jiménez-Alcázar et al. (2017) demonstrated that DNase 1 and DNase 3 are essential for NET clearance, and that mice deficient in DNase 1 and DNase 3 die few days after neutrophil activation, because of blood vessels occlusion caused by persistent NET structures (Jiménez-Alcázar et al., 2017).

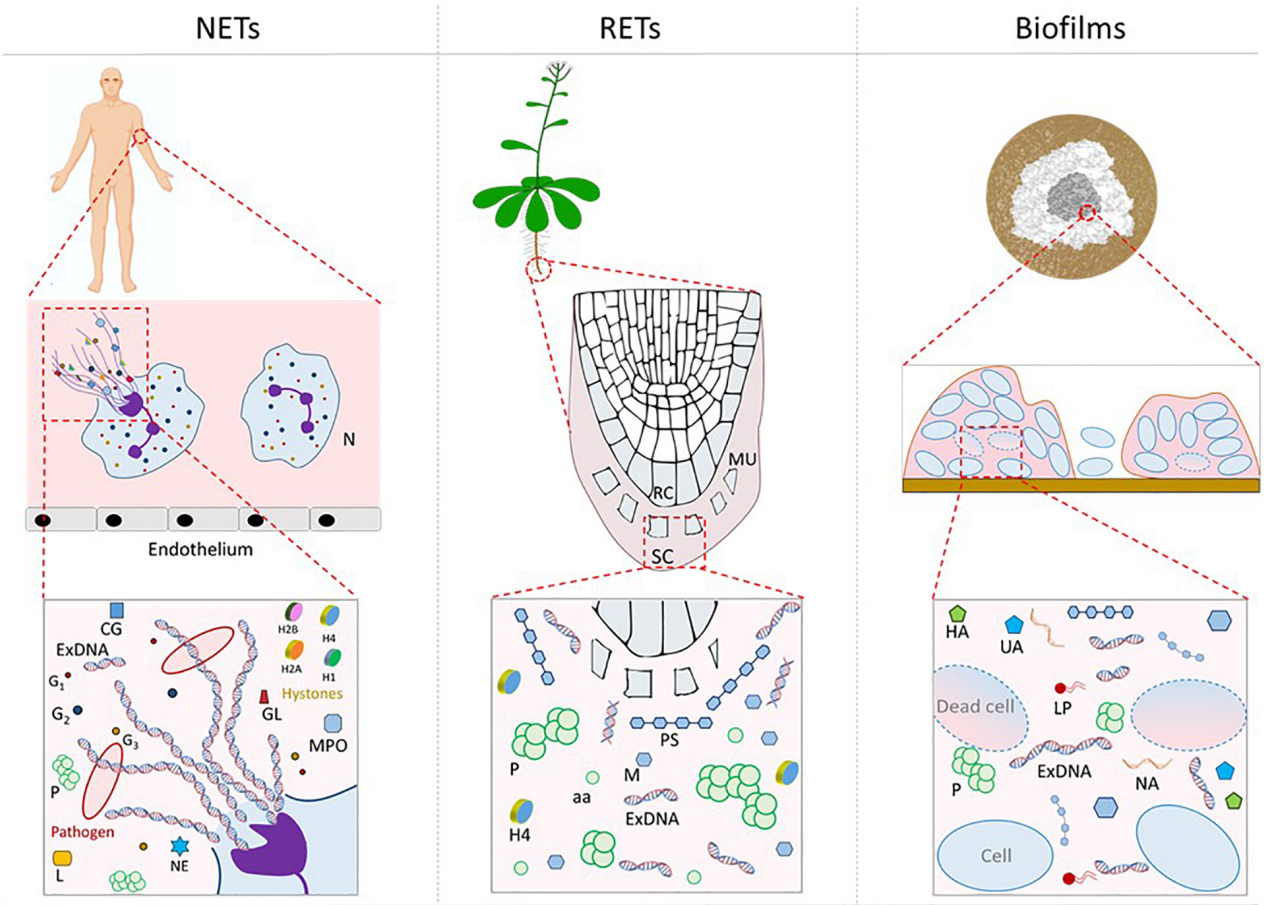

FIGURE 1 | Schematic representation of NETs (animals), RETs (plants), and biofilms (microbial communities) structures. NETs: N, neutrophil; MPO, myeloperoxidase; NE, neutrophil elastase; Gn, neutrophil granules; H1, H2A, HAB, H4, histones; CG, cathepsin G; L, lactoferrin; GL, gelatinase; RETs: RC, root cap cells; MU, mucilage; SC, sloughed cells; aa, aminoacids; Biofilms: NA, nucleic acids; LP, lipids, UA, uronic acid; HA, humic acid; ExDNA, extracellular DNA; M, monosaccharides; PS, polysaccharides; and P, proteins.

Moreover, Savchenko et al. (2014) in their studies on the role of innate immune cells in the early response to myocardial ischemia/reperfusion injury, demonstrated that myocardial injury caused an increase in nucleosomes, neutrophil infiltration, and histone H3 at the site of injury. Treatment with DNase improved cardiac contractile function to a similar degree in both wild type and PAD4-/- deficient mice, which do not produce NETs. This suggested that DNA fragments contribute to cardiomyocyte dysfunction irrespective of NETs, possibly by acting as DAMPs (Savchenko et al., 2014; Shah et al., 2020). Confirming its positive role against NET-mediated pathologies, DNase 1 has been proposed as an enzyme able to attenuate them in mice (Németh et al., 2020). Consistent with the observation in mice, the persistence of NETs can have serious negative consequences in humans, leading to pathologies such as cardiovascular, lung and eye diseases, atherosclerosis, rheumatoid arthritis, thrombosis, diabetes, cancer, and severe COVID-19 (Demers et al., 2012; Arazna et al., 2013; Brinkmann, 2018; Daniel et al., 2019; Erpenbeck et al., 2019; Leppkes et al., 2020).

In 2020, Neumann et al. (2020) traced the evolutionary presence of NET like structures, organized by the extrusion of decondensed chromatin and additional intracellular material, in different phyla: Chordata, Arthropoda, Mollusca, Cnidaria, and the Plantae kingdom included. However, the functional role of their presence is still questionable, as also commented by the authors themselves: "can organisms from other life kingdoms use a similar mechanism as defense strategies against their foes?" (citation by Neumann et al., 2020).

Notwithstanding the great interest and relevant roles of NETs, their release mechanisms are not fully understood and some aspects of the process still remain unclear (Manda-Handzlik et al., 2019). In addition, the structure and possible functional roles of exDNA in NETs organization is still matter of investigation.

RETs

Plant roots provide water and nutrients to the whole plant body. They show a peculiar organization that is also determined

by specific assemblages of extracellular materials, mainly represented by root mucilages. In the external part of the root apex, adjacent to the apical meristem, the plant root cap represents a dynamic and multifunctional tissue. This tissue is extremely resistant to both biotic and abiotic stimuli, in contrast with the internal, highly proliferating, tissue that represents the root elongation zone. The peculiar resistance of the root cap tissue depends on the presence of root border cells at the cap periphery. These cells, in most plant species, separate from the cap as a metabolically active population of cells, that is released into the rhizosphere as free cells or in clump (Brigham et al., 1998; Gunawardena and Hawes, 2002; Wen et al., 2007b; Hawes et al., 2016). Originally, border cells were defined as those cells that are released into suspension by a brief immersion of the root tip into water (Brigham et al., 1995). Proteomic and gene expression profiling studies revealed that these cells are different from their progenitors in the root cap, although they share similarities across diverse plant species (Brigham et al., 1995; Wen et al., 2007b, 2009b). Root border cells were previously referred as "sloughed root cap cells," since they were thought to be a product of tissue disintegration. Subsequently, they were termed "border cells" to emphasize that they are viable after the detaching from the root cap and that they are a specialized tissue, morphologically and physiologically different from the root cap cells (Hawes et al., 2016).

It has been shown that root cap cells and border cells are able to secrete the root mucilage, the high molecular weight sticky matrix that surrounds the plant root cap, through an active continuous process, that piles up materials outside the root (**Figure 1**). The number of border cells and their secretion into the rhizosphere can vary according to many factors [water availability, soil type, physical abrasion, day length, root age, growth rate, the amount of carbon dioxide, of aluminum, of boron, plant pathogens, the altered expression of genes controlling cell cycle or cell wall solubilization at the cap periphery (Hawes et al., 2012)]. Moreover, root mucilage formation also contributes to the whole root network asset, starting from an initial structure surrounding the root cap (Driouich et al., 2019), and in relationships with growth conditions, that determine the root mucilage secretion (Hawes et al., 2012).

The root mucilage is mostly composed by both mono and polysaccharides (mainly galactose, glucose, arabinose, fucose, and xylose), proteins (e.g., proteases, peroxidases, plant defense-related proteins, such as defensins, well-known to be also relevant components of the plant cell wall and of the apoplast) and amino acids. Interestingly, the root mucilage was also revealed to be formed by known intracellular markers, such as histone H4 (Wen et al., 2007b; Weiller et al., 2017). Together with the histone H4, the presence of DNA in the root mucilage was also reported (Wen et al., 2009a). Other molecules could be also part of root mucilage (Vincent et al., 2020).

Plant exDNA in RETs was initially thought to be derived by leakage from dead cells (Levy-Booth et al., 2007). However, currently, there is no observation demonstrating that exDNA in RETs is released by lytic processes (Driouich et al., 2019). Indeed, it has been recently demonstrated that newly synthesized DNA is actively exported into the ECM by vital root cap cells, even if the leakage of nuclear content from dead cells cannot be excluded (Wen et al., 2009a). Once released, the exDNA forms distinctive structures, similar to those produced by neutrophils (Patel et al., 2010; Pilsczek et al., 2010). In addition, initial analyses revealed that the exDNA in RETs is mainly represented by nuclear DNA enriched in repetitive sequences (Hawes et al., 2012) and, moreover, to date there is not yet evidence of presence of mtDNA sequences in these structures (Driouich et al., 2019).

In 1942, Rogers and his colleagues advanced the hypothesis that the border cells producing the root mucilage may represent an "extra-root" digestive system (Rogers et al., 1942), that functions as an exoenzyme system releasing substances, like phosphatases, into the rhizosphere (Driouich et al., 2019). This putative function could resemble the well-known extracellular digestive activity before substrate absorption in fungi, during organic matter decomposition processes (Jennings, 1995; Cole, 1996). Many other roles were associated with the root mucilage, such as: lubricant protecting the root tips while growing into the soil (Greenland, 1979); carrier of gravitropic signals from the root cap to the root tip (Moore et al., 1990); protection of roots from the toxicity of ions such as copper, cadmium, boron, lead, mercury, iron, arsenic, aluminum (Mench et al., 1987; Hawes et al., 2016), or as carbon source for soil microbes (Knee et al., 2001).

The root cap secretion represents a primary site in the root that is colonized by microbial symbionts and pathogens that are present in the rhizosphere. On one hand, it is well known that the rhizosphere sheet surrounding the fine roots is a complex ecosystem, representing the habitat of specific microbial communities interacting with the plant, including bacteria and mycorrhizal organisms in both symbiotic and mutualistic relationships with the root (Lambers et al., 2009; McNear Jr, 2013). On the other hand, similarly to NETs in animals, diverse plant pathogens interact with border cells, which appear to act as a trap against microorganisms, forming aggregates and inhibiting pathogen growth. It has been proposed that the root slime works by "trapping" pathogens to protect the root tip meristem, whose function is critical to root development and plant survival and with a structure that does not show specific resistance to biotic or abiotic stress (Whipps, 2001; Raaijmakers et al., 2009).

Interestingly, the extracellular trapping phenomenon is host-microbe specific, with no aggregation or growth inhibition of non-pathogenic organisms (Jaroszuk-Ściseł et al., 2009). The chemotaxis and the binding of host–specific microbes (bacteria, as well as nematodes, zoospores, and fungi), along the plant cell wall and on the structures of the mucilage secreted by border cells, are always followed by quiescence of the pathogen population (Wen et al., 2017).

All the constituents of the RETs play an important role in the host defense against pathogens. For example, the importance of the involved proteins was documented by the fact that, when the roots are treated with proteases at the time of inoculation with spores of a pathogenic fungus, the normal resistance to root tip infection is abolished (Wen et al., 2007b). Treatment with proteases also results in the disintegration of the surrounding mucilage layer with the subsequent release of bacteria within

the layer (Wen et al., 2007a). This evidence suggests that proteins may play a role in the structural integrity of the matrix (Matsuyama et al., 1999), even though they comprise only a small fraction of the matrix composition, which is mainly composed by carbohydrates (Bacic et al., 1986; Moody et al., 1988; Chaboud and Rougier, 1990; Hawes et al., 2012).

Furthermore, the degradation of exDNA results in loss of root tip resistance to infection. When DNase 1 is added at the time of pathogen inoculation, 100% of root tips becomes necrotic within 48–72 h (Hawes et al., 2011). Moreover, the inactivation of extracellular DNases in the plant pathogen *Ralstonia solanacearum* reduces the virulence, showing that the infection is related to the pathogen capability of dissolving the structural organization of the extracellular trap, thus reducing its protection function (Tran et al., 2016; Wen et al., 2017).

Despite the reported evidence, mechanisms of RETs formation and root mucilage depositions, as well as those promoting the DNA release by the border cells into the extracellular space, still need to be completely elucidated.

BIOFILMs

It is known that the microbial world can appear organized in specific structures composed of sessile cells encased by an ECM, which are called biofilms (**Figure 1**).

The first observation of microbial biofilms was made by Antonie van Leeuwenhoek, in 1684, when he found aggregates of different microbes colonizing his own teeth and tongue (Dobell, 1932). Later, Pasteur observed that aggregates of microbes allowed the fermentation of wine into vinegar (Pasteur, 1864). In the following years, researchers lost interest in biofilms until 1985 (Høiby, 2017), when it was demonstrated the increase in the antimicrobial resistance of biofilm-enclosed bacteria compared to the planktonic counterparts (Nickel et al., 1985). Since then, the interest in biofilm research enormously increased, also because the biofilm life-style was recognized to be the most common mode of growth and survival of microbial species in the environment, with huge implications in ecology, industry, biotechnology, and clinics.

The biofilm formation is a reversible process in which cells can return to planktonic life-style if perturbed by hydrodynamic and repulsive forces, or as a consequence of nutrient depletion (Donlan, 2002). Biofilm development is determined by both intrinsic and environmental factors and consists of different stages. It starts from single cells on a surface showing a stochastic distribution (Kostakioti et al., 2013; Armbruster and Parsek, 2018). On the surface, cells encounter attractive or repelling forces depending on environmental conditions, such as nutrient availability, ionic strength, pH, and temperature. These factors affect the velocity and the direction toward or away from the contact surface (Donlan, 2002). Once microorganisms adhere to the surface, the attachment becomes stable, cells start multiplication and secretion of the ECM, that is also named extracellular polymeric substance (EPS; Flemming and Wingender, 2010). This process leads to the formation of micro-colonies (Costerton et al., 1999). The biofilm architecture can favor different processes such as the exchange of nutrients, the distribution of metabolic products, and of signaling molecules (Jamal et al., 2018). Microbial cells communicate with each other through auto-inducer signals during biofilm maturation, which affect the microbial cell density (Davies et al., 1998; Costerton et al., 1999; Federle and Bassler, 2003). During the maturation, the EPS becomes essential for the biofilm three-dimensional structure organization and for the survival of the micro-colonies. In fact, interstitial channels are embedded in the EPS acting as a circulatory system that favors the distribution of nutrients and the removal of waste products (Jamal et al., 2018). The final stage in the biofilm life cycle includes the production and release of dispersal cells which switch from sessile into motile forms. They leave the original microcolonies and can colonize new surfaces to initiate the surface-association phase of the biofilm formation (McDougald et al., 2012).

The EPS of a biofilm may differ depending on the species, but it is generally composed by several molecules such as carbohydrates, lipids, proteins, and nucleic acids, including DNA (Zhang et al., 1998; Flemming and Wingender, 2010; Kassinger and van Hoek, 2020) as well as by pili, flagella, humic, and uronic acids, which are all considered essential components of the biofilm organization (Nielsen et al., 1996). Extracellular carbohydrates in the biofilm matrix can trap micronutrients, enhance the attachment to the surface and biofilm formation (Harrison et al., 2007; Kassinger and van Hoek, 2020). Extracellular vesicles have also been found in the biofilm matrix of different microbial species, contributing to its lipidic and protein content (Schooling et al., 2009; Zarnowski et al., 2018; Kassinger and van Hoek, 2020). Nevertheless, the role of all these components in the biofilm organization is still under investigation.

Beyond its structural and functional role during cell adhesion and biofilm development, the extracellular DNA has never been discussed for its role as a DAMP in bacteria, also in the context of biofilm formation.

ROLE OF DNA IN THE NETs, RETs, AND BIOFILMs

The presence of exDNA in NET, RET, and biofilm drives the attention on its structure and functional roles in each of the specific contexts (**Table 1**).

DNA in NETs

In NETs, the DNA has been shown to provide a major contribution to the antimicrobial activity. Indeed, it possesses the ability to sequester surface bound cations, disrupt membrane integrity, and lyse bacterial cells (Halverson et al., 2015). The DNA antimicrobial property is determined by its direct contact with the bacterial membrane and by the phosphodiester backbone that is required for the cation chelation (Baums and von Köckritz-Blickwede, 2015). In fact, it has been demonstrated that treatment of NETs with an excess of cations or phosphatase enzyme, and exogenous or secreted microbial DNAses, protects pathogens from the NET antibacterial action (Baums and von

TABLE 1 | Main roles of extracellular DNA in NETs, RETs, and Biofilms and associated bibliographic references confirming (Confirmed), hypothesizing (Hypothesis) the specific role or not available (n/a).

	Extracellular structure		
ExDNA role	**NET**	**RET**	**Biofilm**
Structure	Confirmed[1,2]	Confirmed[3–6]	Confirmed[7–24]
Defense	Confirmed[1,2,25–27]	Confirmed[3,28–31]	Confirmed[32,33]
Pathogen trap	Confirmed[1,2,25,26]	Confirmed[28–31]	n/a
Autotoxicity	Confirmed[2,27,34–43]	Hypothesis[44–48]	Hypothesis[45]
Source of genetic information	n/a	n/a	Confirmed[49–51]
Source of inorganic phosphate	n/a	Confirmed[52]	n/a

Numbers are associated to the corresponding bibliographic references as follows: [1]Brinkmann et al. (2004), [2]Yousefi et al. (2009), [3]Wen et al. (2009a), [4]Hawes et al. (2012), [5]Patel et al. (2010), [6]Pilsczek et al. (2010), [7]Kassinger and van Hoek (2020), [8]Zhang et al. (1998), [9]Ascher et al. (2009), [10]Flemming and Wingender (2010), [11]Flemming et al. (2016), [12]Decho and Gutierrez (2017), [13]Abada and Segev (2018), [14]Mann et al. (2009), [15]Whitchurch et al. (2002), [16]Harmsen et al. (2010), [17]Gödeke et al. (2011), [18]Nguyen and Burrows (2014), [19]Das et al. (2010), [20]Seviour et al. (2019), [21]Gloag et al. (2013), [22]Peterson et al. (2013), [23]Payne and Boles (2016), [24]Gallo et al. (2015), [25]Halverson et al. (2015), [26]Baums and von Köckritz-Blickwede (2015), [27]Tsourouktsoglou et al. (2020), [28]Tran et al. (2016), [29]Driouich et al. (2019), [30]Plancot et al. (2013), [31]Hawes et al. (2011), [32]Chiang et al. (2013), [33]Mulcahy et al. (2008), [34]Savchenko et al. (2014), [35]Shah et al. (2020), [36]Krysko et al. (2011), [37]Demers et al. (2012), [38]Brinkmann (2018), [39]Daniel et al. (2019), [40]Erpenbeck et al. (2019), [41]Leppkes et al. (2020), [42]Arazna et al. (2013), [43]Wang et al. (2015), [44]Mazzoleni et al. (2015a), [45]Mazzoleni et al. (2015b), [46]Barbero et al. (2016), [47]Duran-Flores and Heil (2018), [48]Vega-Muñoz et al. (2018), [49]Lorenz and Wackernagel (1994), [50]Merod and Wuertz (2014), [51]Orwin et al. (2018), and [52]Paungfoo-Lonhienne et al. (2010).

Köckritz-Blickwede, 2015; Halverson et al., 2015). Furthermore, Halverson et al. (2015) demonstrated that the DNA in NETs induces the upregulation of protective surface modifications in bacteria (Halverson et al., 2015). In fact, bacteria co-incubated with NETs, upregulates the expression of the *arn* operone and of spermidine synthesis genes (Baums and von Köckritz-Blickwede, 2015). These two factors stabilize the bacterial envelope and mediate resistance to antimicrobial peptides (Johnson et al., 2012; Gutu et al., 2015). In 2020, Tsourouktsoglou et al. (2020) showed that histones and DNA work together in triggering inflammation, when histone-induced cytotoxicity is not reached. Indeed, at low concentrations, nucleosomes can induce cytokines, and the inflammatory response, whereas at high concentrations they kill the cells (Tsourouktsoglou et al., 2020). Cooperative effects due to histones and DNA are essential for the production of cytokines without killing cells. In fact, histones bind and activate TLR4, whereas DNA recruits TLR4 into endosomes containing histones (Tsourouktsoglou et al., 2020).

Of note, as mentioned above, NETs were demonstrated to be formed from pure mtDNA which can have a potent proinflammatory effect, acting as a DAMP, and directly modulating an inflammatory response (Krysko et al., 2011). This is due to the different methylation pattern of mtDNA when compared to nuclear DNA (Patil et al., 2019), making it detectable as a foreign molecule (bacterial or viral like), rather than a "self" DNA molecule (Yousefi et al., 2019), thus activating an immune response by stimulating the PRRs STING (Lood et al., 2016). Furthermore, in 2015, Wang et al. (2015) demonstrated higher levels of mtDNA in NETs of systemic lupus erythematosus patients when compared with controls, suggesting a possible role of mtDNA in autoimmune diseases (Wang et al., 2015).

DNA in RETs

Similarly to NETs, both the histones H4 and the exDNA in RETs are suggested to have an antimicrobial activity (Tran et al., 2016; Driouich et al., 2019). The former, like the cationic antimicrobial peptides, may bind and disrupt microbial cell membranes. The DNA in RETs is discussed to have a structural role like a scaffold allowing the adhesion of anti-microbial components, being also considered as a trap for pathogens (preventing their spread throughout the organism). In addition, it exerts a direct bactericidal function (Halverson et al., 2015), putatively with the same action discussed for NETs. Evidence suggested that, in RETs, the DNA might be an integral component of plant defense, playing a relevant role in the innate immunity response to pathogen invasion. For instance, it is reported that the DNA is released in the extracellular environment with other molecules, such as callose, ROS, and cell wall extensins, in response to pathogen molecules (Plancot et al., 2013). Moreover, the production of extracellular DNases contributes to pathogens virulence (Hawes et al., 2011). The plant pathogen *R. solanacearum* produces two extracellular DNases that are able to degrade the DNA in pea root mucilage, allowing the pathogen to overcome the border cell trap. Conversely, *R. solanacearum* mutants, lacking both nucleases, remain immobilized in the root matrix, thus showing a reduced virulence (Steichen et al., 2011; Tran et al., 2016). Worthy to note, although the role of self exDNA as a DAMP has been discussed in plants (Barbero et al., 2016; Duran-Flores and Heil, 2018; Vega-Muñoz et al., 2018), as also here reviewed, there is no clear evidence that the exDNA released by root cap cells in the extracellular space and organized in RETs could act as a DAMP too, thus triggering an immunological response in plants.

Despite the evidence listed above, the role of exDNA in RETs remains to be further elucidated. Its release by viable border cells suggests an active role in plant root defense against pathogens in the rhizosphere. However, further analyses are still required to elucidate its possible functions and associated mechanisms.

DNA in Biofilms

ExtracellularDNA is today accepted to be essential during biofilm formation and development (Ascher et al., 2009),

as a component of the ECM in both terrestrial and marine biofilms (Flemming and Wingender, 2010; Flemming et al., 2016; Decho and Gutierrez, 2017; Abada and Segev, 2018), as well as in biofilms of clinically relevant microorganisms such as *Staphylococcus spp., Streptococcus spp., Candida spp., P. aeruginosa* (Mann et al., 2009). Interestingly, DNA is mostly represented by randomly fragmented genomic DNA (Steinberger and Holden, 2005; Wu and Xi, 2009; Kassinger and van Hoek, 2020).

The exDNA is released into the surrounding matrix not only by lysed cells (Sutherland, 2001), but also by an active release, sometimes mediated by membrane vesicles (Kadurugamuwa and Beveridge, 1995; Grande et al., 2015; Kassinger and van Hoek, 2020). In particular, the mechanism of DNA release differs among gram positive and gram negative bacteria. Gram positive bacteria are thought to release DNA in biofilms by autolysis or lytic processes, while the formation of vesicles and the release by the type 4 secretion system appear to be additional processes that are typically described in gram negative bacteria, depicting and active release of DNA in the extracellular space (Ibáñez de Aldecoa et al., 2017).

Multiple functions have been described for exDNA in biofilms (Das et al., 2013; Okshevsky and Meyer, 2015; Ibáñez de Aldecoa et al., 2017). The structural role of exDNA in biofilm formation (Whitchurch et al., 2002; Harmsen et al., 2010; Gödeke et al., 2011) has been demonstrated by the treatment with DNases, that generally lead to biofilm disruption and consequent cell dispersal (Whitchurch et al., 2002; Nguyen and Burrows, 2014). Furthermore, it has been demonstrated that exDNA forms complexes with the amyloid proteins secreted by different species, generating biofilms (Barnes et al., 2012; Schwartz et al., 2016). Apart from the structural role discussed above, other specific functions of exDNA in biofilms have been suggested. ExDNA may act as facilitator of the initial adhesion of cells to the surface (Das et al., 2010), in support of EPS gelification (Seviour et al., 2019), in the maintenance of specific cell orientations (Gloag et al., 2013), in the control of viscoelastic relaxation of the biofilm in mechanical stress conditions (Peterson et al., 2013) and in the induction of the morphological changes from yeast to hyphal growth, during *Candida albicans* biofilm development (Payne and Boles, 2016).

The presence of DNA in the matrix has been also related to biofilm antibiotic resistance (Chiang et al., 2013). For instance, a higher antimicrobial resistance has been detected in the presence of higher concentration of exDNA in biofilms (Mulcahy et al., 2008). Moreover, the chelator action of negatively charged exDNA phosphodiester backbone also plays a role against cationic antimicrobials (Mulcahy et al., 2008). Biofilms have also been shown to stimulate the host innate and the adaptive immune system, and this may cause the development or the progression of host autoimmune responses (Gallo et al., 2015). It has been suggested that these events could be favored by different putative causes. Indeed, the autoimmune response could be triggered or by the release of DAMPs, due to drastic damages of the host structures caused by the bacteria infection, and/or by components of their biofilm structure. As an example, bacteria biofilms may be composed by scaffolds of amyloid proteins that are highly resistant to degradation. For example, curli fibers are amyloids present in biofilms of enteric bacteria. It was shown that the complex between curli fibers and bacterial DNA in enteric biofilms has a higher inflammatory activity when compared with the effects of curli fibers or DNA alone (Gallo et al., 2015). It was demonstrated that curli fibers are detected by the TLR2/TLR1 heterocomplex on the membrane of immune cells. This triggers the internalization of the curli/DNA complex via endosome formation, and the activation of the receptor TLR9 by the bacterial DNA. Since amyloids are expressed also by human cells, their presence could trigger the production of autoantibodies, justifying the possible occurrence of autoimmune responses (Gallo et al., 2015). It has been suggested, however, that the autoimmunity response triggered by the exposure to bacteria biofilm may evolve in autoimmune disease when the individual is already predisposed by genetic factors. Beyond these effects, however, currently there is no further evidence, to our knowledge, that could suggest possible additional roles of biofilm components in causing autoimmune responses, rather than their capability to trigger the development of autoantibodies or trigger adverse reactions due to the detection of their DNA by host cell intracellular receptors.

The role of exDNA as a source of genetic information in the context of HGT within the biofilm has been addressed in several studies (Lorenz and Wackernagel, 1994; Merod and Wuertz, 2014; Orwin et al., 2018). Homologous recombination of foreign DNA into the host chromosome following transformation is believed to play a major role in bacteria evolution (Flemming et al., 2016). Biofilm offers ideal conditions for exchanges of genetic material because of the high cell density, increased genetic competence, and the presence of abundant exDNA.

From a clinical point of view, exDNA also turns out to represent a possible target for antibiotic agents acting against biofilm structural integrity, increasing the susceptibility of its constituents (Koo et al., 2017; Rocco et al., 2017; Ye et al., 2017).

DISCUSSION

Extracellular DNA, whether released in biofilm by bacteria, in RETs by root cap cells or in NETs by neutrophils or other cell types, has key structural, and functional roles that we here reviewed, as summarized in **Figure 1** and **Table 1**. In addition, further intriguing roles of exDNA produced by an organism or by cells from the same species, have also been described in terms of extracellular self-DNA inhibitory effects.

In 2015, Mazzoleni demonstrated for the first time that the exposure to fragments of self-DNA, and not of non-self-DNA, inhibits root growth in plants in a concentration dependent manner (Mazzoleni et al., 2015a). Moreover, Mazzoleni and co-workers suggested that the phenomenon is dependent on the similarity of the "self" DNA fragments with the genome of the treated species, thus explaining the autotoxicity in litters of phylogenetic related species. Indeed, they demonstrated that in a specific plant species the treatment with different non-self-DNA fragments produces different effects: the closer the organisms

phylogenetic distance, the higher the inhibitory effect of non-self-DNA on the treated plant (Mazzoleni et al., 2015a).

Following their experimental evidences, Mazzoleni and co-workers hypothesized that the inhibitory effect of self-DNA, because of its specificity and its occurrence also with DNA of related species, could be the result of a mechanism resembling the "well-known processes of interference based on sequence-specific recognition of small-sized nucleotide molecules" (Mazzoleni et al., 2015a).

Later, the same authors demonstrated that the inhibitory effect of self DNA, compared to non-self DNA, is a general phenomenon (Mazzoleni et al., 2015b) since the growth inhibition was also demonstrated for other species of different taxonomic groups (such as algae, bacteria, and fungi), reinforcing the hypothesis that the mixture of random self-DNA fragments could cause both an interference or exert inhibitory effect on the whole genome functionality (Mazzoleni et al., 2015a).

Duran-Flores and Heil (2015), considering the same topic, suggested two possible mechanisms: a self-specific membrane reception followed by a downstream signaling cascade activation or the direct uptake of fragmented DNA into cells with subsequent interference with essential biological processes. A further hypothesis was suggested to explain the dosage-dependent growth-inhibition by self-DNA as the phenotypic consequence of a costly immune response (Duran-Flores and Heil, 2015).

Cartenì et al. (2016) proposed that the first sensing of exogenous self-DNA could occur at the level of RET and, following its uptake, the cell functionality could be affected in different manners. For example, the self- DNA could interfere with gene expression following a sequence-specific recognition of homologous sequences recognition involving RNA/DNA interactions or the direct interaction with the genome structure through a mechanisms similar to the Small Fragment Homologous Replacement (Cartenì et al., 2016). They suggested that a mixture of random self-DNA fragments, once inside the plant cell, recognizes and anneals with the homologous DNA sequences in the plant genome. This could lead to the formation of structures that activate mechanisms of DNA repair allowing the integration of small DNA fragments into the genomic DNA thus affecting cellular activities (Cartenì et al., 2016).

Currently, however, the mechanisms underlying the specific recognition of either self and non-self-DNA and subsequent responses in plants are still poorly understood (Bhat and Ryu, 2016).

Beyond the structure and functional roles here reviewed for NETs, RETs, and biofilms, novel intriguing aspects arise when considering the specific inhibitory effects of extracellular self-DNA demonstrated by Mazzoleni et al. (2015a,b) and in subsequent efforts (Duran-Flores and Heil, 2018).

Concerning NETs, it is well known the role of the DNases responsible for their degradation that, while avoiding NET diffusion into neighboring tissues, favor their necessary clearance. Indeed, not removed NETs are able to form clots causing vascular occlusion and organ failure (Papayannopoulos et al., 2010; Arazna et al., 2013; Jiménez-Alcázar et al., 2017). Moreover, the evidence that in the absence of a complete NET removal, the complex of chromatin-DNA can be a primary target for autoantibodies leading to the development of autoimmune diseases (Barrat et al., 2005; Wang et al., 2015; Rykova et al., 2017; Vakrakou et al., 2018) or that DNA fragments contribute to cardiomyocyte dysfunction irrespective of NET formation (Savchenko et al., 2014; Shah et al., 2020), or that DNases can attenuate NET-mediated pathologies (Németh et al., 2020), indicates a potential negative effect of extracellular self-DNA present in NETs.

Indeed, the persistence of self-DNA in the context of NETs has been demonstrated to exert pro-inflammatory effects by promoting the formation of autoantibodies against both mitochondrial (Krysko et al., 2011; Wang et al., 2015) and nuclear (Barrat et al., 2005; Tsourouktsoglou et al., 2020) DNA, that can induce the activation of immune cells such as macrophage and neutrophils.

When considering also RETs and biofilms further issues arise: what conditions the exDNA released by root cap cells in the mucilage or while forming biofilms produces? Can they be either positive or negative for the host, either in roots or in microbial organisms, respectively? Could the co-secreted ECM have also the role of decreasing the bio-availability of extracellular self-DNA molecules to limit its potential inhibitory role, thus favoring the formation of RETs, or biofilms, or NETs, and forming self "exDNA traps," thus limiting possible self-inhibitory effects? Although in the context of RETs and microbial biofilms a direct negative role of the exDNA in the matrices against the producing organisms has never been shown, the inhibitory effect of exDNA fragments has been demonstrated to be a general biological process in animal, plants, and bacteria (Mazzoleni et al., 2015b).

Therefore, a major issue remains to be considered on the possible roles of the ECM components in structuring and organizing exDNA. The trapping of exDNA in the ECM offers protective advantages against foreigner attacks, and, in parallel, limits its bio-availability in the environment as a free molecule, affecting its possible effects on the releasing organisms. In this framework the matrices could play an additional role in limiting the extracellular self-DNA self-inhibitory effects.

Studies addressing the roles of DNA in extracellular environments, and specifically in extracellular matrices formation, will shed further light on additional mechanisms and functionalities of these complex systems, ubiquitarian across different kingdoms.

AUTHOR CONTRIBUTIONS

EP, FM, and MC wrote the manuscript. MC conceived the effort and supervised the entire work. PT, PC, EA, and SM gave their intellectual contribution to the work. All the authors read and approved the work.

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REVIEW
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Dampening the DAMPs: How Plants Maintain the Homeostasis of Cell Wall Molecular Patterns and Avoid Hyper-Immunity

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Several oligosaccharide fragments derived from plant cell walls activate plant immunity and behave as typical damage-associated molecular patterns (DAMPs). Some of them also behave as negative regulators of growth and development, and due to their antithetic effect on immunity and growth, their concentrations, activity, time of formation, and localization is critical for the so-called "growth-defense trade-off." Moreover, like in animals, over accumulation of DAMPs in plants provokes deleterious physiological effects and may cause hyper-immunity if the cellular mechanisms controlling their homeostasis fail. Recently, a mechanism has been discovered that controls the activity of two well-known plant DAMPs, oligogalacturonides (OGs), released upon hydrolysis of homogalacturonan (HG), and cellodextrins (CDs), products of cellulose breakdown. The potential homeostatic mechanism involves specific oxidases belonging to the family of berberine bridge enzyme-like (BBE-like) proteins. Oxidation of OGs and CDs not only inactivates their DAMP activity, but also makes them a significantly less desirable food source for microbial pathogens. The evidence that oxidation and inactivation of OGs and CDs may be a general strategy of plants for controlling the homeostasis of DAMPs is discussed. The possibility exists of discovering additional oxidative and/or inactivating enzymes targeting other DAMP molecules both in the plant and in animal kingdoms.

Keywords: growth-defense trade-off, Berberine bridge enzyme-like (BBE-like) proteins, cell wall DAMPs, oligosaccharide oxidase, oxidized oligogalacturonides, oxidized cellodextrins

INTRODUCTION

The cell wall represents the interface between plants and the environment and acts as a physical barrier to protect plants against biotic and abiotic stresses. Main components of the cell wall are the polysaccharides cellulose, hemicellulose, and pectin, which interact among themselves and with lignin to form a complex structure that influences the shape, rigidity, growth, and differentiation of plant cells (Bacete and Hamann, 2020; Zhang and Zhang, 2020). All three types of polysaccharides are also a repository of extracellular damage-associated molecular patterns (DAMPs), potentially released during microbial infections or upon mechanical

damage (De Lorenzo et al., 2018; Hou et al., 2019). DAMPs are likely to be released also upon cell wall remodeling that occurs during plant growth and development. Microlesions caused, for example, by cell expansion, lateral root formation, or organ abscission, may cause cell wall damage and affect the so-called cell wall integrity (CWI; Anderson and Kieber, 2020; Gigli-Bisceglia et al., 2020). In these cases, endogenous cell wall degrading enzymes (CWDEs) can release, at low levels, oligosaccharide fragments of the same nature as those accumulated in larger quantities during more destructive mechanical injuries or plant cell wall degradation caused by pathogen-encoded CWDEs (De Lorenzo et al., 2018, 2019). Some DAMPs are recognized by pattern-recognition receptors and are capable of inducing pattern-triggered immunity (PTI) even in the absence of infection (De Lorenzo et al., 2018).

Unlike mammals, plants do not have an adaptive immune system but, instead, entirely rely on the ability of individual cells to recognize pathogens and to activate defense responses (Gust et al., 2017). The plant cell must be able to discriminate between the possibilities that cell wall degradation is caused by a physiological or a pathological accumulation of DAMPs. The time of formation, the appropriate concentration, and the correct distribution of the active molecules may help distinguish a pathological from a physiological event. In the former case, plants need to respond quickly, intensively, and systemically to the danger, whereas in the latter case, they may only need to activate local immune responses, preventing a massive immune response that may hamper growth (Li et al., 2020). In general, plants cannot dissipate too much energy in defense *vs*. growth and need to maintain the correct growth-defense trade-off. To this purpose, they require continuous balancing of the defense pathways and signaling.

Hyper-immunity generated by DAMPs can cause deleterious effects in plants like in animals. Uncontrolled or prolonged production of DAMPs may promote hyperinflammation or chronic inflammation through the adaptive immune system in animals (Land, 2020), whereas in plants, it mainly causes hypersensitivity and death of individual cells and reduced growth of the entire organism. In this review, we discuss how several extracellular DAMPs released from the polysaccharides of the plant cell wall may be kept under homeostatic control by oxidizing/inactivating enzymes (**Figure 1**) and how a similar mechanism may have general relevance for other DAMPs.

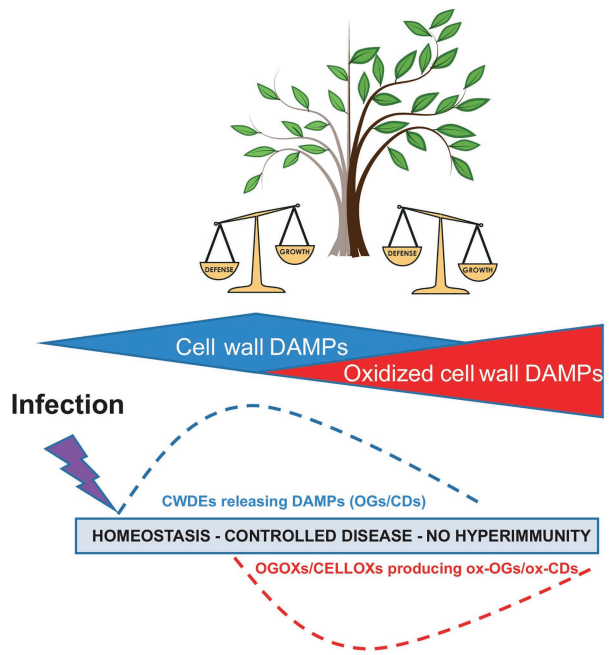

FIGURE 1 | Damage-associated molecular patterns (DAMPs) and oxidized DAMPs in immunity, homeostasis of signals, and "growth-defense trade-off" (modified from Relja and Land, 2020). CWDEs, cell wall degrading enzymes; OGs, oligogalacturonides; CDs, cellulose fragments; ox-OGs, oxidized oligogalacturonides; and ox-CDs, oxidized cellulose fragments.

DYNAMICS OF THE CELL WALL AND RELEASE OF REGULATORY FRAGMENTS

In order to provide structural support during growth and protection against diseases, the composition and structure of the plant cell wall is continuously changing during development or upon biotic and abiotic stresses (Vaahtera et al., 2019). During both development and disease, CWI is often compromised and cell wall components undergo dynamic changes (Gigli-Bisceglia et al., 2020). These changes may cause a direct enzymatic release of fragments possessing growth-regulating/DAMP activity from each of the major wall components. The impairment of CWI itself could function as a mechano-sensory signal of damage that may also regulate cellular events, such as meristem patterning and the cell cycle (Bacete and Hamann, 2020). Notably, microtubule organization, orientation, and deposition of cellulose microfibrils are reorganized, and several CWDEs are activated in response to mechanical stimuli (Vaahtera et al., 2019).

Pectin, which is abundant in the middle lamella and in the primary walls, influences wall porosity and thickness and may play a role in the maintenance of CWI due to its marked sensitivity to mechanical deformation. A recent model indicates that cell shape and expansion as well as the turgor force exerted by the cell wall are driven by 15–30 nm nanofilaments of homogalacturonan (HG), the main component of pectin (Haas et al., 2020). These nanofilaments, located perpendicularly to the cotyledon surface, expand upon demethylation (Haas et al., 2020). Among the cell wall components, pectin appears to be more readily degraded upon tissue damage and microlesions that may arise during growth and development (Gigli-Bisceglia et al., 2020). Pectin also represents the first target of CWDEs produced by invading pathogens. The breakdown fragments of HG, the oligogalacturonides (OGs), act as regulators of plant growth and development and, if accumulated at high concentration, act as elicitors of

plant defense (DAMPs). Interestingly, an alteration of the lignin content of cell walls has been reported to induce the expression of genes encoding CWDEs, such as pectate lyase, xyloglucan (XG) endo-transglycosylases, and ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE (ADPG1). Especially, ADPG1 may be responsible for the release of elicitor-active OGs, which could explain why a reduction in lignin content often enhances disease resistance rather than reducing it (Gallego-Giraldo et al., 2020).

Xyloglucans, hemicelluloses interacting with cellulose through hydrogen bonds, may control the rate of cell wall expansion and plant growth (York et al., 1985; Cosgrove, 2018). XG-derived fucose-containing oligosaccharides have been reported to display growth regulating activity (York et al., 1985; Zablackis et al., 1995). On the other hand, XG-derived fragments have been reported to act as DAMPs (Claverie et al., 2018).

Arabinoxylan (AX), another hemicellulose present in both the primary and secondary walls, consists of copolymers of two pentoses: arabinose and xylose. Very recently, by analyzing mutants in Arabidopsis Response Regulators (ARRs), which mediate cytokinin signaling and modulate the disease resistance (Bacete and Hamann, 2020), it was demonstrated that the AX-derived pentasaccharide 3^3-α-L-arabinofuranosyl-xylotetraose (XA3XX) triggers a strong immune response in Arabidopsis and enhances disease resistance of some crop plants (Mélida et al., 2020).

Cellulose crystalline microfibrils are present at high levels together with hemicelluloses and lignin in the secondary wall (Cosgrove, 2014). The inhibition of cellulose biosynthesis triggers NADPH oxidase-dependent production of hydrogen peroxide and lignin in the elongation zone of the root. Lignin deposition is a compensatory response to reinforce cell walls that occur upon cell wall modifications or degradation (Denness et al., 2011; Vaahtera et al., 2019). Arabidopsis mutants impaired in the perception of cell wall damage and reactive oxygen species (ROS) perception/signaling show decreased or absent lignin deposition in response to cell wall damage, suggesting that the damage causes the release of DAMPs such as OGs that activate lignification to reinforce the secondary wall (Gallego-Giraldo et al., 2018, 2020). Most of the genes involved in cell wall damage are also implicated in responses to biotic stresses (Denness et al., 2011). Cellulose breakdown products, i.e., cellodextrins (CDs), also act as elicitors of plant defense (Aziz et al., 2007; Souza et al., 2017; Johnson et al., 2018).

Recently, a non-branched 1,3-β-D-(Glc)-hexasaccharide has been identified as a major fungal microbe-associated molecular pattern (MAMP). Because linear β-1,3-glucans are present in plants and accumulate as callose papillae at the site of infection (Albersheim et al., 2011; Chowdhury et al., 2014), the possibility exists that this type of oligosaccharide is released during callose synthesis or degradation to act as a DAMP (Mélida et al., 2018).

Mannans are mainly considered as storage polysaccharides that provide energy for the growing seeds, and their presence and functional importance as structural components of both primary and secondary walls has been highlighted by several authors (Schröder et al., 2009; Marcus et al., 2010). Recently, mannan oligosaccharides (MOS) have been reported as novel cell wall DAMPs (Zang et al., 2019).

In conclusion, the plant cell wall is a dynamic structure that during growth and development can depolymerize its components by auto-degradation, recycle the released fragments, and rebuild the entire structure (Barnes and Anderson, 2018). Alterations of cellulose, xylan, glucuronoxylan, pectin, xyloglucans, and lignin often enhance disease resistance due to their recalcitrance to pathogen-mediated degradation, or, as reported in the case of altered lignin content, provoke the release of DAMPs that activate defense responses (Gallego-Giraldo et al., 2020). During growth, in the absence of pathogens, released fragments, like OGs, may play a physiological role as growth regulators and be recruited as DAMPs if a pathological event occurs (**Figure 2**).

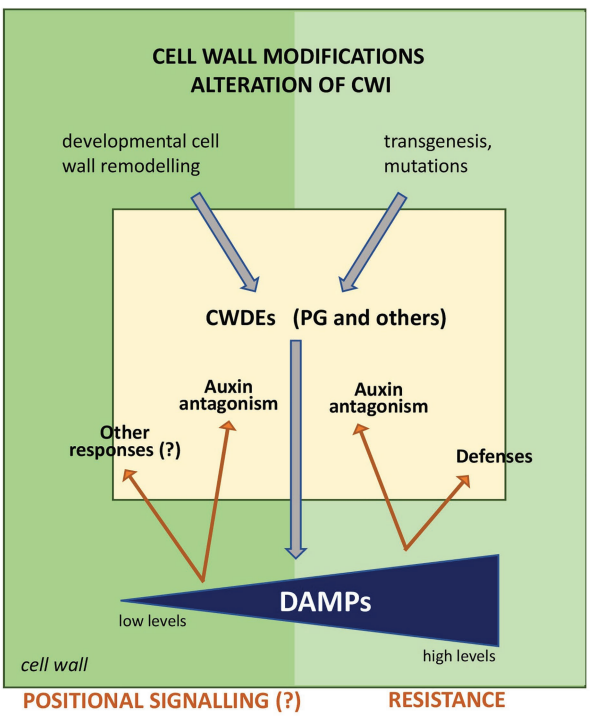

FIGURE 2 | Possible outcomes of the release of DAMPs during alteration of cell wall integrity (CWI). Alterations of the cell wall composition due to transgenesis (for example, reduced lignin content) or mutations lead to a signaling cascade that induces the expression of endogenous cell wall degrading enzymes (CWDEs), among which a polygalacturonase (PG) may be responsible for the release of oligogalacturonides (OGs) that activate defense responses. In normal conditions, i.e., in the absence of pathogens, alterations of CWI during growth, and developmental events, such as cell expansion and division, wall remodeling, secondary root formation, abscission, etc. may be mediated by and/or induce the expression of CWDEs, including PGs. CWDEs release wall fragments that may act as signals for the regulation of growth in a short range cell-cell communication (positional signaling). The question mark indicates events to be elucidated.

THE MODE OF ACTION OF CELL WALL DAMPs

Oligogalacturonides

The best described wall-associated DAMPs are the OGs, which derive from the partial degradation of HG (Cervone et al., 1989; Orozco-Cardenas and Ryan, 1999) and likely act as indicators of CWI under stress conditions (Rojo et al., 1999; Aziz et al., 2004; De Lorenzo et al., 2011; Ferrari et al., 2013; Savatin et al., 2014). OGs antagonize auxin (Branca et al., 1988; Bellincampi et al., 1995) by inhibiting the transcription of auxin-induced genes belonging to the IAA and SAUR families as well as the activity of the auxin-responsive promoter DR5 (Savatin et al., 2011). During microbial infections, the accumulation of OGs is facilitated by the interaction of microbial polygalacturonases (PGs) with specific PG-inhibiting proteins (PGIPs; Kalunke et al., 2015; Haeger et al., 2020). Notably, the transgenic expression of a PGIP-PG chimera referred to as the "OG-machine" is able to produce *in vivo* endogenous OGs, enhancing Arabidopsis resistance against *Botrytis cinerea*, *Pectobacterium carotovorum*, and *Pseudomonas syringae* (Benedetti et al., 2015). OGs bind the extracellular domain of the Arabidopsis wall-associated receptor kinase1 (WAK1; Brutus et al., 2010; Gramegna et al., 2016). Treatments with OGs trigger a wide range of defense responses, including calcium increase, release of ROS and nitric oxide, production of phytoalexins, glucanase, and chitinase, and deposition of callose in the cell wall (Davis et al., 1986; Davis and Hahlbrock, 1987; Broekaert and Pneumas, 1988; Bellincampi et al., 2000; Aziz et al., 2007; Galletti et al., 2008; Rasul et al., 2012). OGs activate the transcription factors WRKY40 and WRKY33, and several genes involved in defense, such as *EDS5/SID1*, *SID2/ICS1*, *NPR1*, *SAG101*, *PAD4*, *ACS7*, *LOX3*, and *LOX4* as well as the indole glucosinolate biosynthetic genes *CYP83B1*, *CYP79B2*, *CYP79B3*, *SUR1*, and *MYB51* (Denoux et al., 2008; Souza et al., 2017). OGs activate phosphorylation of the MAP kinases AtMPK3 and AtMPK6 (Galletti et al., 2011; Mattei et al., 2016) and upregulate phenylalanine ammonia lyase, stilbene synthase (De Lorenzo et al., 1987; Aziz et al., 2007), and serine-protease inhibitors (Ferrari et al., 2003). Treatments with OGs increase the Arabidopsis resistance to *B. cinerea* through the expression of *PAD3*, which is required for the basal resistance to this fungus (Aziz et al., 2004), and, if applied to roots, induce systemic resistance in tomato plants against *B. cinerea* (Gamir et al., 2020).

Cellodextrins

Cellodextrins are linear β-1,4 gluco-oligosaccharides with a degree of polymerization (DP) ranging from 3 to 9 derived from cellulose fragmentation. In grapevine, a CD with a DP of seven induces rapid ROS production and transient elevation of cytosolic calcium levels. Notably, grapevine cells pretreated with CD7 are refractory to a successive treatment with the same oligomer but respond to a subsequent application of OGs, suggesting that CDs and OGs activate different signaling pathways. CDs upregulate the expression of defense genes encoding phenylalanine ammonia lyase, stilbene synthase, chitinases, glucanases, and serine-protease inhibitors, and enhance the resistance of plants against *B. cinerea* (Aziz et al., 2007). In Arabidopsis, cellobiose (DP 2), cellotriose (DP 3), and cellotetraose (DP 4) cause an increase of intracellular calcium in a fast and transitory way, the early activation of MPK3 and MPK6, and the upregulation of some defense-related genes like those involved in the biosynthesis of glucosinolates. Cellotriose derived from the endophytic fungus *Piriformospora indica* also elicits plant defense responses (Johnson et al., 2018). Treatment with cellobiose has been reported to induce resistance to *P. syringae* pv *tomato* DC3000 (Souza et al., 2017). On the other hand, seedlings grown on high levels of cellobiose exhibit an increase in biomass and the induction of β-glucosidase that provides glucose as a useful carbon source for the growth of the plant itself, as well as for microbes. This is in contrast with the notion that the activation of defenses impairs growth and casts some doubts on the activity of cellobiose as a DAMP (Souza et al., 2017). Indeed, in a study, on the activity of a berberine bridge enzyme-like (BBE-like) oxidase (see below), only CDs with DP higher than two showed a significant elicitor activity (Locci et al., 2019).

Xyloglucans

Xyloglucan oligomers have been recently identified as DAMPs in both grapevine and Arabidopsis. In Arabidopsis, they induce phosphorylation of MPK3 and MPK6, PMR4-dependent callose deposition, enhanced expression of defense genes, including *PR1*, *PAD3*, *PR2*, and *PLANT DEFENSIN 1.2* (*PDF1.2*) as well as an increased jasmonic acid (JA)-, salicylic acid (SA)-, and ethylene (ET)-dependent resistance against *B. cinerea*. In grapevine, XG oligomers induce accumulation of the phytoalexin resveratrol and resistance against *B. cinerea* (Claverie et al., 2018; Heloir et al., 2019).

Arabinoxylans

Arabinoxylan oligosaccharides have been recently identified as novel DAMPs (Mélida et al., 2020). This group of hemicelluloses consists of a main backbone of β-1,4-linked D-xylose residues decorated with single L-arabinose residues linked to the C2/C3 position of a D-xylose unit (Ebringerová and Heinze, 2000). The pentasaccharide XA3XX triggers an oxidative burst, a rapid calcium influx, the phosphorylation of MPK3 and MPK6, and the upregulation of genes involved in innate immunity, including several PTI marker genes (*CYP81F2*, *WRKY53*, *PHI1*, *FRK1*, and *NHL10*). Moreover, tomato plants treated with XA3XX are more resistant to *P. syringae* pv *tomato* DC3000, and XA3XX-treated pepper plants are more resistant to *Sclerotinia sclerotiorum* (Mélida et al., 2020).

β-1,3-Glucans

Arabidopsis plants treated with 1,3-β-d-$(Glc)_6$ isolated from the pathogenic fungus *Plectosphaerella cucumerina* show the CERK1-mediated expression of several immunity-associated

responses like elevation of cytoplasmic calcium concentration and MAPK cascades (Mélida et al., 2018). Because β-1,3-glucans are present in the callose of the plant papillae that are formed at the sites of infection, 1,3-β-d-$(Glc)_6$ may be considered as both MAMP and DAMP.

Oligomannans

Oligomannans with DP 2–6 enhance defense responses and expression of genes related with NO and ROS accumulation as well as the expression of *PR-1* and *LOX* in both *Nicotiana benthamiana* and rice. In rice, MOS trigger the upregulation of the expression of *MAPK12* and *MAPK6*, and lead to the accumulation of phytoalexins. Treatments with MOS protect rice and tobacco against *Xanthomonas oryzae* and *Phytophthora nicotianae*, respectively (Zang et al., 2019).

PATHWAYS AND SIGNALS INVOLVED IN THE GROWTH-DEFENSE TRADE-OFF

Due to their energy cost and consequent metabolic limitations, growth and defense are constantly regulated by a balanced trade-off of antithetic pathways, both of which are most likely affected by stimulatory and inhibitory signals (Huot et al., 2014; Vaahtera et al., 2019). As stated above, homeostatic control of the defense response is important to avoid deleterious effects due to a hyper-immune response that causes reduced growth and/or extensive cellular death. Under such circumstances, attenuation of the pathways leading to defense or a reduction in the levels of the signals triggering such pathways is expected to occur (Karasov et al., 2017; Li et al., 2020). The crosstalk between the pathways controlled by ET, JA, SA, and by other growth regulators may influence immune and developmental processes in opposite directions (Denancé et al., 2013; Guo et al., 2018). Over-expression of pathways that activate immunity often enhances pathogen resistance while negatively affecting plant growth and may cause anatomical and physiological responses, such as dwarfism, accelerated senescence, delayed flowering, sterility, or reduced seed production.

Many other signals, besides ET, JA, and SA, are known to influence the growth/defense trade-off. ROS in the apoplast are among these signals as their concentration and homeostasis is critical for maintaining the correct balance between growth and defense (Farvardin et al., 2020). A low level of H_2O_2 in the cell wall, for example, promotes growth and concomitantly suppresses the plant defense (Neuser et al., 2019). Other signals such as OGs antagonize auxin by inhibiting adventitious root formation, stem elongation, and pericycle cell differentiation. At the molecular level, OGs downregulate auxin-induced expression of the *DR5* promoter as well as the early auxin-regulated genes *IAA5*, *IAA19*, *IAA20*, *IAA22*, *SAUR16*, *SAUR AC1*, and *GH3.3*. On the other hand, auxin antagonizes the protection exerted by OGs against *B. cinerea* (Branca et al., 1988; Bellincampi et al., 1993; Ferrari et al., 2008; Savatin et al., 2011). Furthermore, a high level of endogenous OGs strongly reduces growth while a systemic and prolonged accumulation of OGs causes a hypersensitive-like response characterized by extensive cell death (Benedetti et al., 2015). Interestingly, OGs-auxin antagonism is independent of the extracellular accumulation of H_2O_2 produced by NADPH oxidase (Bellincampi et al., 1996, 2000) and is also independent of ET, JA, and SA signaling. The antagonism takes place downstream in the auxin signaling pathway, most likely at the post-translational level (Savatin et al., 2011).

OLIGOGALACTURONIDES AND CELLODEXTRINS ARE ENZYMATICALLY OXIDIZED BY BBE-LIKE PROTEINS

Oxidized OGs were first identified in leaf diffusates of transgenic plants expressing the "OG-machine," a chimeric protein generated by fusing a fungal endo-polygalacturonase with a plant PGIP (Benedetti et al., 2018). The OG-machine is capable of releasing elicitor-active OGs on command under the control of a chemically inducible promoter (Benedetti et al., 2015). The analysis of these transgenic plants revealed the presence of atypical oligomers displaying oxygen at the C1 position of the reducing end due to the conversion of a galacturonic acid into galactaric acid (**Figure 3**; Benedetti et al., 2018). A flavin adenine dinucleotide (FAD)-dependent and sulfite-sensitive enzyme, named oligogalacturonide oxidase (OGOX)1, capable of oxidizing OGs, was subsequently purified and found to belong to the family of BBE-like proteins, which consists of 28 members in Arabidopsis considering that one of the 27 genes, i.e., *At4g20830*, encodes for two OGOX1 isoforms, BBE19 and BBE20 (Benedetti et al., 2018; **Table 1**; **Figure 4**). OGOX1 specifically oxidizes OGs releasing H_2O_2 derived from the oxidization of the reduced FAD cofactor by O_2 that, in turn, restores the activity of the enzyme (**Figure 3**). Three additional BBE-like proteins of the Arabidopsis BBE family, OGOX2, OGOX3, and OGOX4, oxidize OGs at a different pH (Benedetti et al., 2018). Another member of the gene family encodes an enzyme that catalyzes the conversion of indole cyanohydrin to indole-3-carbonyl nitrile, a metabolite with a role in defense (Boudsocq et al., 2010; Rajniak et al., 2015), whereas two other BBE proteins encode monolignol oxidases (Daniel et al., 2015). Finally, a BBE protein member named Cellodextrin Oxidase (CELLOX) was shown to oxidize and convert the glucose at the reducing end of CDs into gluconic acid (Locci et al., 2019). Another BBE protein, BBE23, is characterized by a 62.5% amino acid identity with CELLOX and is likely a potential paralog. CELLOX and BBE23 display the highest amino acid identity with Nectarin V, a glucose oxidase from ornamental tobacco (*Nicotiana langsdorffii x Nicotiana sanderae*; Carter and Thornburg, 2004; **Table 1**; **Figure 4**). Recently, BBE8 was hypothesized to oxidize some wall-derived oligosaccharides in guard cells in order to facilitate stomatal opening in

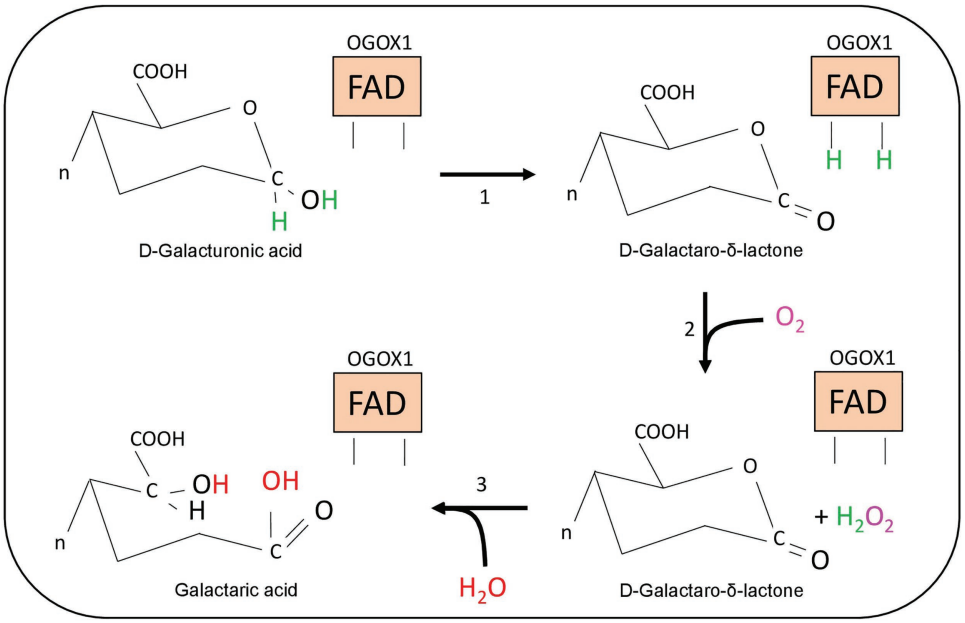

FIGURE 3 | Schematic representation of the enzymatic reaction catalyzed by OGOX1. The oxidized FAD-cofactor of OGOX1 oxidizes the galacturonic acid to a lactone at the reducing-end of OGs (step 1). Molecular oxygen oxidizes the reduced FAD(H_2)-cofactor and releases hydrogen peroxide (step 2). The final hydrolysis transforms the lactone into galactaric acid (step 3).

response to infection by *P. syringae* and *Salmonella enterica* (Rodrigues Oblessuc et al., 2019). However, the activity of BBE8 as an oxidase of oligosaccharides was not proven. Similarly, the substrate specificity of BBE28 has not been identified yet despite the fact that the crystal structure of the enzyme has been solved (Daniel et al., 2016). So far, the substrates of 19 out of the 28 Arabidopsis BBE-like family members have not been identified (**Table 1**). The scenario of undetermined functions of these BBE-like enzymes becomes even more complex if plants with larger BBE-like families are considered such as, for example, the Western Poplar (*Populus trichocarpa*), which comprises 64 BBE-like members (Wallner et al., 2012).

OXIDASES OF MONO- AND OLIGOSACCHARIDES IN PLANTS AND MICROBES

Oxidases of mono- and oligosaccharides are widespread among plants and microbes but only in a few cases their physiological role has been investigated. To date, OGs, CDs, XG, and AX fragments, 1,3-β-d-$(Glc)_6$ and MOS are the oligosaccharides shown to display a DAMP activity (see above). However, FAD-dependent oxidases have only been found for two of these oligosaccharides (OGs and CDs). On the other hand, (oligo)saccharides that apparently do not have DAMP activity have been found to be substrates of enzymatic oxidation both in plants and in microbes, such as *Gibberella zeae*, *Sarocladium strictum*, and *Myceliophthora thermophila*, as well as in the moss *Physcomitrella patens* (**Table 1**; **Figure 4**; Toplak et al., 2018). *Sarocladium strictum*, for example, produces mono- and disaccharide oxidases as well as gluco- and xylo-oligosaccharide oxidases which, despite sharing a high sequence identity with each other, are characterized by different substrate specificities (Lee et al., 2005; Vuong et al., 2013; **Table 1**). An enzyme from *G. zeae* (**Figure 4**; Heuts et al., 2007) oxidizes chito-oligosaccharides derived from fungal cell walls that are known to be powerful elicitors of plant defense (Wan et al., 2008). However, it is not known whether the chito-oligomers lose their elicitor activity upon oxidation as in the case OGs and CDs. Finally, an oxidase from *M. thermophila* exhibits a strong substrate preference toward xylo-oligosaccharides (Ferrari et al., 2016).

A potential physiological role of microbial FAD-dependent oligosaccharide oxidases is to provide H_2O_2 to support the hydrolysis of substrates, such as crystalline cellulose (Villares et al., 2017), chitin, and xylan by enzymes referred to as lytic polysaccharide mono-oxygenases (LPMOs; Nakagawa et al., 2015; Couturier et al., 2018). The copper-containing active site of LPMOs must be reduced after each oxidative-cleavage reaction and electrons may be restored by the H_2O_2 produced by the FAD-depended enzymes (Filandr et al., 2020; Giovannoni et al., 2020).

In conclusion, oxidases of mono- and oligosaccharides are widely distributed among plants and microbes, but their role is difficult to decipher and may depend on the nature of the producing organism, the substrate specificity of the enzyme, and the metabolic role of the substrate. The plant-encoded oxidizing enzymes acting on DAMPs can attenuate

TABLE 1 | Berberine bridge enzyme-like (BBE-like) proteins and FAD-linked oxidases.

Identification code	Gene/protein	Substrate	Organism	Reference
At1g01980	BBE1/OGOX4	Oligogalacturonides	*Arabidopsis thaliana*	Benedetti et al., 2018
At1g11770	BBE2/OGOX3			
At1g26380	BBE3/Fad-linked Oxidoreductase 1	Indole-cyanohydrin		Rajniak et al., 2015
At1g26390	BBE4	nd		na
At1g26400	BBE5			
At1g26410	BBE6			
At1g26420	BBE7			
At1g30700	BBE8			Rodrigues Oblessuc et al., 2019
At1g30710	BBE9			na
At1g30720	BBE10			
At1g30730	BBE11			
At1g30740	BBE12			
At1g30760	BBE13/Monolignol-oxidase 1	Coumaryl alcohol		Daniel et al., 2015
		Sinapyl alcohol		
		Coniferyl alcohol		
At1g34575	BBE14	nd		na
At2g34790	BBE15/Monolignol-oxidase 2	Coumaryl alcohol		Daniel et al., 2015
		Sinapyl alcohol		
		Coniferyl alcohol		
At2g34810	BBE16	nd		na
At4g20800	BBE17			
At4g20820	BBE18			
At4g20830	BBE19-BBE20/OGOX1	Oligogalacturonides		Benedetti et al., 2018
		Tri-galacturonic acid		
		Di-galacturonic acid		
At4g20840	BBE21/OGOX2	Oligogalacturonides		
At4g20860	BBE22/CELLOX	Cellohexaose		Locci et al., 2019
		Cellopentaose		
		Cellotetraose		
		Cellotriose		
		Cellobiose		
At5g44360	BBE23	nd		na
At5g44380	BBE24			
At5g44390	BBE25			
At5g44400	BBE26			
At5g44410	BBE27			
At5g44440	BBE28			Daniel et al., 2016
A0A2K1JP57	BBE1/Cellobiose-oxidase	Lactose	*Physcomitrella patens*	Toplak et al., 2018
		Cellobiose		
Q84N20 + Q84N21	NEC5/Nectarin 5	D-Glucose	*Nicotiana langsdorffii*	Carter and Thornburg, 2004
			X	
			Nicotiana sanderae	
Q8SA60	Carbohydrate oxidase	Cellopentaose	*Lactuca sativa*	Custers et al., 2004
Q8SA59		Cellotetraose	*Helianthus annuus*	
		Cellotriose		
		Cellobiose		
		Lactose		
		Maltose		
		D-Glucose		
		D-Galactose		
		D-Mannose		
		D-Atrose		
P30986	BBE1/Reticuline oxidase	(S)-reticuline	*Eschscholzia californica*	Winkler et al., 2006

(Continued)

TABLE 1 | Continued

Identification code	Gene/protein	Substrate	Organism	Reference
A0A067GII5	CISIN_1g043104mg/uronic acid oxidase	D-Galacturonic acid	*Citrus sinensis*	Wei et al., 2020
Q6PW77	Gluco-oligosaccharide oxidase	Cellopentaose Cellotetraose Cellotriose Cellobiose Maltoheptaose Maltohexaose Maltopentaose Maltotriose Maltobiose Maltose Lactose D-Glucose	*Sarocladium strictum*	Lee et al., 2005
D7PF15	Gluco-oligosaccharide oxidase-VN	Xylotriose Xylobiose Cellotriose Cellobiose Maltose D-Glucose D-Mannose N-acetyl-Glucosamine D-Galactose D-Xylose		Foumani et al., 2011
I1S2K2	Chito-oligosaccharide oxidase	Chitotetraose Chitotriose Chitobiose Cellotetraose Cellotriose Cellobiose Lactose Maltose D-Glucose N-acetyl-Glucosamine	*Gibberella zeae*	Heuts et al., 2007
P0CS93	Galactose oxidase	D-Galactose Methyl-D-Galactose Dihydroxyacetone Lactose Raffinose Melibiose Lactobionic acid		Choosri et al., 2011
G2QG48	Xylo-oligosaccharide oxidase	Xylotetraose Xylotriose Xylobiose Lactose Cellobiose D-Xylose	*Myceliophthora thermophila*	Ferrari et al., 2016

(Continued)

TABLE 1 | Continued

Identification code	Gene/protein	Substrate	Organism	Reference
I1SB12	Carbohydrate oxidase	Maltose	*Microdochium nivale*	Kulys et al., 2001
		Cellobiose		
		Lactose		
		D-Glucose		
		Deoxy-Glucose		
		D-Galactose		
		D-Mannose		
		D-Xylose		
P93762	Hexose (and Disaccharide) Oxidase	Cellobiose	*Chondrus crispus*	Groen et al., 1997
		Maltose		
		Lactose		
		D-Glucose		
		D-Galactose		

Nomenclature of BBE-like proteins from *Arabidopsis thaliana* is in accordance with TAIR database (www.arabidopsis.org/) whereas the other FAD-linked oxidases are identified by their UNIPROT code (www.uniprot.org/). The substrate of each flavoenzyme is indicated. The amino acid sequences were used to generate the protein homology tree shown in **Figure 4**. (CELLOX, Cellodextrin Oxidase; nd, not determined; na, not available; OGOX, Oligogalacturonide Oxidase).

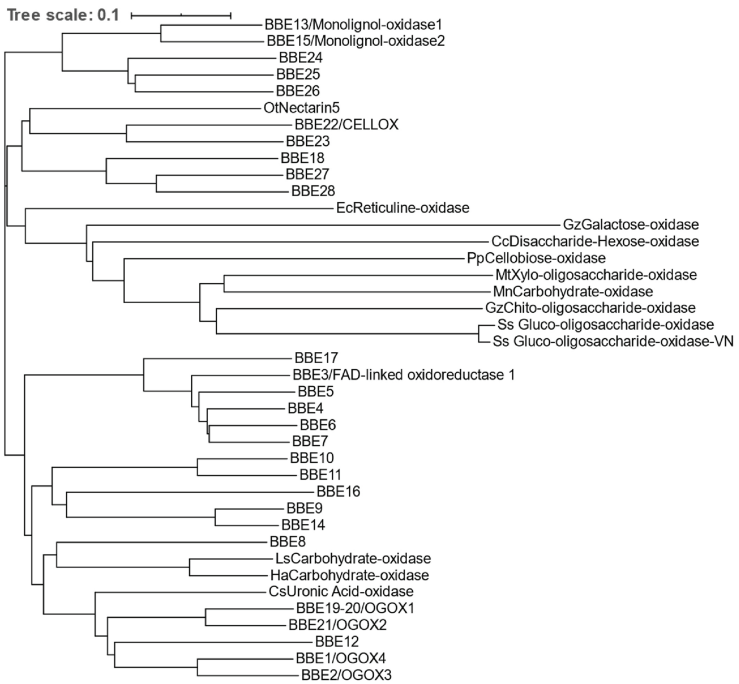

FIGURE 4 | Homology tree of BBE-like proteins and FAD-linked oxidases. The BBE-like superfamily of *A. thaliana* (BBE1-28) and FAD-linked oxidases with mono- and oligo-saccharide oxidase activity from fungi, moss, and plants are indicated in the protein homology tree. Reticuline oxidase from *Eschscholzia californica* is also indicated. The identification code of each flavoprotein is reported in **Table 1**. (Cc, *Chondrus crispus*; Cs, *Citrus sinensis*; Ec, *Eschscholzia californica*; Gz, *Gibberella zeae*; Ha, *Helianthus annuus*; Ls, *Lactuca sativa*; Mn, *Microdochium nivale*; Mt, *Myceliophthora thermophila*; Ot, Ornamental tobacco – *Nicotiana langsdorffii x N. sanderae*; Pp, *Physcomitrella patens*; Ss, *Sarocladium strictum*; CELLOX, cellodextrin oxidase; and OGOX, oligogalacturonide oxidase).

the excessive activity of these elicitors of defense. The same kind of enzymes produced by pathogens may sustain the activity of microbial LPMOs in the degradation of resistant cell wall substrates such as crystalline cellulose. Pathogenic microorganisms may recruit the plant derived FAD-dependent oxidases to sustain the activity of LPMOs and cause a diversion (hijacking) of the host responses into an advantageous trait for the pathogens.

FINAL CONSIDERATIONS: THE ROLE OF OXIDASES IN THE MAINTENANCE OF SIGNAL HOMEOSTASIS AND GROWTH-DEFENSE TRADE-OFF

As a co-product of the enzymatic oxidation of OGs, CDs, and possibly of other DAMPs, H_2O_2 is expected to be present temporally and locally in limited zones of the tissues, where a breach in the wall is generated. This may occur upon local injury caused by biotic or abiotic stress or upon a localized loss of CWI during growth and development like, for example, during the formation of lateral roots (Peretto et al., 1992). It is known that H_2O_2 is involved not only in the strengthening and repairing of the plant tissues (Farvardin et al., 2020) but also in signaling during both immunity and development (Mhamdi and Van Breusegem, 2018; Huang et al., 2019; Sies and Jones, 2020). Spatial distribution of different ROS and their finely-tuned balance drive plant morphogenetic processes (Mhamdi and Van Breusegem, 2018). It can be argued that the enzymatic oxidation of wall polysaccharide fragments by the FAD-dependent oxidases is also a mechanism for a strictly localized production of limited amounts of H_2O_2. This production of H_2O_2 is expected to occur only where breaks are made by CWDEs in the wall, and therefore only in one or few cells, and may have a biological significance, for example, as a signal for a very short range cell-cell communication. In addition, the oxidation of the reducing end of the cell wall polysaccharides may hamper the trans-glycosylation that is critical for cell expansion (Franková and Fry, 2020), thereby contributing to the growth-defense trade-off.

Both OGs and CDs play a dual function as elicitors of plant immunity and as a carbon source sustaining the growth of phytopathogenic microbes. Oxidation of these oligosaccharides protects against hyper-immunity and blocks pathogen growth by making them more difficult to metabolize. Consequently, transgenic Arabidopsis plants overexpressing OGOX1 and CELLOX are more resistant to *B. cinerea* because the fungus does not grow well when fed with a mixture of oxidized oligosaccharides (Benedetti et al., 2018; Locci et al., 2019). It is relevant, in this context, that a marked accumulation of oxidized di-galacturonic acid has been detected as a final product of tissue degradation in Arabidopsis plants infected with *B. cinerea* (Voxeur et al., 2019). The recalcitrance to enzymatic hydrolysis of the oxidized oligosaccharides is also favored by the basification of the apoplastic pH normally occurring as a defense response to microbial attacks. OGOXs and CELLOX display a high optimum pH of activity while the microbial pectinases and cellulases display an optimal activity between pH 3 and 6.

The recognition of DAMPs by pattern recognition receptors is thought to occur ubiquitously across the tree of life (Heil and Land, 2014; De Lorenzo et al., 2018). In mammals and plants, DAMP-mediated immunity exhibits both common and divergent features (De Lorenzo et al., 2018). Elicitors such as DAMPs and MAMPs elicit what is referred to as PTI. Attenuation or suppression of PTI is expected to protect the organism from the deleterious effects of hyperactivated immunity that negatively affect growth (Li et al., 2020). Currently, attenuation of PTI, through phosphorylation and dephosphorylation of the MAPKs, protein degradation by the proteasome, or regulation of WRKY transcription factors is known to occur downstream of MAMP perception (Li et al., 2020). However, not much is known about the attenuation/clearance of DAMPs that must occur to avoid a continuous activation of defense once a corresponding danger has ceased to exist. Moreover, the homeostasis of endogenous molecules that normally have a physiological role and become DAMPs upon pathogen attack needs to be quickly restored, as in the case of OGs that are released in plants not only upon microbial attacks but also in the healthy plants possibly to regulate growth and development in concert with hormones (Pontiggia et al., 2015).

The complexity of the cell wall does not allow an easy *in vivo* investigation of the release, activity, or degradation of oligosaccharide fragments. To this purpose, an excellent tool by which the cause-effect of the biological activity of OGs has been clearly documented is the "OG-machine" expressed in Arabidopsis. Using these plants, it is possible to release elicitor-active OGs under the control of an inducible promoter. Released OGs *in vivo* activate immunity, confer protection against fungal and bacterial pathogens, and, if accumulated in excess, cause an increase of salicylic acid, reduction of growth, leaf discoloration, and chlorosis and, finally, cell death; i.e., a typical hypersensitive response (**Figure 5**; Benedetti et al., 2015). The first oxidase specifically

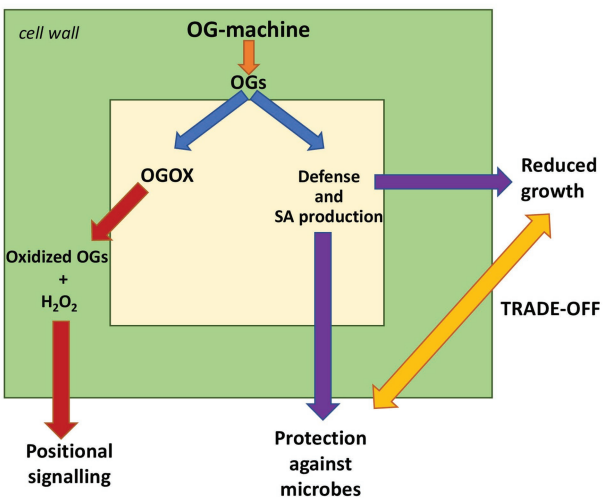

FIGURE 5 | Cellular responses to the "OG-machine" action. The plants expressing the OG-machine under an inducible promoter produce OGs on command. These activate defenses and salicylic acid (SA) formation that, on one side, confer protection against pathogenic fungi and bacteria and, on the other side, reduce the plant growth (trade-off). Excess of accumulated OGs causes cell death (hyper-immunity). Homeostasis of OGs is maintained by an oxidase (OGOX) producing hydrogen peroxide that in turn may act as a transduction signal. Oxidized OGs cannot be utilized by some microbes as a carbon source; this contributes to plant resistance.

acting on DAMPs was identified in these plants, where the enzyme is constitutively expressed (Benedetti et al., 2018). The same plants not only express high levels of OG-oxidases but also an oxidase acting on CDs (Locci et al., 2019). Notably, recent studies show that cellulose and pectin interact (Du et al., 2020; Palacio-Lopez et al., 2020) and that cellulose microfibers and HG nanofilaments form a single cohesive network (Haas et al., 2020; Zhang and Zhang, 2020).

Oxidases can attenuate DAMP activity of OGs and CDs but a relevant question is why plants have evolved such a type of activity if they can potentially mitigate or abolish the DAMP signal by using the vast variety of endogenous degrading enzymes (pectinases and cellulases). One possible answer is found in the evidence that the final products of hydrolysis of pectin and cellulose are utilized as a carbon source by microbes, and therefore may contribute to plant susceptibility. The combination of hydrolases and oxidases, instead, produces oligosaccharide fragments that cannot be readily catabolized by microbes and, consequently, contributes to enhance the plant resistance (Locci et al., 2019).

In humans, inflammation is not only caused by a hyper-accumulation of inducible DAMPs but also by an imbalance of suppression/inhibition of DAMPs or by an insufficient generation of the so-called "SAMPs," where "S" stands for "suppressing DAMPs" (Land, 2018; Relja and Land, 2020). SAMPs include molecules such as prostaglandin E2 (PGE2), annexin A1 (AnxA1), and specialized pro-resolving mediators (SPMs; Land, 2018). The attention on SAMPs is increasing because of their potential therapeutic applications in pathological processes of hyper-inflammation like those currently observed in patients affected by COVID-19, and which, in certain cases, may lead to severe autoimmune disorders (Gallo et al., 2015; Land, 2020). DAMP oxidases can be considered in the broadest sense as DAMP suppressors: i.e. SAMPs that maintain a balanced level of signals in plants. The identification of plant SAMPs may have significant biotechnological applications to overcome the limitations imposed by the growth/defense trade-off and by an excessive accumulation of DAMPs causing a deleterious hyper-immunity.

AUTHOR CONTRIBUTIONS

DP, MB, and SC contributed to writing. FC and GL supervised the work and edited the final version of the paper. All authors contributed to the article and approved the submitted version.

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Effect of Fragmented DNA From Plant Pathogens on the Protection Against Wilt and Root Rot of *Capsicum annuum* L. Plants

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Chili pepper (*Capsicum annuum* L.) production is affected by wilt and root rot, the most devastating disease caused by the pathogen complex of oomycete *Phytophthora capsici* Leon and the fungi *Fusarium oxysporum* Schlecht and *Rhizoctonia solani* Kühn, infecting roots, stems, leaves, and fruits. Fungicides are currently inefficient against this disease and have a high environmental impact. The use of elicitors is a sustainable alternative for inducing resistance to wilting and root rot. DNA fragments of an organism's own origin (conspecific or self-DNA) have shown the ability to inhibit growth and activate defense mechanisms in some plant species. In this investigation, the effect of the fragmented DNA mixture of *Phytophthora capsici* L., *Fusarium oxysporum* S., and *Rhizoctonia solani* K. on the protection against wilt and root rot of *Capsicum annuum* L. plants was evaluated. Changes in plant performance, phenolics, and flavonoids contents, as well as gene expression involved in the production of defense metabolites after the fragmented and unfragmented DNA mixture in three concentrations (20, 60, and 100 μg mL^{-1}) in chili peppers, were studied. The results obtained showed a decrease in plant height in 60 and 100 μg mL^{-1} concentrations in absence of pathogens. Moreover, the treatment with fragmented DNA 100 μg mL^{-1} showed significant increase in the content of phenolic compounds and total flavonoids as well as gene expression associated to plant defense in comparison with control plants. Interestingly, foliar application of DNA fragments of the pathogen complex to a concentration of 100 μg mL^{-1} caused a 40% decrease in the mortality of infected plants with the pathogens at 30 days post-inoculation compared with control plants inoculated with the pathogen complex but not sprayed with DNA fragments. These results suggested a perspective for application of fragmented DNA of these pathogens at the agricultural level in crop protection strategies to cope with wilt and root rot in *Capsicum*.

Keywords: Phytophthora capsici L., Fusarium oxysporum, Rhizoctonia solani, wilt, elicitors, Capsicum annuum

INTRODUCTION

Chili pepper (*Capsicum annuum* L.) belongs to the *Solanaceae* family; according to specialists, it is native to Mexico and one of the main vegetables worldwide (Mejía-Teniente et al., 2013). In Mexico the production of chili pepper in 2018 reached 3.29 million tons (SIAP, 2018). The *Capsicum* genus is made up of 22 species of which five have been domesticated: *Capsicum baccatum*, *Capsicum chinense* (habanero), *Capsicum pubescens* (apple or peron), *Capsicum frutescens*, and *C. annuum* (jalapeño, serrano, black chili, width, walkway, and tree). The latter species is considered the most important because it groups the greatest diversity of cultivated or wild chili peppers (Mejía-Teniente et al., 2019). The chemical composition of *Capsicum annuum* L. fruits has been extensively studied, managing to identify compounds such as capsaicinoids, fatty acids, anthocyanins, glucosides, carotenoids, organic acids, aldehydes, ketones, alcohols, ethers, and sulfur compounds (Villa-Ruano et al., 2019).

The profitability of the crop has been seriously threatened for several decades by the disease known as chili pepper wilt and root rot, which causes premature death of the plant and is one of the most important phytosanitary problems due to the level of devastation and its dispersion in all producing areas of the world (Morán-Bañuelos et al., 2010). It is caused by the pathogen complex of the oomycete *Phytophthora capsici* Leon and the fungi *Fusarium oxysporum* Schlecht and *Rhizoctonia solani* Kühn, infecting both roots, stems, leaves, and fruits (Kim et al., 1997). These pathogens can cause yield losses of up to 100% (García-Rodríguez et al., 2010). The manifestation of the first symptoms is the wilting of the leaves, preserving the color, and hanging from the petioles to the plant; at the root there is a soft, odorless rot, until it reaches its loss or detachment (González-Chavira et al., 2009). At the base of the stem there is a brown stain that as the disease progresses becomes black, causing tissue necrosis and external injuries such as sunken cancers that gradually strangle the stem; the stems are kept upright with the hanging leaves, the nuts, and wrinkled (González-Chavira et al., 2009; Uribe-Lorío et al., 2014).

Due to the etiology of the pathological complex, it is difficult to establish the specific epidemiological cycle of infection in the plant and, therefore, it is difficult to establish a phytosanitary management program; adding to this, the high levels of genetic variability of the pathogens responsible for the disease (Walker and Bosland, 1999; Montero-Tavera et al., 2013). Among the main strategies for the control of this disease, cultivation practices that ensure well-drained soils, crop rotation, soil solarization, and chemical control are recommended (Parra and Ristaino, 2001; Hausbeck and Lamour, 2004; Piccini et al., 2019). However, a certain degree of resistance to fungicides has been developed; thus, these chemicals cannot protect sensitive crops from resistant pathogens (Piccini et al., 2019). Due to this situation, new alternatives for control have been sought, highlighting the use of rhizospheric fungi antagonistic to *Fusarium* spp., *R. solani*, and *P. capsici* L. (Jinag et al., 2006; Ramos-Sandoval et al., 2010; Robles-Yerena et al., 2010), the use of rhizobacteria antagonistic to *P. capsici* (Sang et al., 2008; Sang and Kim, 2012), inoculation with rhizospheric mycorrhizal fungi (Ozgonen and Erkilic, 2007), as well as the combined use of biofumigation and antagonistic microorganisms (Wang et al., 2014) and crop rotation (Lamour and Hausbeck, 2003). Another alternative has been the combined use of compost and grafts in resistant patterns (Gilardi et al., 2013). Currently, there are no varieties of *C. annuum* L. with total resistance to this pathogenic complex and the strategy of applying fungicides is followed, although the use of germplasm with possible resistance to these pathogens has been attempted (Chew et al., 2008; Babu et al., 2011; Koc et al., 2011). Based on the aforementioned, to cope with plant diseases it is important to have a strategy that minimizes or eliminates possible side-effects on other organisms different to the targets, as well as to avoid environmental contamination and the induction of resistance in the organisms to be controlled. A possibility for crop protection from diseases complying to these latter features is the management of the plant immune system using stress factors in an adequate dose (eustressic dose) using a controlled elicitation strategy (Vázquez-Hernández et al., 2019). Controlled elicitation has been successfully used in chili pepper to cope with geminivirus diseases (Mejía-Teniente et al., 2019).

The plant immune response is triggered by the recognition of exogenous molecules called molecular patterns associated with pathogens or microbes (PAMP: Pathogen-Associated Molecular Pattern/MAMP: Microbe-Associated Molecular Pattern) through transmembrane receptor proteins called pattern recognition receptors (PRR), which are found on the surface of plant cells and are responsible for guiding immunity to microbial infection in all plant species (Chisholm et al., 2006; Jones and Dangl, 2006; Bittel and Robatzek, 2007; Boller and Felix, 2009; Postel and Kemmerling, 2009; Bhat and Ryu, 2016; Choi and Klessig, 2016; Ferrusquía-Jiménez et al., 2020). PRR stimulation activates immunity triggered by PAMP (PTI: Pattern Triggered Immunity) (Dodds and Rathjen, 2010). Beside PAMPs and MAMPs, the plant defense system can also recognize endogenous signal molecules derived from the plant called "damage-associated molecular patterns" (DAMP) inducing immune responses (Seong and Matzinger, 2004; Mackey and McFall, 2006; Boller and Felix, 2009). PRRs perceive DAMPs or PAMPs/MAMPs and play an important role in resistance to pathogens (Zipfel, 2014), triggering responses mediated by jasmonic acid (JA) and salicylic acid (SA), thus also presenting resistance to herbivores (Duran-Flores and Heil, 2014; Ross et al., 2014).

A novel and interesting approach to be tested in controlled elicitation strategies is the use of randomly fragmented nucleic acids as suggested by Mazzoleni et al., 2014. Studies with litter decomposition identified that a general occurrence of litter autotoxicity was related to the exposure to self-DNA fragments, displaying species-specific inhibitory effects on plants by reduction of conspecific root growth and seed germination without affecting heterospecifics (Mazzoleni et al., 2015a). Further, these effects were also probed in bacteria, fungi, algae, protozoa, and insects, thus seeming to be a general biological process (Mazzoleni et al., 2015b). Moreover, based on these findings, the use of fragmented self-DNA for biological control has been proposed (Mazzoleni et al., 2014). Since that discovery, relevant evidence of the immunogenic function of extracellular DNA (eDNA) as immunity activator has been reported in

plants (Barbero et al., 2016; Duran-Flores and Heil, 2018; Vega-Muñoz et al., 2018; Kawasaki, 2019). Based on this idea, on one hand, plants could recognize exogenous DNA from pathogen microorganisms and activate immunity (i.e., DNA acting as PAMP) (Ferrusquía-Jiménez et al., 2020). On the other hand, fragments of nuclear or mitochondrial DNA molecules that appear in the extracellular or cytosolic compartment indicating cellular dysfunction or damage, including loss of integrity of nuclei, mitochondria, or whole cells, (i.e., DNA acting as DAMP) can also be recognized (Vega-Muñoz et al., 2018). Duran-Flores and Heil (2018) reported that the application of fragmented self-DNA, but not heterologous DNA, generated a stronger immune response than the application of DNA from other species.

There are several genes related to plant defense, among which are phenylalanine ammonium lyase (*pal*), chalcone synthase (*chs*), manganese superoxide dismutase (Mn-*sod*), peroxidases (*pox*), glucanases (*glu*), and chitinases (*chi*) (Fernández-Herrera et al., 2012; Sudisha et al., 2012; Villar-Luna et al., 2015). Phenylalanine ammonium lyase (PAL) and chalcone synthase (CHS) are regulatory enzymes for the synthesis of phenylpropanoids and flavonoids in different plant tissues, respectively. Higher gene expression and activity of both enzymes is strongly related to resistant genotypes in several plant species against several diseases (Richins et al., 2010; Mejía-Teniente et al., 2013, 2019; Chávez-Díaz and Zavaleta-Mejía, 2019). Additionally, in response to stress, plants usually increase gene expression and activity of enzymes producing reactive oxygen species (ROS) (as superoxide dismutases) and those scavenging them (peroxidases or catalases) as a modulating system to establish an adequate stress response (Rodríguez-Calzada et al., 2019). In the present work, the effect of the fragmented DNA mixture of the pathogenic complex including *Phytophthora capsici* L., *Fusarium oxysporum* S., and *Rhizoctonia solani* K. on the protection against wilt and root rot of *Capsicum annuum* L. plants was evaluated. The results are discussed in regard the potential application of this strategy in chili pepper protection against wilt and root rot disease.

MATERIALS AND METHODS

Biological Material

In vivo tests were carried out with seedlings of jalapeño pepper (*Capsicum annuum* L.), Gladiator MSC 983 variety from the MARSEED Company seed house, a variety susceptible to chili pepper wilt and root rot. Seedlings 45 days post germination were used, with 10–12 true leaves. They were transplanted into 1-L capacity unicel cups; sterile Sunshine 3M was used as substrate in greenhouse conditions, and their acclimatization was 7 days after transplantation.

Pathogen complex used in this study were isolated from Guanajuato state in Central Mexico affecting chili pepper plants with wilt and root rot disease. The pathogens were identified as *Phytophthora capsici* L. (identified using the method reported by Mitchell and Kannwischer-Mitchell, 1995 and the PCR strategy for species as reported by Silvar et al., 2005), *Fusarium oxysporum* F. Sp. capsici (Velarde-Félix et al., 2018), and *Rhizoctonia solani* (anastomosis group GA4, Montero-Tavera et al., 2013), provided by the Molecular Markers Laboratory of the National Institute of Agricultural and Livestock Forest Research (INIFAP) Campo Experimental Bajío.

Extraction of Genomic DNA From *Phytophthora capsici* L, *Fusarium oxysporum*, and *Rhizoctonia solani*

Five disks (8 mm diameter) of potato dextrose agar medium with mycelium of each pathogen were placed in potato dextrose broth medium acidified with lactic acid at pH 4.0. Growth of the pathogens was carried out using incubation at 28°C, 180 rpm for 4 days for *P. capsici* and *R. Solani*. In the case of *F. oxysporum*, it was grown in aeration at room temperature. The mycelium was subsequently filtered through a nylon blanket, allowed to dry to remove excess moisture, and frozen. DNA extraction was performed using the CTAB method (Doyle and Doyle, 1987). From the frozen mycelium, it was macerated in a mortar with a 1:2 ratio of extraction buffer (CTAB 2%, 50 mM tris–HCl pH 8.0, 1.4 M NaCl, and 10 mM EDTA). The concentration was verified in the spectrophotometer (Multiskan GO) at 260 and 280 nm, and DNA integrity was verified by horizontal electrophoresis in 1.5% agarose and SB 1X buffer at 100 V for 20 min stained with SYBR Safe (Invitrogen), and displayed on the photo documentation (MiniBIS Pro).

Genomic DNA Fragmentation

DNA fragmentation was performed using the Sonics Vibra Cell VCX130 sonotrode with a pulse emission per second of 26 KHz at 10 W, with an amplitude of 50%, for 10 min. The fragments were visualized in a 1.5% agarose gel in SB 1X buffer and stained with Gel red (Biotium) to determine the range of fragmentation obtained.

Application of DNA Fragments

A mixture of fragmented DNA of *Phytophthora capsici* L., *Fusarium oxysporum* S., and *Rhizoctonia solani* K. was applied once by direct spraying on the leaves in three different concentrations (20, 60, and 100 $\mu g\ mL^{-1}$). Fifteen plants with 10–12 true leaves and 7 days of transplant were used for each treatment. A treatment with unfragmented DNA consisting only with the genomic DNA obtained with the aforementioned methodology and not sonicated in order to have high-weight genomic DNA was used as another experimental control in the work. **Table 1** shows the distribution of treatments.

Inoculation of Plants With the Pathogenic Complex *Phytophthora capsici* L., *Fusarium oxysporum*, and *Rhizoctonia solani*

After 24 h of the application of the mixture of DNA fragments, the inoculation with the pathogen complex (CF) was carried out, for which a spore suspension of *F. oxysporum* was prepared based on the methodologies described by Kim et al. (1997) and Fernández-Herrera et al. (2007). The fungus was seeded in Petri dishes with

TABLE 1 | Design of experiments for the application of DNA fragments to pathogens.

	DNA fragments of the pathogen complex (DNA_{FCF})						
	20 $\mu g\ mL^{-1}$	60 $\mu g\ mL^{-1}$	100 $\mu g\ mL^{-1}$	20 $\mu g\ mL^{-1}$	60 $\mu g\ mL^{-1}$	100 $\mu g\ mL^{-1}$	
Pathogen complex	DNA_{FCF20} + CF	DNA_{FCF60} + CF	DNA_{FCF100} + CF				P_{CF}
Pathogen free				DNA_{FCF20}	DNA_{FCF60}	DNA_{FCF100}	P_h

Subscript nomenclature: FCF, mixture of DNA fragments from the pathogen complex; CF, pathogen complex; P_{CF}, Control of plants infected with the pathogen complex; P_h, Control healthy plants; 20, 60, and 100, concentration of the mixture of the DNA fragments of the pathogen complex given in $\mu g\ mL^{-1}$. As controls, the same fungi DNAs were applied unfragmented in the same concentrations onto the plants to compare results.

potato dextrose (PD) agar medium; once it covered the entire box, 20 mL of sterile distilled water were added, and then with a scalpel the mycelium was scraped to separate the conidia; it was filtered to retain the mycelium and only allow the passage of the conidia; the filtrate product was collected and adjusted to a concentration of 1×10^6 spores, determining the concentration with a hemacytometer. From *P. capsici*, a suspension of 1×10^5 zoospores was added. The production of zoospores consisted of placing a disk of mycelium in PD broth, and then incubated in the dark for 15 days at 25°C with shaking at 120 rpm. Then, the mycelium was separated and washed three times with sterile distilled water, and incubated in sterile distilled water with agitation for 5 days; finally the concentration was adjusted with a hemocytometer Ezziyyani et al. (2004). In the case of *R. solani*, it was performed according to the methodology described by Büttner et al. (2004) and Berdugo et al. (2010). In 250 mL of PDA medium, four 8-mm-diameter mycelial disks were placed, with constant agitation at 100 rpm and 21°C, under darkness for 4 days; the mycelium was extracted and homogenized in a mortar, and the suspension of 2 mg of mycelium per mL of sterile distilled water was adjusted. Finally a concentration of 20 mg per plant was placed. For inoculation, 1 mL of the spore suspensions of *P. capsici* (final concentration of 1×10^6 zoospores) and *F. oxysporum* (final concentration of 1×10^5 spores) was placed on the stem base (neck) of the plant, as well as 20 mg of collected mycelium of *R. solani* (suspended in 10 mL of sterile distilled water). Once the three pathogens were placed, the plant neck was covered with the substrate.

Measurement of Plant Morphological Variables

The height of the plant was measured in centimeters at 7, 14, 22, and 30 days after the application of the mixture of DNA fragments, using a flexometer, measuring from the base to the apex of the plant. Thirty days after the application of the mixture of DNA fragments, the length (using a flexometer) and the weight of the roots of the chili plants were determined in the different treatments. The dry weight of the root of the different treatments was determined, drying the tissue of the plant using an oven at 60°C for a time of 24–48 h until it reached a constant weight. In all cases, three biological replicates were carried out in the study.

Severity and Incidence of the Disease

Plants were evaluated at 0, 7, 14, 22, and 30 days post inoculation (dpi) and application of treatments, to determine the proportion of infected plants in the different treatments (incidence). To measure severity, a gradual scale from 0 to 9 was used, reported by Bosland and Lindsey (1991), which takes into account the coloration of the plant, the state of the leaves, signs of disease, or if the plant is dead. On this scale, the interaction phenotype was standardized in 9 levels, where 0 = no response, vigorous, healthy (as uninoculated control); 1 = slight root darkening, vigorous, healthy; 3 = brown roots, slight stunting, very small lesions on stems; 5 = brown roots, small lesions stems, lower leaves wilted, stunted plants; 7 = brown roots, large lesions on stems, girdling, whole plant wilted, and stunted; and 9 = death. Even numbers were used for intermediate responses. A disease index value of 2 or less was considered resistant, and a value greater than 2 was susceptible. In all cases, three biological replicates were carried out in the study.

Determination of Phenols and Total Flavonoids

The determination of phenolic compounds for all the evaluated treatments was carried out at 0, 1, 5, 15, and 30 days post-application of the mixture of fragmented or unfragmented DNA, according to Mejía-Teniente et al. (2013). Briefly for the sample preparation, 50 mg of leaf tissue was collected, ground with liquid nitrogen and homogenized in 2.5 mL of absolute methanol, and protected from light, with constant stirring at 150 rpm, 20°C for 24 h. After this time, it was centrifuged for 10 min at 5000 rpm, and the supernatant was recovered and stored at −20°C in the dark. After extraction, the total phenol content was determined by the Folin-Ciocalteu method, adapted for use in microplates. The reaction mixture consisted of 20 μL of the extract, 230 μL of distilled water, and 125 μL of Folin-Ciocalteu 1N reagent; the sample was homogenized and left to stand for 5 min, and 625 μL of 20% $NaCO_3$ was added. The mixture was homogenized and left to stand for 2 h in the dark. After the resting time, 250 μL were taken to place on the microplate, and then the absorbance at 760 nm was determined on the Thermo Scientific Multiskan GO spectrophotometer. The amount of total phenols was expressed in micrograms equivalents of gallic acid per gram of fresh weight. For the determination of total flavonoids, it was performed according to Iqbal et al. (2015). For this method, 125 μL of the extract was mixed with 25 μL of 10% aluminum chloride, 25 μL of 1M potassium acetate, 375 μL of 80% methanol, and 700 μL of distilled water. This mixture was homogenized and allowed to stand 30 min at room temperature. Subsequently, the absorbance at

415 nm was determined on the Thermo Scientific Multiskan GO spectrophotometer. The amount of total flavonoids was expressed in micrograms of quercetin equivalents per gram of fresh weight. Three biological replicates were carried out in the study for these measurements.

Gene Expression Analysis

Gene expression analysis was performed using RT-qPCR of two molecular markers of phenylpropanoids and flavonoid biosynthesis (phenylalanine ammonium lyase, *pal*, GenBank accession number AF081215) and chalcone synthase, *chs* (GenBank accession number FJ705842.1), as well as a gene marker of oxidative stress response (mitochondrial manganese superoxide dismutase, Mn-*sod*, GenBank accession number AF036936.2). As a housekeeping gene control, beta-tubulin (β-*tub* GenBank accession number EF495259.1) was used. For the extraction of total RNA, the TRIzol® Reagent method (Ambion, Life Technologies) was used, following the manufacturer's methodology. Complementary DNA (cDNA) synthesis was performed using the Clontech PCR-Select cDNA subtraction package (BD Biosciences). For the amplification of these genes in the treated chili pepper plants, primers used were previously reported by Rodríguez-Calzada et al. (2019); 200 ng of cDNA was used to perform qPCR, using Maxima SYBR Green qPCR Master Mix Thermo Scientific, in the C100 Touch Thermal Cycler device, BIORAD CFX96 Real Time System brand. Amplification conditions for *pal* and β-*tub* were 5 min at 94°C, 40 cycles of 94°C for 1 min, 55°C for 1 min, and 65°C for 5 s; while for Mn-*sod* and *chs*, it was 5 min at 94°C, 40 cycles of 94°C for 1 min, 58°C for 1 min, and 65°C for 5 s. This analysis was performed on chili pepper plants treated at 0, 1, and 5 days post application of the DNA fragments. Three biological replicates were analyzed in this study for all treatments. Relative gene expression was determined using the ΔΔCt methodology (Rodríguez-Calzada et al., 2019).

Statistical Analysis

A completely randomized block design was carried out to evaluate the effect of DNA fragments of the pathogen complex as biocontrol agents of phytopathogens and the activation of defense mechanisms of chili plants in different concentrations. For gene expression, a two-way analysis of variance (ANOVA) was performed (two-way ANOVA) and the differences between treatments will be performed using the Tukey test $p = 0.05$, using the GraphPad Prism 8.0.2 software. In all the measured variables in this study, three biological replicates were analyzed and used in the statistical analysis.

RESULTS

Genomic DNA Fragmentation

DNA fragment sizes were obtained from *P. Capsici* L., *F. oxysporum* S., and *R. Solani* K. in a size range of 100–1650 bp by direct sonication (**Figure 1**), which were applied in mixture to chili pepper plants.

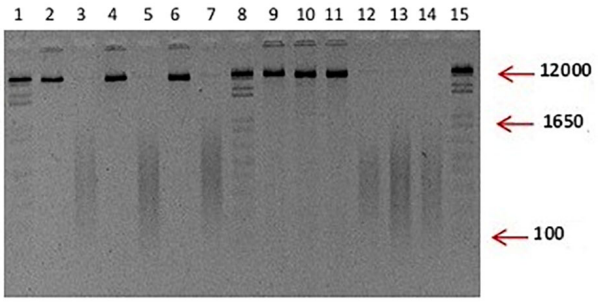

FIGURE 1 | Typical visualization of unfragmented and fragmented DNA used in the study. Integral DNA of *P. capsici* and *F. oxysporum* in lanes 2, 4, 6, 9, 10, 11. Fragmented DNA is observed in lanes 3, 5, 7, 12, 13, and 14; Lane: 1, 8, and 15 100 ng of 1 Kb plus. Although not included, the DNA of *R. solani* used in the experiments displayed the same characteristics as the ones showed for *P. capsici* and *F. oxysporum*.

Effect of Mixture DNA Fragments Application on Plant Performance, Severity, and Incidence of "Wilt and Root Rot" Disease in Chili Pepper

Plant height was determined at 7, 14, 22, and 30 days after DNA fragments application (dpa). As controls, unfragmented DNA of this pathogen complex was also evaluated in the study. The height of the plants treated with the mixture of DNA fragments of the pathogen complex (F_{CF}) is presented in **Figure 2A**. At 7 dpa, there was no significant difference among the treatments. However, at 14 dpa, a significant decrease in height for the DNA_{FCF60} + CF and DNA_{FCF100} + CF treatments was observed (**Figure 2A**). Interestingly, at 22 and 30 dpa, a significant decrease in plant height was also observed in the treatment DNA_{FCF20} + CF (**Figure 2A**).

The length and weight of the roots were also evaluated as another indicator of the disease in the chili pepper plants (**Figures 2B–D**, **3**). Treatments with the mixture of DNA fragments of the pathogen complex (DNA_{FCF20}, DNA_{FCF60}, and DNA_{FCF100}) showed that in root length and weight the plants did not show a significant difference compared to the control healthy plants (P_h) (**Figures 2B–D**, **3**). However, significant decreases in length and weight of roots were displayed in the latter treatments when the pathogen complex was inoculated onto the plants (**Figures 2B–D**, **3**).

The symptoms of the disease were observable at 5 days post-inoculation (dpi) in control plants inoculated with the pathogen complex (disease control or P_{CF}) (not shown). The percentage of dead plants at 30 days post-inoculation of the pathogen complex was 65% in disease controls (**Figure 4A**). A significant reduction in plant mortality at the same time was observed for the three fragmented DNA concentrations evaluated, showing 100 $\mu g\ mL^{-1}$ as the best treatment causing a reduction of 40% in plant mortality in comparison with disease control (**Figure 4A**). Neither control healthy plants (P_h) nor the treatments only with DNA (fragmented or not) caused plant mortality (**Figure 4B**).

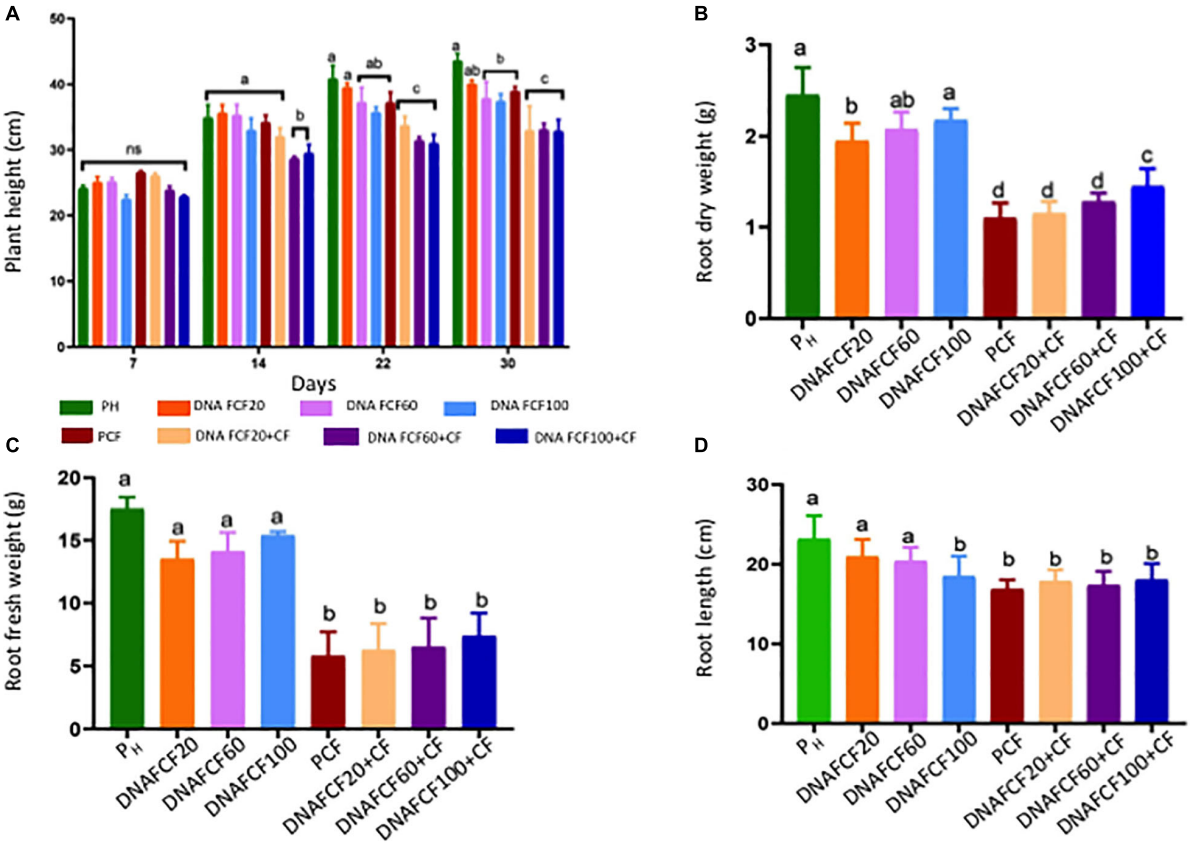

FIGURE 2 | Morphological variables in chili pepper plants evaluated in the present study. **(A)** Plant height was evaluated in different times indicated in the figure and the rest of the variables (**B–D**, respectively, corresponding to dry weight, fresh weight, and length of roots) were evaluated at 30 dpi. Average ± standard deviation data of three biological replicates is shown. Two-way ANOVA statistical analysis, Tukey test $p = 0.05$. Equal lower case letters in each bar means no significant statistical difference for each analyzed variable. Symbology: FCF, mixture of DNA fragments from the pathogen complex; CF, pathogen complex; P_{CF}, control plants infected with the pathogen complex; P_h, control healthy plants; FCF 20, 60, and 100, different concentrations of the mixture of the DNA fragments of the pathogen complex given in $\mu g\ mL^{-1}$ evaluated in the study. The expression "ns" indicates no significant difference.

In the case on non-dead plants, at 30 dpi a severity level of 8 was observed in disease control plants (P_{CF}) (**Figure 5**). In the treatments inoculated with the pathogen complex and the mixture of fragmented DNAs of the three pathogens, the severity of disease at the same time was 7, 6, and 3 for the treatments with 20, 60, and 100 $\mu g\ mL^{-1}$, respectively (**Figure 5**). Thus, the treatment 100 $\mu g\ mL^{-1}$ caused a significant reduction in symptoms severity of 60%. Moreover, plants treated with unfragmented DNA of the pathogen complex and the controls without pathogen complex displayed no severity of the fungi disease as expected (**Figure 5**). A typical phenotype of wilt and root rot symptoms in the plants as well as resistant plants in each fragmented pathogen complex DNA treatments evaluated at 30 dpi is shown in **Figure 6**.

Total Phenol and Flavonoid Levels in *C. annuum* Plants Treated With the Fragmented DNA of Pathogens

A common defense mechanism displayed by plants to cope with the attack of pathogens is the production of phenols and flavonoids and the strength of cell membranes and walls (Dotor-Robayo and Cabezas-Gutierrez, 2014). Thus, the levels of phenols and flavonoids were measured in chili peppers treated with fragmented DNA of the pathogen complex (**Figures 7**, **8**). As shown in **Figure 7**, all DNA fragment treatments as well as the pathogen complex significantly increased the total phenols in comparison with pH control plants from 1 to 30 dpa displaying Gaussian curve behavior. The DNA fragment concentration of 100 $\mu g\ mL^{-1}$ increased the highest level of phenols in all times evaluated (**Figure 7**). In the case of flavonoids, the DNA fragments treatments displayed a similar behavior as in the one described for phenol levels throughout the evaluated times (**Figure 8**). Again the concentration of 100 $\mu g\ mL^{-1}$ showed the highest increase in flavonoids (**Figure 8**).

Gene Expression Levels

The expression patterns of the *pal*, *chs*, and Mn-*sod* genes were analyzed at 0, 1, and 5 dpa in the treatments P_S, P_{CF}, DNA_{FCF100}, and DNA_{FCF100} + CF (**Figure 9**). The time

FIGURE 3 | Root of plants infected with the pathogen complex at 30 dpi. From left to right are the Positive control (C+) pathogen complex control (P_{CF}); (20), DNA_{FCF20} + CF; (60), DNA_{FCF60} + CF; (100), DNA_{FCF100} + CF; (Ctrl-), P_h; (E20) DNA_{FCF20}; (E60), DNA_{FCF60}, and (E100), DNA_{FCF100}. Symbology: FCF, mixture of DNA fragments from the pathogen complex; CF, pathogen complex; P_{CF}, control plants infected with the pathogen complex; P_h, control healthy plants; FCF 20, 60, and 100, different concentrations of the mixture of the DNA fragments of the pathogen complex given in $\mu g\ mL^{-1}$ evaluated in the study.

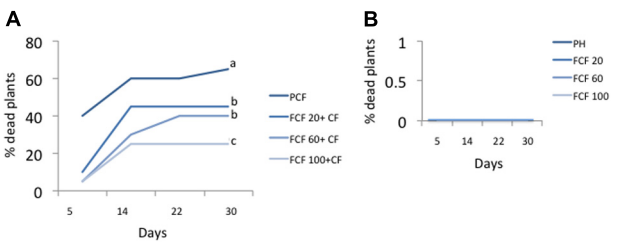

FIGURE 4 | Percentage of dead plants at 5, 14, 22, and 30 days post-inoculation. **(A)** Percentage of dead plants in treatments inoculated with the pathogenic complex (CF) and treated with pathogens DNA (FCF) at 3 different concentrations. **(B)** Percentage of dead plants in healthy plant control (uninoculated with pathogenic control) and treated with pathogens DNA. Different letters in each line at day 30 in **(A)** indicates significant statistical difference according to Turkey test ($p = 0.05$). Simbology: FCF, mixture of DNA fragments from the pathogen complex; CF, pathogen complex; P_{CF}, control plants infected with the pathogen complex; P_h, Control healthy plants; 20, 60, and 100: concentrations of the mixture of the DNA fragments of the pathogen complex given in $\mu g\ mL^{-1}$. The percentage of dead plants for the treatments with un-fragmented DNA had the same values as those shown in **(B)** (data not shown).

intervals evaluated were chosen based on previous studies in our group in chili pepper in response to other stress response factor (hydrogen peroxide) protecting chili peppers against geminivirus disease (Mejía-Teniente et al., 2019). The highest level of *pal* gene expression at 1 dpa was shown by the DNA_{FCF100} treatment with significant difference compared with control healthy plant (Ph), control plants inoculated with the pathogen complex (P_{CF}), and the DNA_{FCF100} + CF treatment (**Figure 9A**). Comparing the treatments DNA_{FCF100} + CF with control treatment P_{CF} no significant difference was shown. However, control of healthy plants (P_h) displayed a significant difference (**Figure 9A**). At 5 dpa the gene expression level of *pal* decreases more than 50% for the DNAFCF100 treatment, but it presents a significant difference compared to the control of healthy plants (P_h), the control of plants inoculated with the pathogen complex (P_{CF}), and the DNA_{FCF100} + CF treatment, with DNA_{FCF100} treatment showing a higher level of expression. When comparing the DNA_{FCF100} + CF treatment with the control Ph and the control P_{CF}, there was no significant difference (**Figure 9A**). For *chs* expression, a higher level was observed at 1 dpa in the DNA_{FCF100} treatment, showing a significant difference compared with the control P_h, the control P_{CF}, and the DNA_{FCF100} + CF treatment (**Figure 9B**). Comparing treatment DNA_{FCF100} + CF with control P_{CF} and the control P_h, a significant difference was observed, showing the highest level of expression for DNA_{FCF100} + CF treatment. At 5 dpa, the level of *chs* gene expression decreased for the DNA_{FCF100} treatment, with no significant difference compared to the control P_h, but showing significant difference when compared to the control P_{CF} and the DNA_{FCF100} + CF treatment. When comparing the DNA_{FCF100} + CF treatment with the control Ph, a significant decrease in the expression level was shown for the DNA_{FCF100} + CF treatment. Comparing

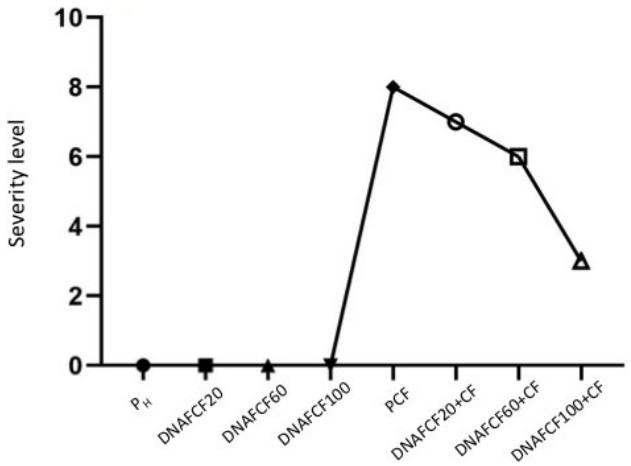

FIGURE 5 | Maximum average severity level at 30 dpi. Simbology: FCF, mixture of DNA fragments from the pathogen complex; CF, pathogen complex; P_{CF}, control plants infected with the pathogen complex; P_h, control healthy plants; FCF 20, 60 and 100, different concentrations of the mixture of the DNA fragments of the pathogen complex given in $\mu g\ mL^{-1}$ evaluated in the study.

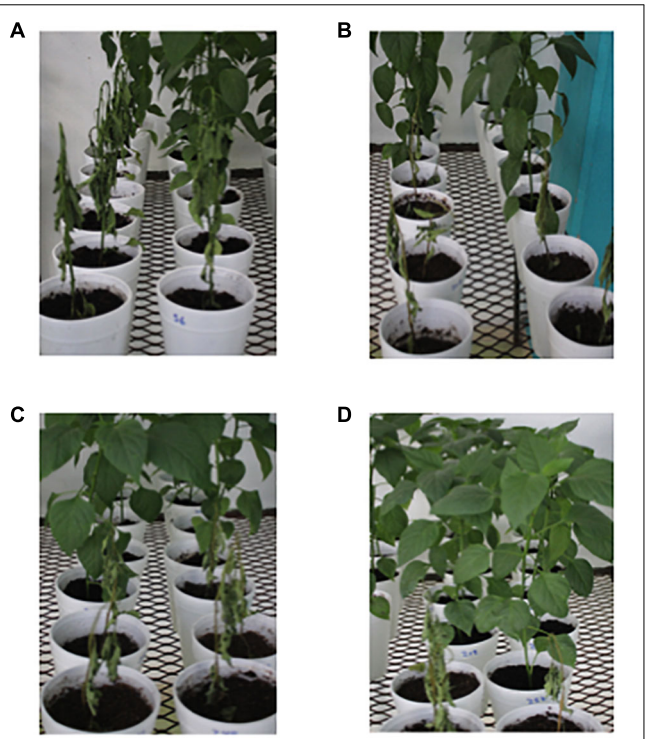

FIGURE 6 | Plants treated with the pathogen complex. **(A)** Control plants plus pathogen complex (P_{CF}) at 30 dpi. **(B)** Plants at 30 dpa of the DNA_{FCF20} + CF treatment. **(C)** Plants at 30 dpa of the DNA_{FCF60} + CF treatment. **(D)** Plants at 30 dpa of the DNA_{FCF100} + CF treatment. Symbology: FCF, mixture of DNA fragments from the pathogen complex; CF, pathogen complex; P_{CF}, control plants infected with the pathogen complex; P_h, control healthy plants; FCF 20, 60, and 100, different concentrations of the mixture of the DNA fragments of the pathogen complex given in $\mu g\ mL^{-1}$ evaluated in the study.

the DNA_{FCF100} + CF treatment with the control P_{CF}, no significant difference was shown (**Figure 9B**). Mn-*sod* did not show changes in the expression levels in control plants P_h and P_{CF} at 1 dpa, but it presented a significant difference compared with the DNA_{FCF100} and DNA_{FCF100} + CF treatments (**Figure 9C**). At 5 dpa, the DNA_{FCF100} control showed an increase in expression level with respect to 1 dpa. The level of Mn-*sod* gene expression for DNA_{FCF100} + CF decreased from 1 to 5 dpa (**Figure 9C**). In general, it was observed that Mn-*sod* showed a decrease in the expression levels in plants treated with the mixture of DNA fragments of the pathogen complex (**Figure 9**).

DISCUSSION

Recent studies have reported that extracellular self-DNA stimulates plant responses, activating the immune response such as PTI, by activating MAPKs, production of reactive oxygen species, depolarization of the membrane, activating ion flows of calcium, and thus providing resistance to plants against pathogens (Duran-Flores and Heil, 2014, 2018; Barbero et al., 2016; Hou et al., 2019). It has been reported that once the immune responses are activated in plants, there is an energy consumption affecting their growth, since several of the plant's resources are used to defend itself of the disease (Walters and Heil, 2007; Yakushiji et al., 2009; Duran-Flores and Heil, 2018; Quintana-Rodríguez et al., 2018). In our work, there was a decrease in the height and root density (root length and weight) of the plants in the treatments inoculated with the pathogens alone or together with the DNA fragments of the pathogenic complex, possibly because their resources are being used to cope with the disease and generate resistance or disease management as suggested elsewhere (Bhat and Ryu, 2016). Recent advances in uncovering signal transduction networks have revealed that defense trade-offs are often the result of regulatory "decisions" by the plant, enabling it to fine-tune its phenotype in response to diverse environmental challenges (Züst and Agrawal, 2017).

In another study, Duran-Flores and Heil (2018), with the use of DNA fragments of *Phaseolus vulgaris*, significantly decreased infections caused by *Pseudomonas syringae* in *Phaseolus vulgaris*. This latter study is a case of using DNA as DAMP for inducing plant immunity. Our study is a case of DNA as PAMP with the same goal as the report of Duran-Flores and Heil (2018) in a different plant species (*Capsicum annuum* L.). With the application of the DNA fragments of the pathogen complex, a decrease of 40% was obtained in the control of wilt and root rot in chili peppers at a concentration of 100 $\mu g\ mL^{-1}$. Our study also showed that the higher the concentration of fragmented DNA the higher the control of the disease at severity and dead plant levels. Both PAMPs and DAMPs have the ability to reprogram plants transcriptomically and metabolomically acting as amplifiers for PTI (Hou et al., 2019). The phenylpropanoid route is one of the main defense mechanisms. In this work, with the application of the DNA fragments from root rot

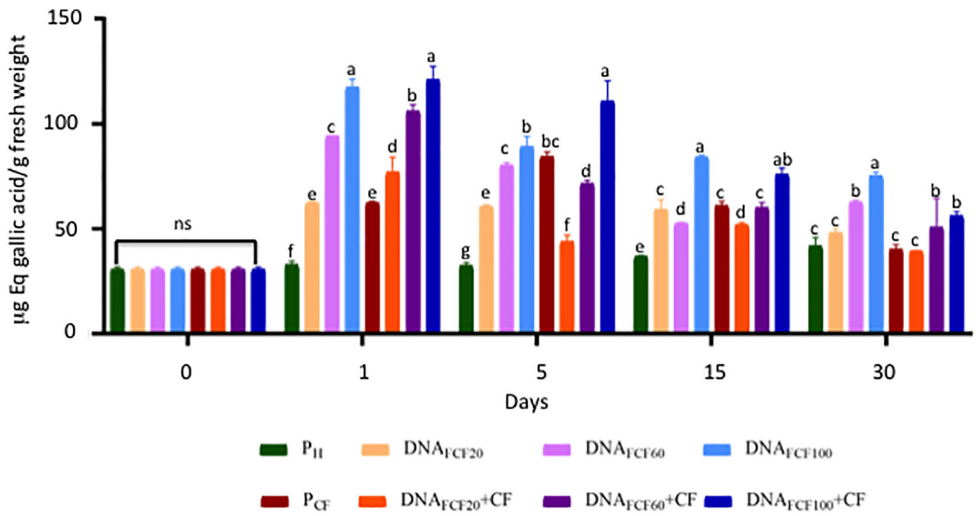

FIGURE 7 | Total phenols (equivalents of gallic acid) of the plants treated with a mixture of DNA fragments of the pathogen complex at 0, 1, 5, 15, and 30 days post-DNA application. Average ± standard deviation data of three biological replicates is shown. Equal lower case letters in each bar for each time indicates significant statistical difference according to Tukey test ($p = 0.05$). Symbology: FCF, mixture of DNA fragments from the pathogen complex; CF, pathogen complex; P_{CF}, control plants infected with the pathogen complex; P_h, control healthy plants; FCF 20, 60, and 100, different concentrations of the mixture of the DNA fragments of the pathogen complex given in µg mL^{-1} evaluated in the study. The expression "ns" indicates no significant difference.

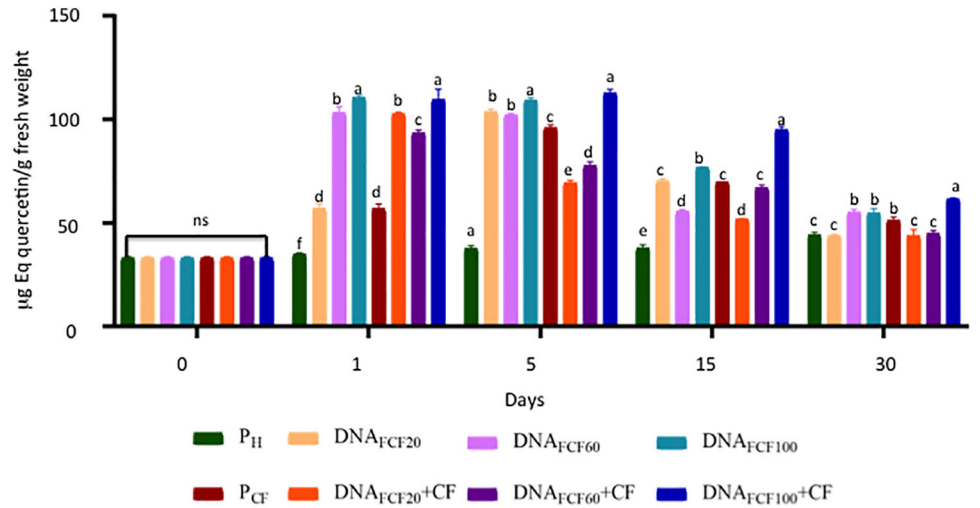

FIGURE 8 | Total flavonoids (equivalents of quercetin) of plants treated with a mixture of DNA fragments of the pathogen complex at 0, 1, 5, 15, and 30 days post-DNA application. Average ± standard deviation data of three biological replicates is shown. Equal lower case letters in each bar for each time indicates significant statistical difference according to Tukey test ($p = 0.05$). Symbology: FCF, mixture of DNA fragments from the pathogen complex; CF, pathogen complex; P_{CF}, control plants infected with the pathogen complex; P_h, control healthy plants; FCF 20, 60, and 100, different concentrations of the mixture of the DNA fragments of the pathogen complex given in µg mL^{-1} evaluated in the study. The expression "ns" indicates no significant difference.

phytopathogens onto chili plants, it was possible to increase the levels of phenolics and flavonoids. In recent studies it has been reported that with the exogenous application of hydrogen peroxide to protect chili peppers against geminivirus diseases, the protection level was directly related to increases in phenols and flavonoids levels (Mejía-Teniente et al., 2019). Moreover, the increase of the phenol and flavonoid levels displayed a Gaussian curve behavior during the time evaluated in the present study. Similar results were obtained in other works studying several elicitors as salicylic acid, chitosan, and hydrogen peroxide in chili peppers (Mejía-Teniente et al., 2013). In another study, Vega-Muñoz et al. (2018) reported that DNA fragments of the lettuce plant (*Lactuca sativa* L.) increased the concentrations of phenols and total flavonoids, activating defense responses to oxidative stress and the synthesis of secondary metabolites for stress management.

Koc et al. (2011) reported that *pal* activity increased when *P. capsici* infection occurs, becoming one of the first responses

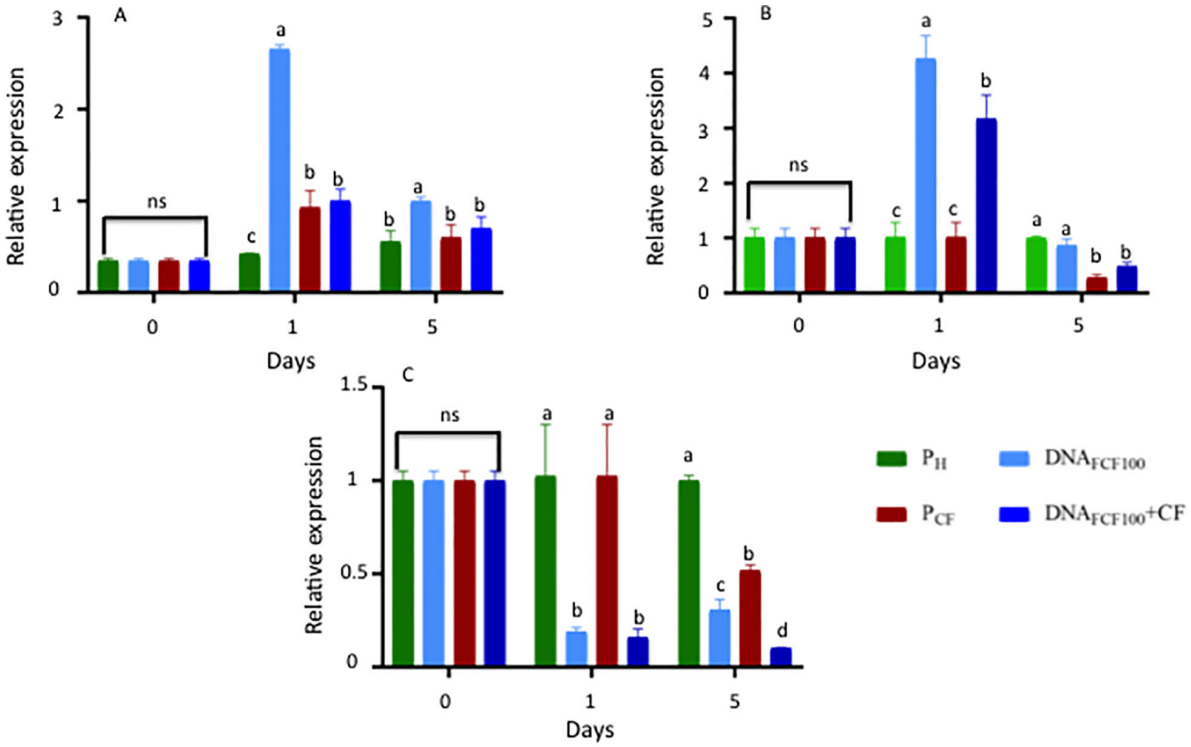

FIGURE 9 | Relative gene expression of *pal*, *chs*, and Mn-*sod* at 0, 1, and 5 dpi in samples treated with a mixture of DNA fragments from the pathogen complex. **(A)** Relative expression of the phenylalanine ammonium lyase (*pal*). **(B)** Relative expression of the chalcone synthase (*chs*). **(C)** Relative expression of the Manganese Superoxide dismutase (Mn-*sod*). Average ± standard deviation data of three biological replicates is shown. Equal lower case letters in each bar for each time indicates significant statistical difference according to Tukey test (p = 0.05). Symbology: FCF, mixture of DNA fragments from the pathogen complex; CF, pathogen complex; P_{CF}, control plants infected with the pathogen complex; P_h, control healthy plants; FCF 20, 60, and 100, different concentrations of the mixture of the DNA fragments of the pathogen complex given in μg mL^{-1} evaluated in the study. The expression "ns" indicates no significant difference.

after infection, since it participates in the biosynthesis of phenolic compounds and phytoalexins. PAL is correlated with the defense in plants of *Capsicum annuum* against pathogens (Jung et al., 2004). In this work, with the application of the DNA fragments, a greater expression of *pal* and *chs* was achieved at 1 dpa was directly related to the increase in the concentrations of phenols and flavonoids of the samples analyzed as aforementioned. Chávez-Díaz and Zavaleta-Mejía, 2019 reported that the expression of *pal* in *Capsicum* plants, presenting an accumulation of phenols and phytotoxic compounds that activate the hypersensitive response and the synthesis of salicylic acid. Interestingly, the protection levels of chili pepper treated with fragmented DNA of the pathogenic complex at 100 μg mL^{-1} was correlated with higher levels of phenylpropanoids and gene expression associated to stress response in plants. Finally, our results also suggested that only one application of fragmented DNA of the pathogenic complex at 100 μg mL^{-1} onto juvenile chili peppers (10–12 true leaves) was enough to significantly diminish the wilt and root rot disease in 40%. Thus, fragmented DNA from pathogenic-origin used as PAMP in chili peppers might be an interesting sustainable strategy to be included in the management of this disease worldwide. Mazzoleni et al. (2014) suggested that self-DNA applications into soil might be a strategy to cope with soil phytopathogens. In our research, we use DNA from soil-living phytopathogens applied onto chili pepper plants (i.e., as PAMP) not into the soil having significant crop protection. It is plausible supposedly that a combined strategy in which an adequate dose of DNA from these pathogens used as PAMP (as in the present work) and applied into the soil to inhibit the growth of these phytopathogens (i.e., as DAMP) might increase the protection of chili peppers against wilt and root rot disease. These strategies are currently being tested in our group in experiments at open field and greenhouse levels, trying to provide a sustainable strategy to protect chili peppers against this important disease thus increasing plant growth and yields with minimal side-effects to nature.

CONCLUSION

The mixture of DNA fragments of the three phytopathogens evaluated in this research applied in a concentration of 100 μg mL^{-1} induced the immune system of the chili plants and caused a control on the severity of the wilt of the chili caused by these phytopathogens, reducing mortality by 40%. In addition, a significant decrease of 60% in symptom severity compared to control plants inoculated with the pathogen complex was also obtained.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

LS-J carried out all the experimentation as part of her Master in Science thesis. EV-P and MG-C carried out the design and advisory in phytopathological tests in the greenhouse. GM-R and RG-G conceived the research and participated in gene expression and biochemical tests carried out in the research, additionally both wrote the manuscript. All authors contributed with critical review of the manuscript before submission.

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REVIEW
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Pars Pro Toto: Every Single Cell Matters

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Compared to other species, plants stand out by their unparalleled self-repair capacities. Being the loss of a single cell or an entire tissue, most plant species are able to efficiently repair the inflicted damage. Although this self-repair process is commonly referred to as "regeneration," depending on the type of damage and organ being affected, subtle to dramatic differences in the *modus operandi* can be observed. Recent publications have focused on these different types of tissue damage and their associated response in initiating the regeneration process. Here, we review the regeneration response following loss of a single cell to a complete organ, emphasizing key molecular players and hormonal cues involved in the model species *Arabidopsis thaliana*. In addition, we highlight the agricultural applications and techniques that make use of these regenerative responses in different crop and tree species.

Keywords: wounding, regeneration, callus, single cell, crops

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INTRODUCTION: TISSUE DAMAGE FROM THE INSIDE OUT

Tissue damage can present itself in a wide spectrum of severity, depending on how it was inflicted. This can range from death of a single cell, a group of cells from the same or different cell type, to even loss of an entire organ. A single dead cell can arise from a spontaneously occurring event, triggered by pathogens, or can be induced artificially through laser-mediated cell ablation (Hoermayer and Friml, 2019; Marhavý et al., 2019). Similarly, death of a group of cells can be generated by both exogenous as well as endogenous factors. For example, heavy-metal-contaminated soils can trigger cell death through the induction of DNA damage (Jalmi et al., 2018), which can be mimicked by the use of radiometric DNA damage-inducing compounds, such as zeocin or bleomycin (Fulcher and Sablowski, 2009). Herbivory or harsh environmental conditions can result in tissue shearing or the loss of partial or entire organs. For each type of damage, plants have evolved elegant strategies to repair the damage sustained, for which the activated response is dictated by the type of havoc inflicted. Ultimately, complete plant bodies can be regenerated starting from tissue explants or even a single cell, including pollen, root hair cells, or protoplasts. In this review, we aim to address these different types of regeneration that have been predominantly studied in the model plant *Arabidopsis thaliana* (Arabidopsis), and highlight the possible agricultural applications in economically interesting species, including poplar, that have originated from the described responses. To conclude, we implicate the role of callus in the regeneration process, how this can be generated in an artificial setting and how this knowledge is translated in order to facilitate *in vitro* culturing and transformation of recalcitrant crop species, including maize, wheat, and rice.

A SINGLE CELL IS ALL IT TAKES: DEATH OF A SINGLE CELL

Modes of Single Cell Replenishment

Even loss of a single cell is sufficient to initiate a localized regenerative response. Although single cell damage can be inflicted naturally by nematodes, small insect larvae or invading necrotrophic microbes, it has been technically challenging to reproducibly target specific cells in order to study the regenerative response. However, fine-tuning of the laser-mediated single cell ablation technique allowed significant progression in our understanding of this regenerative process. In the Arabidopsis root, ablation of a single cell results in the local activation of cell division in the neighboring cells (van den Berg et al., 1995; Heyman et al., 2016; Marhavý et al., 2016; Marhava et al., 2019). Typically, cells from the contacting innermost located tissues rather than cells from the same tissue are being called upon to replenish the lost cell. This was already observed 25 years ago upon ablation of a quiescent center (QC) cell, located within the root stem cell niche (SCN) that is composed of a cluster of pluripotent cells possessing a high proliferative capacity. Laser-ablated QC cells are replaced by division of vascular stem cells that are located directly upward from the QC (van den Berg et al., 1995; Xu et al., 2006). Later, studies focusing on the tissues located higher up in the root meristem revealed a similar inside-out cell replacement mechanism. Here, loss of an endodermal cell results in the activation of cell division in the adjacent, inward located pericycle cells, which through periclinal cell division provide new cells that transdifferentiate, thereby adopting the fate of an endodermal cell (Marhavý et al., 2016; Marhava et al., 2019). In turn, loss of a cortex cell is replenished *via* periclinal cell division and subsequent transdifferentiation of the inward located neighboring endodermis cells, providing an inward-out mode of tissue regeneration (**Figure 1A**; Marhava et al., 2019). This mode of regeneration appears to be strongly correlated with the division potential of the responding cells. The closer the cells are to the SCN, the more efficiently a lost cell can be replaced. Venturing away from the SCN toward the end of the root meristematic zone, the regenerative potential steadily decreases, suggesting that the proliferative capacity of the cells is key for their regenerative potential (Marhava et al., 2019).

Hormonal Response Following Single Cell Loss

Even at this single cell level, different hormones and their respective cross-talks are indispensable for damage perception and elicitation of the key downstream responses (Vega-Muñoz et al., 2020). Jasmonic acid (JA) is probably considered to be the most important wounding-responsive phytohormone, as its accumulation can be detected within seconds following damage (Glauser et al., 2009). Downstream of JA perception by the CORONATINE-INSENSITIVE PROTEIN 1 (COI1) receptor, MYC-type transcription factors, such as MYC2, initiate the expression of key downstream response genes (**Figure 1A**; Kazan and Manners, 2013). Following ablation of even a single root meristem cell, a swift JA response can be observed, visualized using the JAS9-VENUS reporter (Larrieu et al., 2015; Zhou et al., 2019). This JA response enables regeneration, as plants mutated for the JA receptor COI1 are unable to recover from laser-ablated QC cell loss, whereas application of JA increases the QC cell regeneration rate following ablation (Zhou et al., 2019).

Contrary, whereas the regenerative cell division response within the root meristematic zone has been proposed to be facilitated mainly by JA signaling (Zhou et al., 2019), ethylene signaling appears to be the predominant hormone involved in transmitting the single cell death signal in differentiated non-dividing root cells (Kong et al., 2018; Marhavý et al., 2019). Contrary to JA, ethylene accumulates around 30 min following wounding (Boller and Kende, 1980). Damage sustained through single cell ablation has been proposed to elicit a similar response as observed for nematode infection and can therefore be used as a proxy for studying cellular damage sustained upon nematode entry. Correspondingly, death of a single differentiated cell causes an ethylene-dominated stress response. This observation is evidenced by the robust induction of *ACS6* and *PR4*, reporting ethylene biosynthesis and signaling, respectively, following root cortical cell ablation. Contrastingly, JA does not appear to play a clear effect following mature cortex ablation, as shown by the lack of a response of the JAS9-VENUS sensor (Marhavý et al., 2019). Next to ethylene and JA, although not being a primary wound-responsive hormone, auxin plays an indispensable role during the subsequent regeneration process in the root meristematic region. Following single cell ablation, strictly localized auxin signaling, independent of biosynthesis or active transport, coordinates the regeneration response (**Figure 1A**). Application of the synthetic auxin 1-naphtaleneacetic acid (NAA) upon ablation increases the regenerative cell division rate and results in overproliferation of the roots, which can be interpreted as uncontrolled regenerative divisions, indicating the importance of auxin in single cell replenishment (Hoermayer et al., 2020).

Single Cell Replenishment at the Genetic Level

To date, only a handful of genetic players have been appointed a role in the single cell regeneration process, including SCARECROW (SCR) and members of the PLETHORA (PLT) family of transcription factors. SCR represents a member of the GRAS-type family of transcription factors, involved in tissue patterning, whereas PLTs, being AP2-type transcription factors, play a predominant role in SCN specification (Shimotohno et al., 2018). It was found that plants lacking a functional SCR or a combination of PLT1 with PLT2, are unable to re-specify a new QC upon its ablation, visualized by the use of the QC-specific WUSCHEL-RELATED HOMEOBOX 5 (WOX5) marker (Haecker et al., 2004; Blilou et al., 2005), resulting in a failure to recover from the damage inflicted (Xu et al., 2006). Another key player, ETHYLENE RESPONSE FACTOR 115 (ERF115), also a member of the AP2-type transcription factors, was found to play a predominant role in the initial activation of regenerative cell divisions. Although originally identified as a rate-limiting factor controlling stress-induced QC cell divisions, *ERF115* represents an important wound-responsive gene whose

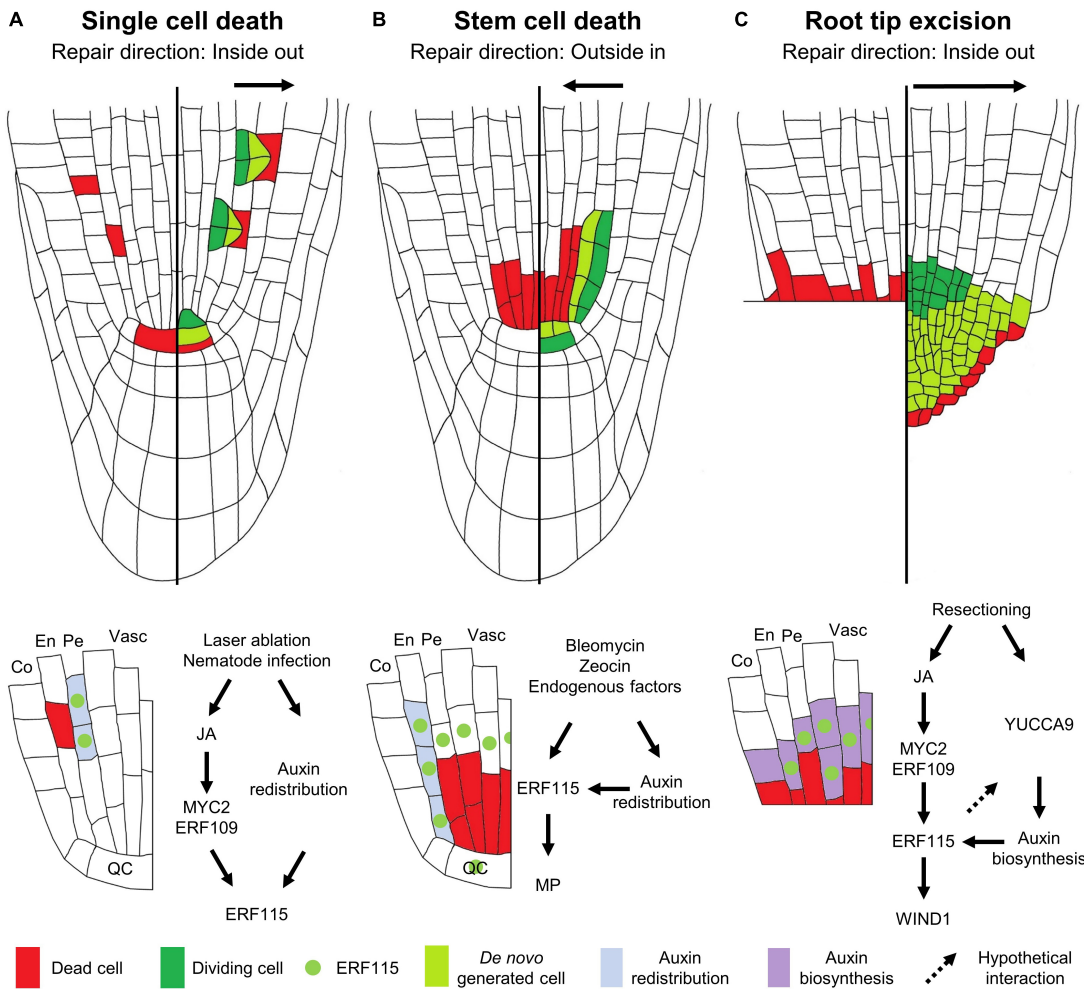

FIGURE 1 | Modes of regeneration in the Arabidopsis root apical meristem following **(A)** single cell death, **(B)** vascular stem cell death, or **(C)** root tip excision. The left sides of the roots show the type of damage inflicted, the right sides illustrate the regenerative response, and the schemes below the roots indicate the molecular response. **(A)** Upon single cell damage, activated JA signaling results in the induction of the *ERF115* transcription factor (green dot) in cells neighboring the dead cell, *via* the action of MYC2/ERF109. In parallel, a local auxin response is activated in these cells (light blue). The co-occurrence of ERF115 and activated auxin signaling stimulates these cells to engage into regenerative divisions in order to replace the outward located cell. **(B)** Vascular stem cell death results in the transcriptional activation of *ERF115* in the surrounding cells, including endodermal cells and QC cells. Due to the stem cell death, the perturbed auxin flow results in the establishment of a new auxin maximum in the neighboring endodermal cells, triggering regenerative cell divisions in cells expressing *ERF115* and downstream target gene *MP*, thereby replacing the inward located cells. **(C)** Loss of the root tip, including stem cells, results in activated JA signaling that, *via* the action of MYC2/ERF109, triggers *ERF115* expression in the vascular and endodermal cells in close contact to the wound site. YUCCA9-mediated auxin biosynthesis at the wound site ensures ample auxin levels required for enforcing ERF115-mediated regenerative divisions. WIND1, directly downstream of ERF115, facilitates root tip regeneration. QC, quiescent center; En, endodermis; Co, cortex; Pe, pericycle; Vasc, vasculature.

activation is highly responsive to cell death (Heyman et al., 2013, 2016). It was found that death of a single root meristem cell induces *ERF115* transcription in the adjacent cells within a time frame of less than 2 h. Following its activation, ERF115 together with its interaction partner PHYTOCHOME A SIGNAL TRANSDUCTION 1 (PAT1), a member of the GRAS-type transcription factors, stimulates these cells to activate their cell division program, resulting in regenerative divisions within 5–7 h post damage (Heyman et al., 2016; Marhava et al., 2019; Zhang et al., 2019). Although auxin boosts ERF115 activity following cell ablation, application of NAA alone is not sufficient to activate *ERF115* expression (Canher et al., 2020; Hoermayer et al., 2020).

In accordance with its predominant role during wound signaling, JA has been put forward to be involved in *ERF115* activation, as induction of *ERF115* could be observed in protoxylem and QC cells following JA application (**Figure 1A**). Furthermore, lack of *ERF115* induction upon removal of the MYC2 *cis*-regulatory element in the *ERF115* promoter revealed *ERF115* expression is activated in a MYC2-dependent way. In addition, ERF109, an ERF115 homolog and JA-responsive transcription factor, was also found to be involved in JA-dependent *ERF115* induction (Wang et al., 2008; Zhou et al., 2019). Indeed, upon laser ablation of QC cells, *ERF115* activation was reduced in seedlings defective for *COI1* (Zhou et al., 2019). Strikingly, although regenerative

divisions could be detected in the cortical and epidermal cell files, *ERF115* expression was found to be confined to the endodermis and stele cells, indicating that ERF115 is not the sole factor facilitating cell replenishment and leaving the question which other factors drive the regeneration process in these tissues (Heyman et al., 2016; Marhava et al., 2019).

A DEADPOOL OF CELLS: REPOPULATING A COMPROMIZED STEM CELL NICHE

Environmental stresses, including hypoxia, of high light, temperature or elevated ozone levels, can trigger the generation of reactive oxygen species that in turn activate a cell death program (Van Breusegem and Dat, 2006; Beaugelin et al., 2019). Similarly, exposure of the root to high concentrations of heavy metals, such as cadmium, can result in the accumulation of dead cells in the root (Panda et al., 2008; Ye et al., 2013), probably arising due to inflicted DNA damage (Filipič, 2012; Cui et al., 2017). Likewise, it was demonstrated that when roots are exposed to near-freezing temperatures, a DNA damage-dependent apoptotic program of columella cells is executed. Sacrificing these columella cells upon exposure to low temperatures allows the release of auxin from these cells that helps to maintain quiescence and survival of the root QC (Hong et al., 2017). Additionally, endogenous factors, such as loss of MERISTEM DISORGANIZER/TEN or TOPOISOMERASE α have been shown to result in cell death, more specifically of the vascular stem cells (Hashimura and Ueguchi, 2011; Zhang et al., 2016). Such a cell death pattern can be mimicked through the application of zeocin or bleomycin (Fulcher and Sablowski, 2009) and is used to experimentally assess the activated regeneration response (Cruz-Ramírez et al., 2013; Heyman et al., 2013, 2016; Takahashi et al., 2019; Canher et al., 2020). When plants are allowed to recover from bleomycin- or zeocin-induced vascular stem cell death, regenerative divisions were originally observed in the adjacent QC cells (Cruz-Ramírez et al., 2013; Heyman et al., 2013). Whereas QC cells normally reside, as their name suggests, in a proliferation-quiescent state, they appear to be called upon in order to help to replenish the lost vascular stem cells, thereby serving as a backup pool of stem cells (Heyman et al., 2014). However, it seems unlikely that this extensive loss of vascular stem cells can be completely replenished solely by QC cell division activity. Accordingly, through time-lapse imaging and cell-lineage tracking experiments, a major contribution of the endodermal cells located directly next to the damaged vascular stem cells was observed, engaging these cells in periclinal divisions to repopulate the compromised vascular tissue (Heyman et al., 2016; Canher et al., 2020; **Figure 1B**). Thus, contrary to the regeneration orientation accounting for single cell replenishment, repopulation of the vascular stem cell pool occurs *via* an outside-in direction (Canher et al., 2020).

Molecular Response

Similar to single cell damage, auxin plays an important role in orchestrating the regeneration response following loss of the vascular stem cell pool. Through a combination of cellular imaging with mathematical modeling, it was demonstrated that the activation of regenerative endodermal cell divisions correlates with a local redistribution of auxin due to a loss of auxin transporters (known as PINs), resulting in the establishment of an auxin maximum in the endodermal cells neighboring the dead vascular stem cells. The combination of a new auxin maximum with the induction of *ERF115* in the same endodermal cells, engages these cells in regenerative divisions. *MONOPTEROS* (*MP*), encoding an auxin-responsive transcription factor required for vascular development, was identified as a direct ERF115 target gene and therefore represents a key nexus point in the integration of both wound- and auxin-signaling cues for tissue regeneration (Canher et al., 2020; **Figure 1B**). The importance of ERF115 to allow recovery from bleomycin-induced vascular stem cell death is demonstrated by the observation that impaired ERF115 activity disenables plants to activate periclinal endodermal cell divisions, resulting in an inability to replenish the lost vascular cells. This results in a collapse of the root meristem in an upward proceeding direction (Heyman et al., 2016; Canher et al., 2020). Although JA was shown to trigger *ERF115* expression in the protoxylem tissue in the root (Zhou et al., 2019), *ERF115* induction following stem cell death could still be observed in a JA-independent way, suggesting a still yet to be identified signal that contributes to the cell death-dependent activation of *ERF115* expression (Canher et al., 2020).

THE TIP OF THE REGENERATING ICEBERG

Regeneration of an Arabidopsis Root Tip

Regeneration of the Arabidopsis root tip following extensive tissue damage has been the topic of several studies. Over a decade ago, the first study of root tip regeneration by means of excision using a fine needle was reported by Sena et al. (2009). Upon removal of the complete root tip, including the SCN, plants were found to regenerate a *de novo* tip within three to 4 days following excision. This process requires the complete reformation of the SCN from the remaining meristematic cells. A detailed single cell transcriptomics approach combined with lineage tracking of a regenerating root tip revealed that root tip re-establishment occurs *via* rapid cell identity transitions. Here, cells from the endodermal cell files assume a stem cell-like identity in order to generate a new epidermal layer and lateral root cap. Contribution of the pericycle in the regenerating root appeared to be restricted to generating the new cortex and endodermis tissues, whereas the new SCN is derived from pre-existing stele cells adjacent to the cut site. Whereas the newly formed stem cells generate new cell files from the inside out, tissue markers revealed that cell identities are restored following an outside-in manner (Efroni et al., 2016; **Figure 1C**).

Identical to regenerative cell divisions following laser ablation, root tip regeneration efficiency is linked to the cell division potential of the tissues, as excision of a small root tip fragment, close to the SCN, results in a higher regeneration frequency compared to the removal of a larger tip fragment (Sena et al., 2009; Durgaprasad et al., 2019). A recent report states the

presence of a regeneration competence zone, marking a clear-cut boundary beyond which no tip is able to regenerate. This competence zone appears to be marked by the presence of endogenous stem cell marker genes, such as *PLT2*, rather than the actual meristem size (Durgaprasad et al., 2019). Indeed, similarly to that observed for impaired QC cell respecification following ablation, *plt1 plt2* double mutants are largely compromized in their ability to regenerate a *de novo* root tip upon its excision (Xu et al., 2006; Sena et al., 2009).

Molecular Components Driving Root Tip Regeneration

Similar to the previously described types of damage, excision of the root tip results in a swift *ERF115* induction that is required for regeneration, because plants with impaired ERF115 activity display a nearly complete lack of root tip regeneration potential (Heyman et al., 2016; Johnson et al., 2018; Zhou et al., 2019). Similar to that observed for single cell damage, JA appears to play a role in inducing *ERF115* upon root tip excision (Zhou et al., 2019). Directly downstream of ERF115, WOUNDING INDUCED DEDIFFERENTIATION 1 (WIND1), another member of the AP2-type of transcription factors, was identified (Heyman et al., 2016). As its name suggests, WIND1 plays a role in cellular dedifferentiation during the regeneration process (Iwase et al., 2011). Correspondingly, a role for WIND1 during root tip regeneration could be attributed (Heyman et al., 2016; **Figure 1C**), but surprisingly, not following single cell ablation (Marhava et al., 2019). Although being an ERF115 target gene, *WIND1* induction could be observed outside of the *ERF115* expression domain, suggesting additional mechanisms triggering WIND1-mediated cellular dedifferentiation upon wounding (Heyman et al., 2016). For example, whereas induction of both *ERF115* and *WIND1* could be observed following wounding of roots and hypocotyls, *WIND1* induction appeared to be more transient in roots, adding a putative tissue-specific preference of the regeneration response to the equation (Rymen et al., 2019).

Again, auxin is involved in root tip regeneration. However, whereas "minor" single cell damage and stem cell death result in a very localized redistribution of the available auxin pool, "extensive" loss of the entire root tip triggers a different response. Here, YUCCA9-dependent auxin biosynthesis was found to be indispensable to provide the adequate levels of auxin required to allow *de novo* root tip regeneration (**Figure 1C**). This auxin biosynthesis cascade appeared to be downstream of ERF115, as application of IAA was able to restore the regeneration potential of ERF115-impaired plants (Matosevich et al., 2020). The difference in auxin response upon ablation or stem cell death versus root tip excision remains an open question, but likely depends on the type of damage and corresponding intensity. Upon minor damage, the available auxin pool present in the surrounding tissues might be sufficient to initiate the regeneration program and a local redistribution will suffice. Upon stem cell death, auxin redistribution, due to the "rocks" being present in the auxin flow, triggers SCN replenishment until the normal auxin flow is re-established. However, upon more extensive damage, such as loss of the entire root tip, the main auxin biosynthesis machinery, being located in the tip, is no longer available and the remaining available auxin pool might no longer be adequate to instigate regeneration, or to respecify a novel QC, making auxin biosynthesis an essential part of the regeneration response (Canher et al., 2020).

Regeneration of the Shoot Apical Meristem

Whereas most research has focused on the regeneration response in the Arabidopsis root tip, the response following stem cell loss in the shoot remains largely unexplored, probably due to it being experimentally less easily accessible. Although DNA damage-inducing compounds are able to induce stem cell death in the shoot (Fulcher and Sablowski, 2009), laser ablation is predominantly used to microdissect shoot stem cells. Following microdissection of the entire Arabidopsis shoot organizing center (OC), being the shoot counterpart of the root QC, a re-establishment of the OC could be detected 3 days after dissection. This is shown by the regeneration of *WUSCHEL* (*WUS*)-expressing cells, a key transcription factor marking shoot OC cells, similar to *WOX5* in the root QC cells (Mayer et al., 1998; Adibi et al., 2016). A similar laser-mediated dissection of the tomato shoot revealed that organ formation was not affected and that meristem repair was executed within 2 days, again reflected by the reformation of a *WUS*-expressing cell cluster (Reinhardt et al., 2003). Contrary to the root, re-establishment of the shoot OC is mainly driven by cytokinin signaling rather than auxin (Adibi et al., 2016).

CUT FOR REPAIR

Partial Incision Repair

Herbivore attack or harsh weather conditions can result in more extensive tissue damage, such as shearing or cuts. This type of wounding results in the activation of cell division, not in order to (re)generate tissues as such, but rather to regain a reconnection between the incised tissues. Such tissue reunion following incision requires a reactivation of cell division to allow reconnection of the severed vascular tissue, needed for water and nutrient transport throughout the plant (**Figure 2A**). Upon incision of the inflorescence stem, different hormonal and transcriptional changes could be observed in the top compared to the bottom part of the cut site, which together will result in tissue reunion (Asahina et al., 2011). Among these, the NAC-type ANAC071 and AP2-type RAP2.6L transcription factors are activated in order to assist in the reunion process. On the one hand, *RAP2.6L*, also known as *ERF113* and representing a homolog of *ERF115* (Heyman et al., 2018), is induced within 1 day following incision at the bottom part of the incision site in a JA-dependent manner. However, recent data using hypocotyls instead of inflorescence stems suggests that *RAP2.6L*, although being induced following a hypocotyl cut, is not required to allow cell proliferation and tissue healing to

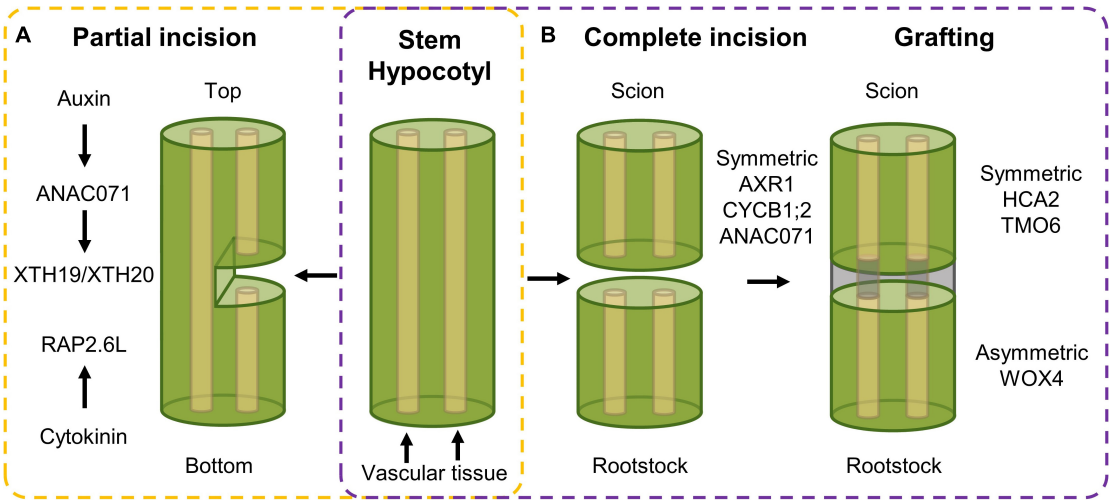

FIGURE 2 | Transcriptional response following **(A)** partial incision in stem tissue and **(B)** complete incision and following tissue reuniting. **(A)** Upon partial incision of the inflorescence stem, a different response in the top versus bottom side of the cut can be observed. In the top, auxin accumulation activates expression of *ANAC071*, that in its turn induces the *XTH19* and *XTH20* cell wall modifying genes, that, in combination with cytokinin-dependent *RAP2.6L* expression in the bottom side, ensure vascular tissue reconnection. **(B)** Complete incision results in an induction of auxin-related, cell division and wound healing genes, being *AXR1*, *CYCB1;2* and *ANAC071*, respectively, in both scion and rootstock. Following grafting, symmetric and asymmetric responsive genes, being *HCA2* and *TMO6*, and *WOX4*, respectively, facilitate tissue reconnection in the hypocotyl.

occur (Matsuoka et al., 2018). On the other hand, *ANAC071* is induced in the top part of the incision between 1 and 3 days as a result of auxin accumulation (Asahina et al., 2011). Following induction, ANAC071 activates the cell wall modifying genes *XYLOGLUCAN ENDOGLUCOSYLASE/HYDROLASE 19* (*XTH19*) and *XTH20*, which appear to catalyze the tissue reattachment process *via* hydrolysis of cell wall glucans (Pitaksaringkarn et al., 2014).

Grafting: Tissue Reuniting Following Complete Separation

Tissue reuniting can happen as well between tissues that were completely severed, a process used in horticulture in the form of grafting, an efficient means of asexual propagation and even enabling the generation of chimeric organisms. For example, a root system (rootstock) from a plant with pathogen-resistant traits can be combined with the aerial part (scion) obtained from a plant with high crop yield, resulting in a chimeric crop having both high yield and pathogen resistance characteristics (Melnyk, 2017). It allows the combination of beneficial traits from different plant species into a single entity without the need of time-consuming breeding. Here, a similar top-versus-bottom differential response, similar to partial tissue reconnection, is observed. Transcript profiling of the bottom and top part of cut Arabidopsis hypocotyls revealed both symmetric and asymmetric gene expression responses. Genes related with auxin response [e.g., *AUXIN RESISTANT 1* (*AXR1*)], cell division [e.g., *CYCLIN B1;2* (*CYCB1;2*)], and wound healing (e.g., *ANAC071*) were activated in a symmetrical manner in grafted rootstock and scion. Contrastingly, when scion and rootstock were kept separate, the symmetrical responses were mostly preserved in the separated scion but abolished in the separated rootstock, which likely originates from the rootstock being deprived of auxin and sugars produced in the scion. Grafting-activated genes related to vascular formation can be grouped in two subsets displaying either symmetrical [e.g., *HIGH CAMBIAL ACTIVITY 2* (*HCA2*) and *TARGET OF MONOPTEROS 6* (*TMO6*)] or asymmetrical expression (e.g., *WOX4*) between the scion and the stock, referring to genes that are activated similarly in both scion and rootstock, or preferentially in only one of the two, respectively (**Figure 2B**). Among the symmetrically responsive genes, *HCA2* seems to be required to facilitate phloem reconnection as its impairment results in delayed reconnection rates. The asymmetrical responses peak around 72 h after grafting and disappear gradually, which is believed to result from phloem reconnection. However, genes related to the sugar response remain asymmetrically responsive even in the grafted scion and rootstocks accompanied by the formation of starch granules predominantly in the scion but also in the rootstock at later stages. Addition of exogenous sucrose lowered the grafting efficiency, pointing to the necessity of this differential sugar response between rootstock and scion, which might be important for vascular tissue reconnection (Melnyk et al., 2018). At this moment, the involvement of sugar in the tissue-reuniting process remains to be elucidated.

Recently, a major advance in grafting efficiency was found through the use of an interscion from a β-*1,4-glucanase*-overexpressing *Nicotiana benthamiana* (tobacco) plant. Here, the β-1,4-glucanase secreted from the tobacco interscion facilitates cell wall reconstruction, thereby improving cell–cell adhesion. Using this tobacco interscion as bridge, successful grafting of a tomato scion onto an Arabidopsis rootstock, otherwise graft-incompatible species, could be facilitated, again suggesting that

the cell wall plays an important role in determining grafting compatibility (Notaguchi et al., 2020).

DIVIDE ET IMPERA

Molecular Responses at the Cut Site

Whereas minor damage results in the activation of local cell divisions in order to replace the lost cells or reconnect severed tissues, such a local regeneration response will no longer suffice upon loss of an entire organ. Rather than attempting to repair or replace the lost tissue, the plant invests in generating entire *de novo* organs from the cut site, such as roots or shoots, which can be achieved directly or indirectly, but both rely on cellular reprogramming (Kareem et al., 2016). In the case of direct regeneration, cells transdifferentiate, e.g., root cells can be reprogramed to shoot cells and *vice versa*. The indirect mode relies on the generation of an intermediate regenerative mass of cells, which requires dedifferentiation of somatic cells near the wound site, allowing these cells to regain a cellular proliferation competence (Iwase et al., 2011; Kareem et al., 2016). This mass of undifferentiated, pluripotent cells is referred to as callus and serves as a base of origin from which novel organs can subsequently be formed (Stobbe et al., 2002). Although wounding-induced callus formation, generated from undifferentiated xylem cells near the wound site, is thought to prevent infection and water loss at the wound site, for example, following debarking of trees, in some cases this callus can regenerate new organs or tissues as well (Stobbe et al., 2002). For example, spontaneous wound-induced callus formation can be observed upon hypocotyl and petiole excision (Iwase et al., 2011, 2017; Liu et al., 2014).

Rooting Following Leaf Blade Excision

Upon excision of the leaf between the blade and petiole, callus is generated locally at the cut site and adventitious roots can subsequently sprout within 8 days following excision (**Figure 3A**; Liu et al., 2014). Following leaf excision, auxin accumulation at the wound site, possibly provided by YUCCA4-dependent biosynthesis (Chen L. et al., 2016), directly activates expression of *WOX11*, working redundantly with *WOX12*, which enables the generation of callus from local cambium cells, that are known to contain adult stem cell populations (Lachaud et al., 1999). Root founder cells are specified from this callus within 4 days (Liu et al., 2014). Following root founder cell establishment, activity of WOX11 and WOX12 induces *WOX5* and *WOX7*, in turn initiating root primordia (Hu and Xu, 2016). However, the rooting capacity within excised leaves appears to diminish with increasing age. This "age sensing" is transmitted by SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors (Xu et al., 2016) that act as negative regulators of root regeneration by inhibiting wound-induced auxin biosynthesis (Ye et al., 2020). SPL10 appears to regulate several ERF/AP2-type transcription factor genes, including *ERF109*, being both a close homolog and putative upstream regulator of *ERF115* in the root meristem, thereby possibly imposing an age-dependent control on tissue regeneration (Heyman et al., 2018; Zhou et al., 2019; Ye et al., 2020).

Propagation Through Cuttings

Analogously, certain tree species, including poplar, with agricultural beneficial traits can also be propagated clonally through cuttings, allowing a fast propagation of cultivars with specific traits without the need to wait for seed set or possible seed germination difficulties. Although many tree species are propagated from tissues of juvenile specimens, cloning of mature trees is generally preferred because often it is not possible to determine if selected embryos or seedlings have the genetic potential to develop the desired qualities later in their life cycle. Here, a piece of stem is excised and allowed to regenerate a *de novo* root system from the cut site (**Figure 3B**). Typically, the inflicted damage triggers signaling pathways that eventually result in the activation of stem cell activity at the cut site, resulting in the *de novo* formation of root primordia. Rooting on stem tissue depends on auxin, either triggered through *de novo* biosynthesis or by accumulation as a result of cutting off the basal auxin drain (Cai et al., 2014; Chen X. et al., 2016; Druege et al., 2019; Zhang et al., 2019; Matosevich et al., 2020). In Arabidopsis, early auxin maxima in etiolated hypocotyl cuttings were identified by accumulation of the *GRETCHEN-HAGEN 3-2* (*HG3-2*) auxin-response marker (Sukumar et al., 2013). Subsequent rooting is promoted by the application of indole-3-butyric acid (IBA), being an endogenous auxin, and is always preceded by callus formation (Ludwig-Müller et al., 2005). Here, the auxin maxima result in the activation of the AUXIN RESPONSE FACTOR 6 (ARF6) and ARF8, being positive regulators of the rooting process, together with ARF17, a negative regulator (Gutierrez et al., 2012). The combination of hormonal input and a complex molecular network of genetic and epigenetic changes (Jing et al., 2020) likely explains why rooting efficiency not only depends on the species and genotype, but also on growth conditions and seasonality, as well as the decrease in *de novo* rooting potential over age (Batista et al., 2015). Because of the observed drop in regeneration potential over age, even the propagation of good rooting species can become problematic over time. Such loss of competence for *de novo* rooting represents a frequently occurring issue in breeding programs, impairing the propagation of genotypes with interesting attributes for commercial production. Although several hormone-containing rooting compounds are commercially available in order to boost a cutting's rooting potential, several agriculturally important crops or ecotypes remain recalcitrant to *de novo* root initiation.

Root Pruning: Cut a Root to Make a Root

The regenerative response where organs are formed *de novo* following injury or even arising from the cut site, is similarly widely used in agriculture. One of these applications relies on the mechanical removal of a large mass of roots in order to stimulate formation of a denser lateral root network (**Figure 3C**). This process is commonly referred to as root pruning and has been shown in Arabidopsis to be facilitated, at least in part, by the action of WOX11

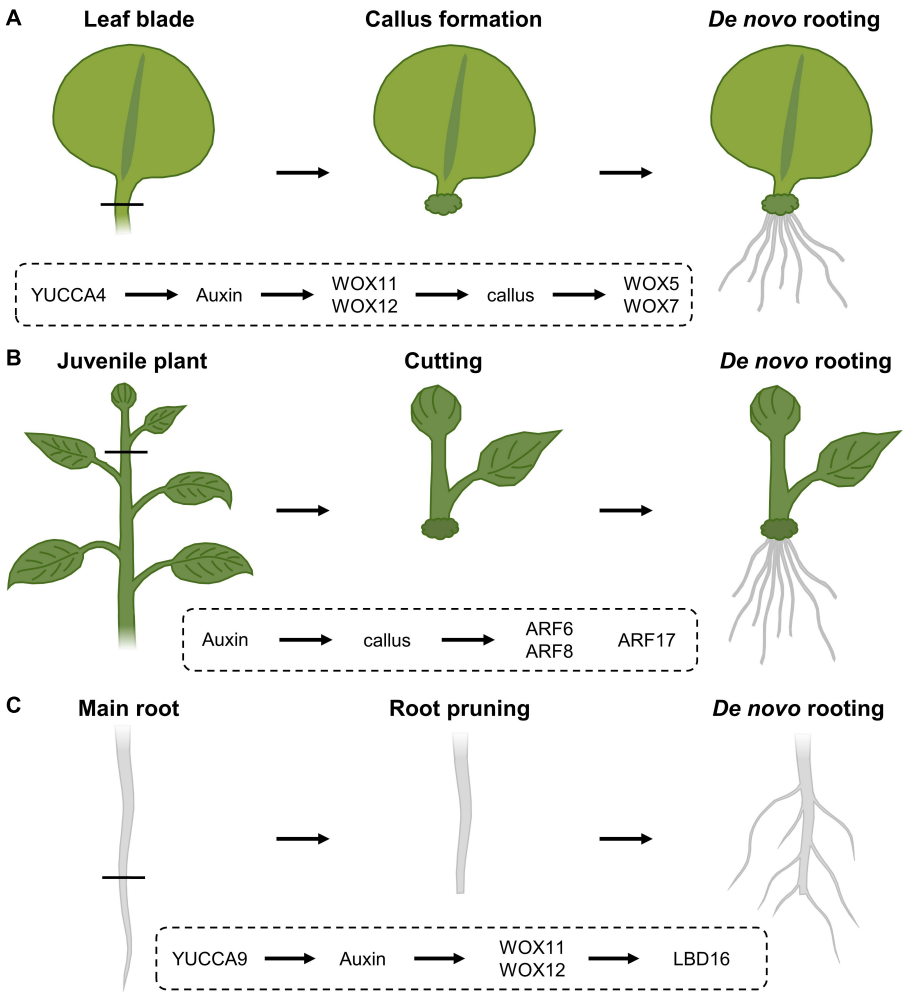

FIGURE 3 | Regeneration response following **(A)** leaf blade excision, **(B)** stem cuttings and **(C)** root pruning, and the corresponding molecular cascades involved. **(A)** Upon leaf blade excision, YUCCA4-mediated auxin biosynthesis results in the induction of *WOX11* and *WOX12*, resulting in callus generation from local cambial cells. Following callus induction, WOX11 and WOX12 activate the expression of *WOX5* and *WOX7* that will initiate root primordia. **(B)** In cuttings from stems, auxin triggers callus formation and the expression of *ARF6* and *ARF8*, being positive regulators of the rooting process, as well as *ARF17*, a negative regulator of *de novo* rooting. **(C)** Following cutting of the main root system, YUCCA9-dependent auxin biosynthesis activates the expression of *WOX11* and *WOX12*, that in turn regulate *LBD16* expression, being needed to induce rooting.

and again its partially redundant WOX12. Although lateral root initiation in seedlings grown under standard tissue-culturing conditions occurs independently of WOX11/12, the primary root is able to produce both WOX11-dependent and -independent roots when grown in soil or following wounding through direct transcriptional activation of *LATERAL ORGAN BOUNDARIES DOMAIN 16* (*LBD16*). Although the WOX11-mediated lateral root initiation appears to be independent of the developmentally controlled lateral root production by AUXIN RESPONSE FACTOR 7 (ARF7) and ARF19, both pathways appear to converge on LBD16 in order to initiate rooting (Lee et al., 2009; Goh et al., 2012; Sheng et al., 2017). Following removal of the main root, generation of a new root system appears to depend on the elevation of endogenous auxin levels, as the formation of this root system is abolished in mutants defective in YUCCA9-mediated auxin biosynthesis or in the presence of the polar auxin transport inhibitor naphthylphthalamic acid, commonly known as NPA (Xu et al., 2017).

In agriculture, root pruning is usually performed for economically important species such as tomato, potato, soybean, and apple trees and often stimulates a variety of phenotypic responses, depending on the species. For example, for fruit trees, in addition to increased lateral root formation, root pruning results in an increased number of flower buds, the production of smaller fruit with a higher quality, a reduction in pre-harvest fruit drop and an overall increased plant size and vigor. In potato, root pruning 2 weeks prior to harvesting leads to the formation of a firmer skin around the potatoes, resulting in a longer shelf life. In tree species like poplar, root pruning is mostly performed to create a denser mass of lateral roots, leading to a reduced shock when the plant is

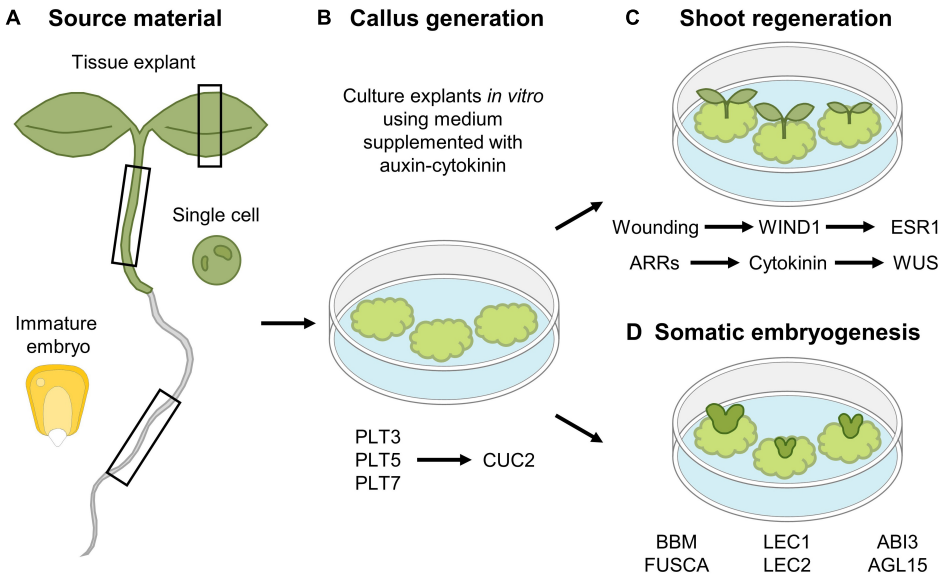

FIGURE 4 | *In vitro* tissue culturing using **(A)** various tissue explants to **(B)** generate callus and subsequent **(C)** shoot regeneration or **(D)** somatic embryogenesis. **(A,B)** Different tissue explant material can be used for *in vitro* callus generation, including leaf, hypocotyl and root explants, immature maize embryos and single cultured cells. **(C)** Shoot regeneration occurs in a two-step mechanism, where callus is first generated by the action of the PLT3, PLT5, and PLT7 transcription factors, whereas in a second step, expression of *CUC2* is required for shoot induction. In parallel, a new shoot organizing center is established by the action of ARRs that facilitate cytokinin production, that in turn activates *WUS* expression. In addition, wounding-induced expression of *WIND1* allows in turn induction of *ESR1* that contributes to regeneration of shoots. **(D)** Somatic embryogenesis from callus occurs by the expression of a network of transcription factors, composed of *BBM*, *LEC2*, *FUSCA*, *ABI3*, and *AGL15*.

transported to another location, but also to expand the absorption area of the root and to improve the rhizosphere soil fertility (Jing et al., 2017, 2018).

COMING FULL CIRCLE, ONCE MORE A SINGLE CELL IS ALL IT TAKES

Molecular Insights Into Callus Formation and Shoot Regeneration

Besides spontaneously being generated following wounding, callus can also be generated artificially through the exogenous application of hormones, which is often the preferred *modus operandi* to study the callus formation process and which is extensively used in laboratory conditions and biotechnical applications, including plant transformation. Using this *in vitro* approach, callus can be generated from different tissue types, including leaves, cotyledons, hypocotyls, root explants, or even single cultured cells (**Figure 4A**). However, callus induced through wounding does not appear to display the same molecular and physiological properties compared to tissue-culture generated callus. Regardless of the source explant tissue used, hormone-induced callus rather contains root-like properties, as suggested by the expression of root meristem marker genes and the inability to be generated from mutants defective in lateral root initiation, which is not observed for wound-induced callus (Sugimoto et al., 2010; Iwase et al., 2011; Ikeuchi et al., 2013). By placing explants, or even single differentiated cells (Steward et al., 1958; Nagata and Takebe, 1971), on culture medium supplemented with a specific auxin-cytokinin phytohormone ratio, a pool of pluripotent cells is generated (Skoog and Miller, 1957), a process that is preceded by extensive epigenetic reprograming (Iwase et al., 2017; Kim et al., 2018; Rymen et al., 2019). Once the callus-like tissue is formed, roots or shoots can subsequently be induced by transferring the callus to medium supplemented with a high or low auxin-to-cytokinin ratio, respectively. In Arabidopsis, *de novo* shoot induction is facilitated in a two-step mechanism. In a first step, the PLT transcription factors PLT3, PLT5, and PLT7 are required for the induction of the root stem cell regulators PLT1 and PLT2. In a next step, PLT3/5/7 regulate the required shoot-promoting-factor CUP-SHAPED COTELYDON2, being a NAC-type transcription factor, again highlighting the importance of PLTs in callus formation and subsequent organ induction (Kareem et al., 2015; **Figure 4B**). Besides the role for PLTs, the induction of *WUS* is indispensable to allow shoot regeneration from callus. By placing callus on shoot-inducing medium, activity of ARABIDOPSIS RESPONSEREGULATORS (ARRs), such as ARR12, facilitate the transduction of the cytokinin signal, which is indispensable for *WUS* induction and subsequent shoot regeneration (Boutilier et al., 2002; Edgar et al., 2002; Dai et al., 2017; Meng et al., 2017; **Figure 4C**).

ENGHANCER OF SHOOT REGENERATION 1 (ESR1) and its paralog ESR2 were identified as key regulators of shoot formation, as ectopic expression of either *ESR1* or *ESR2* is sufficient to trigger shoot regeneration (Banno et al., 2001; Iwase et al., 2017). Recently, it was found that WIND1 acts as a direct

transcriptional activator of *ESR1*, indicating that the wound-induced expression of *WIND1*, and subsequent *ERS1* activation, is required to initiate shoot regeneration (Iwase et al., 2017; **Figure 4C**).

The knowledge concerning this *de novo* shoot formation following callus induction was translated into an elegant tool allowing the generation of CRISPR-edited plants from tobacco leaves (Maher et al., 2020). Here, wound-free callus is generated from tobacco leaves by Agrobacterium-mediated introgression of callus-inducing factors, together with a CRISPR-Cas9 editing module. The subsequent regeneration of shoots from this edited callus allows a rapid generation of genome-edited tobacco plantlets.

Somatic Embryogenesis and Epigenetic Control of Regeneration

Although callus formation represents a naturally occurring response to wounding or can be induced artificially through hormone supplementation, callus can also be obtained through ectopic expression of key regeneration-driving or stem cell-specific transcription factor-encoding genes, including the aforementioned *WIND1* (Iwase et al., 2011), *ERF115* in combination with its dimerization partner *PAT1* (Heyman et al., 2016), or *PLTs* such as *BABYBOOM* (*BBM*) (Boutilier et al., 2002). Contrary to the use of WIND1 and ERF115-PAT1 in the generation of callus, likely resulting from uncontrolled cellular dedifferentiation or regenerative divisions, respectively, BBM can be used to boost the *in vitro* tissue-culturing potential. Here, rather than the generation of a mass of undifferentiated cells, BBM contributes to the generation of complete plant bodies originating from only a small group of cells or even a single cell, which very well may represent the "ultimate" regeneration response, commonly known as somatic embryogenesis (**Figure 4D**; Fehér, 2019; Ikeuchi et al., 2019). In Arabidopsis, a transcription factor network composed of BBM, LEAFY COTYLEDON 1 (LEC1), LEC2, FUSCA, ABSCISIC ACID INSENSITIVE3, and AGAMOUS-LIKE15 has been shown to play a central role during somatic embryogenesis (Radoeva and Weijers, 2014; Horstman et al., 2017b). This network is also activated upon seed germination, but not during zygotic embryogenesis, indicating that somatic embryogenesis rather resembles the seed germination pathway. Indeed, ectopic expression of the key *BBM*, *LEC1*, or *LEC2* genes results in somatic embryogenesis (Lotan et al., 1998; Stone et al., 2001; Boutilier et al., 2002; Zuo et al., 2002). In seedlings, activity of these transcription factors is epigenetically repressed in order not to interfere with normal development (Holdsworth et al., 2008), as ectopic expression of these transcription factors results in somatic embryogenesis in vegetative tissues, such as cotyledons (Radoeva and Weijers, 2014; Horstman et al., 2017a). Similarly, failure to transcriptionally repress (some of) these key players results in the induction of somatic embryos (Ikeuchi et al., 2015), or using a more artificial tissue-culturing setting, whole plants can be generated from pollen or single protoplasts through somatic embryogenesis (Takebe et al., 1971; Zhu et al., 1997; Maraschin et al., 2005; Chupeau et al., 2013).

Besides the activity of key transcription factors, accumulating evidence indicates that the transcription of many reprogramming genes involved in regeneration and somatic embryogenesis are epigenetically regulated. Specific histone modifications play important roles in determining the activation or repression of gene expression (Ikeuchi et al., 2019). For example, expression of the aforementioned embryonic regulators *LEC2* and *BBM*, and *WIND3*, is developmentally repressed by the evolutionary conserved POLYCOMB REPRESSIVE COMPLEX 2 (PRC2), in order to prevent spontaneous somatic cellular dedifferentiation, callus formation and ectopic onset of embryogenesis, as observed in single root hair cells in *prc2*-deficient plants (Chanvivattana et al., 2004; Bouyer et al., 2011; Ikeuchi et al., 2019). Contrary, HISTONE ACETYL TRANSFERASE OF THE GNAT/MYST SUPERFAMILY (HAG1), also known as GENRAL CONTROL NONREPRESSED 5, plays a pivotal role in the acquisition of shoot regeneration competence. By facilitating histone acetylation and subsequent activation of gene expression, HAG1 is thought to be responsible for activating the expression of *WOX5* and *SCR* in order to confer cellular pluripotency (Kim et al., 2018).

PUTTING THINGS TO USE: TISSUE CULTURING FOR GENETIC TRANSFORMATION

Genetic modification allows for the generation of new varieties with improved traits *via* knowledge transfer from model to crop species. Central in the search for an efficient transformation system is the need for tissue that is susceptive to transformation and capable of whole plant regeneration. In Arabidopsis, these demands are met by the female gametes, allowing easy and rapid transformation using the Agrobacterium-mediated floral dip method (Clough and Bent, 1998), whereas many other plant species require an elaborate tissue-culturing period as to provide proliferative cells with the capacity to regenerate (Kausch et al., 2019). Important food crops, such as maize, rice or wheat, are monocotyledonous plants and are especially recalcitrant to *in vitro* culturing, conceivably due to their scattered vascular structures that lack the meristematic cell types that are susceptible to culturing (Benson, 2000). Consequently, cereal transformation is a labor- and time-intensive process with a generally low efficiency, which is genotype and explant dependent (Ji et al., 2013). Most reported transformation protocols require the use of immature embryos (IE) or IE-derived callus, for example for the transformation of maize (Ishida et al., 2007; Frame et al., 2011), rice (Hiei and Komari, 2008), and wheat (Ishida et al., 2015). However, the frequency of embryogenic callus induction and further regeneration into transgenic plants is strongly influenced by the tissue-culture media components and culturing conditions.

A more generic, cultivar- and even species-independent approach to improve tissue culturing is to utilize the aforementioned genetic factors from Arabidopsis that play a role in callus formation and following plant regeneration,

with a special preference toward factors whose overexpression results in somatic embryogenesis. For example, orthologs of the Arabidopsis embryonic regulators LEC1 and LEC2 have been shown to improve transformation efficiency in maize and wheat (Lowe et al., 2003). Similarly, orthologs of SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) originally identified in carrots (Schmidt et al., 1997) have been linked with somatic embryogenesis in maize, rice, and rye (Baudino et al., 2001; Singla et al., 2009; Gruszczyńska and Rakoczy-Trojanowska, 2011). Recently, also the maize ortholog of the developmental regulator GROWTH-REGULATING FACTOR 5 (GRF5), and the wheat ortholog of GRF4 together with its cofactor GRF-INTERACTING FACTOR 1 (GIF1) have been linked with increased regeneration (Debernardi et al., 2020; Kong et al., 2020). Many transformation protocols currently available rely on the embryonic regulators BBM and WUS. Combined *BBM* and *WUS* overexpression leads to growth stimulation of embryogenic tissue in recalcitrant maize, rice, and sorghum, and expands the range of successful explants from IE or IE-derived callus to mature seeds and even leaf tissue (Lowe et al., 2016). However, there is a trade-off as constitutive expression of these factors results in developmental defects and therefore needs to be excised from engineered plants. Therefore, the recently reported GRF-GIF module may emerge as an interesting alternative. GRFs and GIFs interact to form a complex, therefore combined expression can be used to drastically improve regeneration efficiencies, as observed in *GRF4-GIF1* expressing "chimeras" from wheat (Debernardi et al., 2020). The GFR-GIF chimera approach appears to work efficiently to increase regeneration in both monocotyledonous species (including durum wheat, common wheat, rice, and triticale, being a hybrid between wheat and rye), as well as dicotyledonous species, including citrus and, contrary to BBM-WUS, does not result in developmental defects (Luo and Palmgren, 2021).

CONCLUSION

Researchers have been drawn to explore and utilize the regenerative power of plants for more than 100 years (Haberlandt, 1902). Although a vast number of phenotypic responses and molecular data have already been gathered to date, plants have not revealed all their regenerative secrets yet. The further fine-tuning of microscopic techniques, such as laser-mediated cell ablation, and the development of high-resolution single cell transcript profiling have provided the possibility to investigate new and more detailed aspects of the plants' response following tissue damage. Ranging from a plant that needs to regenerate a single cell to a single cell that needs to regenerate a plant, and everything in between, nothing appears to be impossible. This once again highlights that even a differentiated plant cell possesses the capacity to regain the potential to form all different cell types that constitute a plant, with or without a minor biotechnological intervention. However, some crop species or genotypes remain more recalcitrant when it comes down to regeneration efficiency or tissue culturing. For many crops, tissue culturing is still an inherent step to genetic modification. Central in the search for an efficient genome editing system is the need for an accessible and susceptible tissue that is receptive to transformation and capable of subsequent regeneration into fertile plants. Finding the key to unlock *in vitro* culturing, regardless of the explant tissue used, or the full regeneration potential of recalcitrant (crop) species, will undoubtedly have a significant impact on their transformation efficiency and agri- and horticultural applications.

AUTHOR CONTRIBUTIONS

JH, FC, BC, FL, and LDV wrote the manuscript. AB and SS provided critical suggestions for the manuscript. JH created the figures. All authors contributed to the article and approved the submitted version.

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Mixed Linkage β-1,3/1,4-Glucan Oligosaccharides Induce Defense Responses in *Hordeum vulgare* and *Arabidopsis thaliana*

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Plants detect conserved microbe-associated molecular patterns (MAMPs) and modified "self" molecules produced during pathogen infection [danger associated molecular patterns (DAMPs)] with plasma membrane-resident pattern recognition receptors (PRRs). PRR-mediated MAMP and/or DAMP perception activates signal transduction cascades, transcriptional reprogramming and plant immune responses collectively referred to as pattern-triggered immunity (PTI). Potential sources for MAMPs and DAMPs are microbial and plant cell walls, which are complex extracellular matrices composed of different carbohydrates and glycoproteins. Mixed linkage β-1,3/1,4-glucan (β-1,3/1,4-MLG) oligosaccharides are abundant components of monocot plant cell walls and are present in symbiotic, pathogenic and apathogenic fungi, oomycetes and bacteria, but have not been detected in the cell walls of dicot plant species so far. Here, we provide evidence that the monocot crop plant *H. vulgare* and the dicot *A. thaliana* can perceive β-1,3/1,4-MLG oligosaccharides and react with prototypical PTI responses. A collection of Arabidopsis innate immunity signaling mutants and >100 Arabidopsis ecotypes showed unaltered responses upon treatment with β-1,3/1,4-MLG oligosaccharides suggesting the employment of a so far unknown and highly conserved perception machinery. In conclusion, we postulate that β-1,3/1,4-MLG oligosaccharides have the dual capacity to act as immune-active DAMPs and/or MAMPs in monocot and dicot plant species.

Keywords: DAMP-triggered immunity, MAMP-triggered immunity, elicitor, Arabidopsis, barley, β-1, 3/1, 4-mixed-linkage glucans

INTRODUCTION

Plants are constantly exposed to a variety of potential pathogens, including oomycetes, fungi and bacteria. To counteract potential intruders, plants have evolved a complex immune system. The presence of potentially harmful microbes can be recognized by plants through the perception of conserved microbe associated molecular patterns (MAMPs) that are absent from plants

(Bigeard et al., 2015; Hückelhoven and Seidl, 2016). Besides the perception of these "non-self" molecules, plants are also able to sense altered plant-derived molecules that are only present upon pathogen attack or cell damage. These "self" molecules with eliciting activity are referred to as danger or damage associated molecular patterns (DAMPs) (Bigeard et al., 2015; Hou et al., 2019). The perception of MAMPs and DAMPs is mediated by plasma-membrane localized pattern recognition receptors (PRRs) that can be classified as receptor-like kinases (RLKs) or receptor-like proteins (RLPs) (Couto and Zipfel, 2016; Boutrot and Zipfel, 2017). Upon perception of MAMPs and DAMPs a signaling cascade is induced which results in the activation of pattern-triggered immunity (PTI). Typical PTI responses include intracellular Ca^{2+} elevation, generation of reactive oxygen species (ROS), phosphorylation of mitogen activated protein kinases (MAPKs) and transcriptional reprogramming (Bigeard et al., 2015; Hückelhoven and Seidl, 2016; Hou et al., 2019).

In recent years, various bacterial and fungal MAMPs as well as host-derived DAMPs of protein and carbohydrate origin have been identified (Saijo et al., 2018; Hou et al., 2019). The plant immune system is e.g., able to sense the two bacterial proteins flagellin and elongation factor TU (EF-Tu) as well as the plant-derived plant elicitor peptide 1 (PEP1) and its homologs (Gómez-Gómez et al., 1999; Gómez-Gómez and Boller, 2000; Kunze et al., 2004; Huffaker et al., 2006; Zipfel et al., 2006; Bartels et al., 2013). The homopolymer chitin and β-1,3-glucans derived from fungal and oomycete cell walls as well as peptidoglycan from bacterial cell walls represent carbohydrate-based MAMPs (Klarzynski et al., 2000; Aziz et al., 2003; Ménard et al., 2004; Kaku et al., 2006; Gust et al., 2007; Miya et al., 2007; Mélida et al., 2018). Interestingly, recent studies have revealed the carbohydrate-rich plant cell wall as source for several DAMPs. Oligosaccharides derived from the plant cell wall components homogalacturonan, cellulose, xyloglucan, mannan, and arabinoxylan have been identified as DAMP molecules (Aziz et al., 2007; Ferrari et al., 2013; Benedetti et al., 2015; Davidsson et al., 2017; de Azevedo Souza et al., 2017; Claverie et al., 2018; Locci et al., 2019; Zang et al., 2019; Mélida et al., 2020). These immunogenic cell wall fragments are likely to be released through the action of secreted microbial cell wall degrading enzymes (CWDEs) (Bacete et al., 2018).

While leucine-rich repeat (LRR) RLKs play a role in the perception of proteinaceous MAMPs and DAMPs, the wall-associated kinase 1 (WAK1) and lysin-motif (LysM) domain containing RLKs mediate perception of carbohydrate-based ligands (Brutus et al., 2010; Bigeard et al., 2015; Zipfel and Oldroyd, 2017). In Arabidopsis, chitin is detected by the LysM-RLKs CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1), LYSIN MOTIF RECEPTOR KINASE 4 (LYK4) and LYK5 which form a heteromeric complex upon ligand perception (Kaku et al., 2006; Miya et al., 2007; Petutschnig et al., 2010; Liu et al., 2012; Wan et al., 2012; Cao et al., 2014; Erwig et al., 2017). Moreover, CERK1 was shown to be required for the perception of lipopolysaccharides (Gimenez-Ibanez et al., 2009; Desaki et al., 2018). CERK1 also mediates recognition of linear β-1,3-glucans as well as peptidoglycans together with the LysM RLPs LYSIN MOTIF DOMAIN-CONTAINING GLYCOSYLPHOSPHATIDYLINOSITOL-ANCHORED PROTEIN 1 (LYM1) and LYM3 (Willmann et al., 2011; Mélida et al., 2018; Wanke et al., 2020). However, the receptors involved in the perception of oligosaccharides derived from cellulose, xyloglucan, mannan, and arabinoxylan still remain elusive (de Azevedo Souza et al., 2017; Claverie et al., 2018; Locci et al., 2019; Zang et al., 2019; Mélida et al., 2020). Although several bacterial and fungal MAMPs, host-derived DAMPs and several cognate receptor (complexes) have been identified, it seems likely that plants are able to perceive more carbohydrate-based structures and that more receptors and co-receptors are involved in signal perception and transduction.

In contrast to the linear plant β-1,3- or β-1,4-glucose homopolymers callose and cellulose, mixed linkage β-1,3/1,4-glucans (β-1,3/1,4-MLGs) are linear polymers composed of glucosyl residues that are connected through both β-1,3- and β-1,4-glycosidic linkages (Burton and Fincher, 2009). In the plant kingdom, the presence of β-1,3/1,4-MLG is distributed asymmetrically. β-1,3/1,4-MLGs are found in the cell walls of most members of the monocot order *Poales* including the crop plant barley (*Hordeum vulgare*) as well as in evolutionary older plant lineages such as brown algae, liverworts and *Equisetum* spp., but are absent in dicot plants such as *Arabidopsis thaliana* (Zablackis et al., 1995; Popper and Fry, 2003; Fry et al., 2008; Sørensen et al., 2008; Burton and Fincher, 2009; Salmeán et al., 2017). Interestingly, β-1,3/1,4-MLGs have also been identified in lichen, namely *Cetaria islandica*, as well as in fungal, oomycete and bacterial species (Honegger and Haisch, 2001; Pettolino et al., 2009; Pérez-Mendoza et al., 2015; Rebaque et al., 2021). Thus, it is tempting to speculate that β-1,3/1,4-MLGs are more widespread cell wall components than previously thought.

As β-1,3/1,4-MLGs are present in the cell wall of monocotyledonous plants but are also abundant in bacterial and fungal species, they represent potential DAMPs and MAMPs in monocots and dicots, respectively. Thus, we analyzed the eliciting capacity of β-1,3/1,4-MLG oligosaccharides in *H. vulgare* and *A. thaliana* in this study. We found that β-1,3/1,4-MLG oligosaccharides derived from the hydrolysis of the *H. vulgare* β-1,3/1,4-MLG polysaccharide trigger canonical PTI responses in both, the monocot crop plant *H. vulgare* as well as the model dicot *A. thaliana*, suggesting a potential dual function as both DAMP and/or MAMP in a plant lineage-dependent manner. Reverse genetics and an accession screen in Arabidopsis revealed that known receptors and co-receptors of PTI are not involved in β-1,3/1,4-MLG oligosaccharide perception and that yet to be identified conserved molecular components mediate β-1,3/1,4-MLG oligosaccharide-induced signaling.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The Arabidopsis accession Col-0 was the background for all transgenic and mutant lines used in this study. Further Arabidopsis accessions that were used are listed in **Supplementary Table 1**. Seeds were surface sterilized by washing three times for 2 min with 70% EtOH and 0.05% Tween-20

with agitation. Seeds were afterward washed two times for 1 min with 100% EtOH and dried. The dry seeds were either sown on soil or grown on aqueous ½ Murashige and Skoog (MS) medium. For RNA extraction and MAPK experiments, 7-days old seedlings were transferred into individual wells (two seedlings per well) of a transparent 24-well plate and grown for seven further days. Plants were grown in a growth cabinet (CLF Plant Climatics, Wertingen, Germany) under short day conditions (12 h light, 12 h darkness).

H. vulgare was grown on soil in a growth chamber (Johnson Controls, Milwaukee, WI, United States) with long day conditions (16 h light, 26°C, 200 m^{-2} s^{-1}, 65% relative humidity).

Elicitors

Polymeric chitin of shrimp shells was obtained from Sigma-Aldrich (C9752-5G, Sigma), oligogalacturonides (OGs) were kindly provided by Simone Ferrari (Sapienza University of Rome) and the flg22 peptide (Felix et al., 1999) was synthesized by Thermo Scientific at a purity level of 98%. β-1,3/1,4-MLG tetra- and -trisaccharides used in this study were obtained from Megazyme (O-BGTETB, O-BGTETC, O-BGTRIA, and O-BGTRIB) and dissolved in ultrapure water at a concentration of 10 mg ml^{-1}. Fourteen-day-old Arabidopsis seedlings or leaf discs from second leaves of 14-days old *H. vulgare* plants were treated with elicitors added to liquid ½ MS plus sucrose medium. Unless otherwise stated, 100 μg ml^{-1} chitin, 100 nM flg22, 150 μM, or 190 μM of the β-1,3/1,4-MLG tetra- and -trisaccharides, were used for calcium and ROS burst assays, 10 μg ml^{-1} chitin, 10 μg ml^{-1} OGs, 50 nM flg22, 15 μM, or 10 μM of the β-1,3/1,4-MLG tetra- and -trisaccharides, were used for MAPK experiments and gene expression analyses.

To generate *H. vulgare* -derived β-1,3/1,4-MLG oligosaccharides, 10 mg ml^{-1} *H. vulgare* β-1,3/1,4-MLG polysaccharide (P-BGBL, Megazyme) was dissolved in 100 mM sodium phosphate buffer (pH = 6.5). 0.025 or 1 U ml^{-1} lichenase (E-LICHN, Megazyme) was added to the solution and incubated for different times at 40°C with agitation. The hydrolysis was stopped by incubation in boiling water for at least 15 min. The hydrolysis products were tested via Thin Layer Chromatography (TLC). 5 μl of the analytes were applied onto a TLC Silica gel plate (TLC silica gel 60, 1.05721.0001, Merck). Upon drying, the plate was put into running buffer (isopropanol:ethylacetate:water, 2:2:1). Upon drying of the plate, the plate was wetted with a TLC staining solution (10% sulfuric acid in methanol) and incubated on a heating plate at 99°C. Seedlings were treated with a 1:10 dilution of the hydrolysis products.

Calcium Measurements

Intracellular calcium was measured using an aequorin-based calcium assay (Ranf et al., 2012). Calcium responses in the absence of an elicitor was included as negative control. The Ca^{2+} concentrations were calculated and normalized according to Rentel and Knight, 2004 and are depicted as L/Lmax with L representing the luminescence at any time point upon β-1,3/1,4-MLG oligosaccharide or MAMP treatment and Lmax representing the total remaining aequorin. To calculate Lmax, the luminescence obtained upon treatment with the discharge solution was integrated.

ROS Measurements

The generation of ROS was determined using a luminol-based assay. Leaf discs (4 mm diameter) of 5-7 weeks old Arabidopsis plants or 10–12 days old *H. vulgare* plants were incubated in water overnight in a flat-bottom 96-well plate. The water was replaced with a luminol solution [10 μg ml^{-1} Horseradish peroxidase (P6782, Sigma), 100 μM L-012 (120-04891, WAKO Chemicals)] containing no elicitor, elicitors at the indicated concentrations or a 1:10 dilution of *H. vulgare* β-1,3/1,4-MLG polysaccharide hydrolysis products. Luminescence was recorded with a TECAN infinite® M200 plate reader for 60 min in 1 min intervals with an integration time of 150 ms.

MAPK Assays

Arabidopsis seedlings were grown *in vitro* as described above. One day before the treatment, the medium was replaced with 500 μl ½ fresh MS medium to ensure equal volumes. 14-days old seedlings were treated with the elicitors for 12 min and directly frozen in liquid nitrogen. *H. vulgare* plants were grown on soil as described above, 12–14 leaf discs of 4 mm diameter were harvested from second leaves of 14-days old plants and incubated for 16 h in 2 ml ultrapure water. The leaf discs were transferred to fresh ultrapure water and incubated for 30 min. Subsequently, elicitor solutions were added to the indicated final concentrations. Negative control samples were treated with an equivalent volume of water. The leaf discs were incubated for 12 min and then directly frozen in liquid nitrogen. The frozen seedlings or leaf discs were homogenized in 600 or 200 μl extraction buffer, respectively (Petutschnig et al., 2010). After centrifugation for 10 min at 4°C at 13.000 rpm, the protein concentration was determined via Bradford Assay with BSA as standard and protein concentrations were equalized. Protein extracts were frozen at −20°C. Samples were separated on a 10% SDS gel. Immunoblot analysis were performed using Phospho p44/42 (#9101, Cell Signaling Technology, 1:5000) as primary antibody and a goat anti-rabbit IgG (A3687, 1:5000, Sigma Aldrich) as secondary antibody.

RNA Isolation and qRT-PCR

Arabidopsis seedlings were grown *in vitro* as described above. One day before the treatment, the medium was replaced with 500 μl ½ MS medium to ensure equal volumes. 14-day-old seedlings were treated with the elicitors for 30 min and directly frozen in liquid nitrogen. Total RNA was extracted from seedlings using Qiazol (Qiagen, Hilden, Germany) and digested with DNase (EN0521, Thermo Scientific). 1 μg RNA per 20 μl reaction were used to generate cDNA using RevertAid™ H Minus M-MulVRT (EP0451, Thermo Scientific). For qRT-PCRs, 3 μL of 1:500 diluted cDNA was used to analyze gene expression with SsoFast EvaGreen supermix (1725204, BioRad) using the following PCR conditions: 95°C for 30 s, 45 cycles of 95°C for 5 s, and 55°C for 10 s, followed by fluorescence reading. For normalization, *UBIQUITIN5* was amplified in

parallel on each plate. Aliquots of cDNAs used within one experiment were pooled and a dilution series was prepared from the pool to calculate primer efficiencies. Primer pair efficiencies were determined to be 98.53% (*WRKY33*), 100.06% (*WRKY53*) and 103.58% (*Ubiquitin5*) for the experiment shown in **Figures 1D,E** and 92.91% (*WRKY33*), 96.56% (*WRKY53*) and 104.82% (*Ubiquitin5*) for the experiment shown in **Figures 2G,H**. Melting curves and no-template controls were analyzed to rule out primer-dimer formation and contaminations. Primers are listed in **Supplementary Table 2**.

Seedling Growth Inhibition

Arabidopsis seedlings were grown for 5 days on ½ MS medium and then transferred to 24-well plates (one seedling per well). Each well contained 500 μL ½ MS medium with either no elicitor, or one of the following substances:1 μM flg22, 10 mM Sodium Phosphate buffer (pH = 6.5) or a 1:10 dilution of the enzymatically generated *H. vulgare* β-1,3/1,4-MLG oligosaccharides. Pictures were taken of 13-day-old seedlings. To determine the dry weight, seedlings were dried for 1 day at 65°C and the total weight of all eight seedlings was determined.

Carbohydrate Analysis

High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was performed using a Dionex ICS-5000 HPLC system equipped with an AS-AP autosampler in a sequential injection configuration using the Chromeleon software version 7. 10 μl of the samples were injected on a 3 × 250 mm Dionex Carbopac PA200 column (Thermo Scientific, Waltham, United States). 56 μM of the β-1,3/1,4-MLG tetrasaccharide or 45 μM of the β-1,3/1,4-MLG trisaccharide were loaded onto the column. The gradient was used as follows: 0–5 min, 10% B, 3.5% C (initial conditions); 5–12 min 10% B, linear gradient from 0–30% C; 12.0–12.1 min, 50% B, 50% C; 12.1–13.0 min, exponential gradient of B and C, back to initial conditions, 13–17 min initial conditions. Solvent A was ultrapure water, solvent B was 1 M sodium hydroxide and solvent C was 1 M sodium acetate.

Matrix Assisted Laser Desorption Ionization–Time of Flight (MALDI-TOF) analysis of mixed-linkage glucans was performed with a Bruker Autoflex system (Bruker Daltonics) operated in reflectron mode. 10 mg/ml of the oligosaccharide were mixed 1:5 with 2,5-dihiydroxybenzoic acid in 1:1 H_2O:MeOH on a Bruker MTP 384 grounded steel MALDI plate. The samples were allowed to dry and directly analyzed.

Accession Numbers

The Arabidopsis mutant lines used in this study were: *cerk1*-2 (GABI_096F09), *lyk5*-2 *lyk4*-2 (SALK_131911C × GABI_897A10), *lym2*-1 (SAIL_343B03), *lym2*-4 (GABI-Kat 165 H02), *efr*-1 (SALK_044334), *fls2c* (SAIL_691_C4), *bak1*-4 (SALK_116202), *bak1*-5 (Schwessinger et al., 2011), *sobir1*-12 (SALK_050715), and *sobir1*-14 (GABI-Kat_643F07).

RESULTS

Enzymatically Generated β-1,3/1,4-MLG Oligosaccharides Induce Immune Responses in *H. vulgare* and *A. thaliana*

To test the potential MAMP or DAMP activities of β-1,3/1,4-MLG polymer and enzymatically derived oligosaccharides in the monocot and dicot model plants *H. vulgare* and *A. thaliana*, we first used commercially available *H. vulgare* β-1,3/1,4-MLG polysaccharide that we incubated with *Bacillus subtilis* lichenase (**Supplementary Figure 1**). *H. vulgare* β-1,3/1,4-MLG is composed of β-1,3-linked cellotriosyl or cellotetrasyl units (Burton and Fincher, 2014) and *B. subtilis* lichenase hydrolyses β-1,4-bonds that immediately follow β-1,3-linkages in β-1,3/1,4-MLG polymers (Planas, 2000). Thus, enzymatic end products are *H. vulgare* β-1,3/1,4-MLG tri- and tetrasaccharides such as G4G3G and G3G4G4G, where G represents glucose and 3 and 4 indicate the β-1,3- and β-1,4-linkages, respectively. We stopped lichenase-mediated hydrolysis at different incubation times (0, 15, 30, 45, 60, 120, and 240 min), to obtain hydrolyzates containing β-1,3/1,4-MLG oligosaccharides with different degrees of polymerization (DP). The hydrolysis of the β-1,3/1,4-MLG polysaccharide was confirmed with TLC and revealed an increase in abundance of β-1,3/1,4-MLG oligosaccharides with a shorter DP over time (**Supplementary Figure 1**).

The activation of MAPKs upon MAMP or DAMP perception is a conserved immune response in *H. vulgare* and *A. thaliana* (Bigeard et al., 2015; Hückelhoven and Seidl, 2016), which can be monitored by Western blot analysis (**Figures 1A,B**). Our experiments revealed that only enzymatically generated β-1,3/1,4-MLG oligosaccharides induced the phosphorylation of MAPKs in *H. vulgare* and *A. thaliana*, whereas application of the β-1,3/1,4-MLG polysaccharide, lichenase enzyme and mock controls did not trigger MAPK activation (**Figures 1A,B**). Interestingly, only MAPK6 and MAPK3 but not MAPK4/11 were phosphorylated upon elicitation with β-1,3/1,4-MLG oligosaccharides in *A. thaliana* (**Figure 1B**). Next, we used transgenic Arabidopsis plants producing the Ca^{2+}-sensor aequorin to analyze treatment-dependent influx of calcium (**Figure 1C**), another MAMP/DAMP-induced immune response (Bigeard et al., 2015). Again, only enzymatically generated hydrolysis products of the β-1,3/1,4-MLG polysaccharides, but not the β-1,3/1,4-MLG polysaccharide led to a fast and transient influx of Ca^{2+} which peaked at 30 s and lasted for about 3 min (**Figure 1C**). Finally, the expression of the two transcription factors *WRKY33* and *WRKY53*, which were previously shown to be up-regulated in response to MAMPs (Cao et al., 2014), was analyzed by qRT-PCR. The expression of both, *WRKY33* and *WRKY53*, was upregulated upon β-1,3/1,4-MLG oligosaccharides elicitation (**Figures 1D,E**). Notably, the intensity of the tested immune responses in *A. thaliana* and *H. vulgare* was more pronounced upon treatment with hydrolyzates containing a high amount of β-1,3/1,4-MLG oligosaccharides with a short DP (60, 120, or 240 min incubation time, **Figure 1** and **Supplementary Figure 1**). These results indicate that lichenase-generated β-1,3/1,4-MLG oligosaccharides can be perceived by

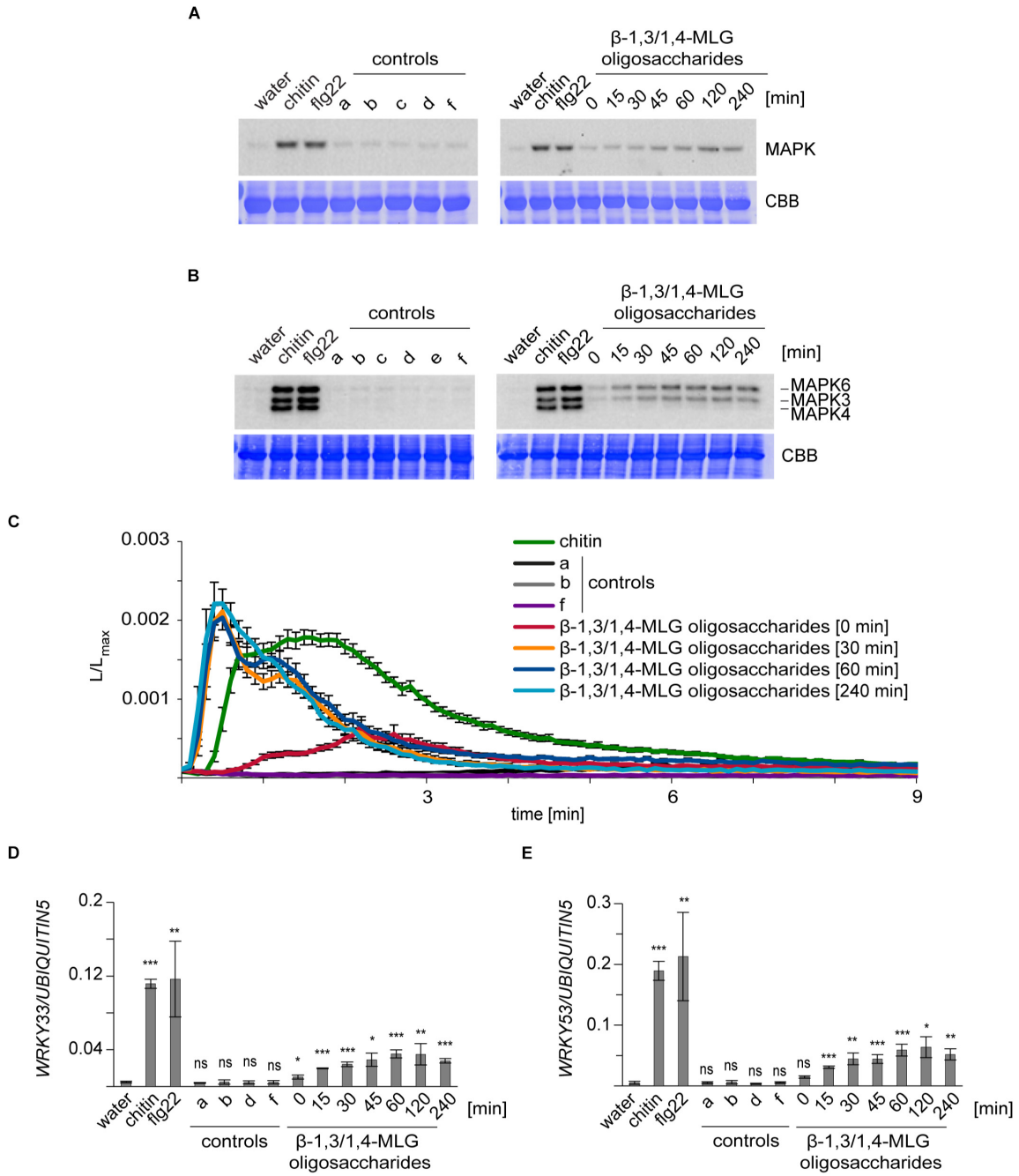

FIGURE 1 | Enzymatically-generated β-1,3/1,4-MLG oligosaccharides induce immune responses in *A. thaliana* and *H. vulgare*. β-1,3/1,4-MLG oligosaccharides were generated by incubation of the β-1,3/1,4-MLG polysaccharide (10 mg ml^{-1}) with the *B. subtilis* lichenase (0.025 U/ml) and was stopped at several time points (0, 15, 30, 45, 60, 120, or 240 min). For the treatment, a 1:10 dilution of the hydrolysis products was used. Chitin (10 μg ml^{-1} for MAPK activation and defense gene expression; 100 μg ml^{-1} for calcium elevation) and flg22 (50 nM) served as positive controls. As negative controls, water, sodium phosphate buffer (a, 10 mM), β-1,3/1,4-MLG polysaccharide (b, 1 mg ml^{-1}), active lichenase (c, 0.0025 U ml^{-1}), heat-inactivated lichenase (d, 0.0025 U ml^{-1}; e, 0.00125 U ml^{-1}) and β-1,3/1,4-MLG polysaccharide (1 mg ml^{-1}) and heat-inactivated lichenase (f, 0.0025 U ml^{-1}) were used. **(A,B)**: MAPK phosphorylation in **(A)** leaf discs of 12–14 day-old *H. vulgare* plants and **(B)** 14-day-old Arabidopsis Col-0 seedlings upon elicitation with β-1,3/1,4-MLG oligosaccharides. As loading control, the PVDF membrane was stained with Coomassie Brilliant Blue (CBB). The data shown are derived from one of three biological replicates that gave similar results. **(C)** Calcium elevation in 8–10 day-old Arabidopsis Col-0 aequorin lines in response to enzymatically generated β-1,3/1,4-MLG oligosaccharides. The luminescence was assessed immediately after elicitor treatment (L) and was normalized to total luminescence measured upon addition of calcium (L_{max}). The data show mean of twelve seedlings with SEM from one of three biological experiments that produced similar results. **(D,E)**: Expression of the defense genes *WRKY33* **(D)** and *WRKY53* **(E)** in 14-day-old Arabidopsis Col-0 seedlings upon treatment with enzymatically generated β-1,3/1,4-MLG oligosaccharides. Data are presented as means of three biological replicates (with three technical replicates each) ± SD. *UBIQUITIN5* was used as reference gene. Statistical significance (unpaired student's *t*-test) of elicitor treatment compared to water treatment: ns = $p > 0.5$; * = $p \leq 0.5$, ** = $p \leq 0.05$, *** = $p \leq 0.001$.

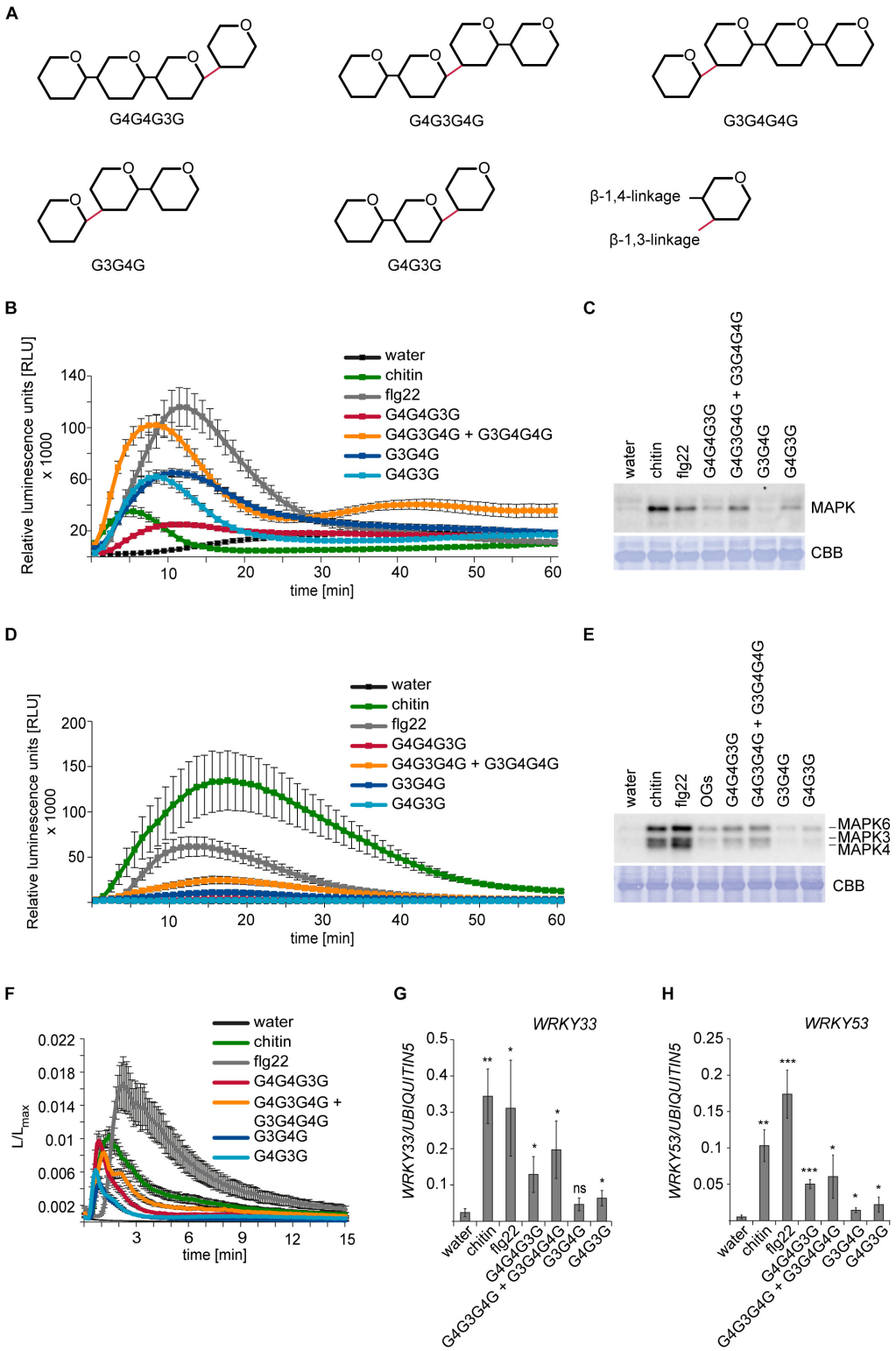

FIGURE 2 | Activation of PTI in *H. vulgare* and *A. thaliana* by β-1,3/1,4-MLG tetrasaccharides and trisaccharides. For ROS production and calcium elevation, 150 μM of the β-1,3/1,4-MLG tetrasaccharides and 190 μM of the β-1,3/1,4-MLG trisaccharides were used. Water, chitin (100 μg ml^{-1}) and flg22 (100 nM) served as controls. For MAPK activation and calcium elevation, 15 μM and 19 μM of the β-1,3/1,4-MLG tetrasaccharides and β-1,3/1,4-MLG trisaccharides were used, respectively. As controls, water, chitin (10 μg ml^{-1}) and flg22 (50 nM) and oligogalacturonides (OG; 10 μg ml^{-1}) were used. **(A)** Structure of the β-1,3/1,4-MLG MLG tetrasaccharides and -trisaccharides used in the experiments. **(B)** ROS production in leaf discs of 12–14-days old *H. vulgare* plants upon elicitation with

(Continued)

FIGURE 2 | Continued
β-1,3/1,4-MLG tetrasaccharides and trisaccharides. Data represent the mean of eight leaf discs from one out of three biological replicates that gave similar results. Error bars represent SEM. **(C)** Phosphorylation of MAPK in leaf discs of 14-day old *H. vulgare* plants in response to β-1,3/1,4-MLG tetrasaccharides and -trisaccharides. As loading control, the PVDF membrane was stained with Coomassie Brilliant Blue (CBB). Data show one biological replicate. The experiment was repeated three times with similar results. **(D)** Generation of ROS in Arabidopsis leaf discs of 5–7 weeks old Arabidopsis Col-0 plants upon treatment with β-1,3/1,4-MLG tetra- and trisaccharides. Data shown the mean of eight leaf discs from one biological replicate. Error bars represent SEM. The experiment was repeated three times with similar results. **(E)** MAPK activation in 14-day old Arabidopsis Col-0 seedlings upon elicitation with β-1,3/1,4-MLG tetra- and trisaccharides. As loading control, the PVDF membrane was stained with Coomassie Brilliant Blue (CBB). Data show the result of one out of three biological replicated that showed similar results. **(F)** Calcium elevation in 8–10 day-old Arabidopsis Col-0 seedlings expressing aequorin in response to β-1,3/1,4-MLG tetra- and trisaccharides. The luminescence was assessed immediately after elicitor treatment (L) and was normalized to total luminescence upon addition of calcium (L_{max}). The data show means of twelve seedlings + SEM from one of three biological experiments that gave similar results. **(G,H)** Expression of the defense genes *WRKY33* **(G)** and *WRKY53* **(H)** in 14-days old Arabidopsis Col-0 seedlings upon treatment with β-1,3/1,4-MLG tetra- and trisaccharides. Data are presented as means of three biological replicates (with three technical replicates each) ± SD. *UBIQUITIN5* was used as reference gene. Statistical significance (unpaired student's *t*-test) of elicitor treatment compared to water treatment: ns = $p > 0.5$; * = $p \leq 0.5$, ** = $p \leq 0.05$, *** = $p \leq 0.001$.

H. vulgare and *A. thaliana* and induce canonical pattern-triggered immune responses.

β-1,3/1,4-MLG Tetra- and Trisaccharides Activate PTI Responses

To confirm the ability of β-1,3/1,4-MLG oligosaccharides to trigger innate immune responses in the monocot and dicot models *H. vulgare* and *A. thaliana*, commercially available β-1,3/1,4-MLG preparations with defined linkage sequences were tested for MAMP/DAMP activity: a β-1,3/1,4-MLG tetrasaccharide (G4G4G3G), a β-1,3/1,4-MLG tetrasaccharide mixture (G4G3G4G + G3G4G4G), as well as two β-1,3/1,4-MLG trisaccharides (G3G4G and G4G3G) (**Figure 2A**). The purity and the masses of the obtained β-1,3/1,4-MLG oligosaccharides were confirmed using HPAEC-PAD and MALDI-TOF (**Supplementary Figure 2**).

First, we tested the generation of ROS, which represents another canonical and early MAMP/DAMP-inducible plant response (Bigeard et al., 2015; Hückelhoven and Seidl, 2016). In *H. vulgare*, ROS production was induced upon treatment with the β-1,3/1,4-MLG tetrasaccharide mixture and the two β-1,3/1,4-MLG trisaccharides. The generation of ROS in response to β-1,3/1,4-MLG oligosaccharides was weaker than the flagellin (flg22)-induced ROS burst, but stronger than the chitin-induced ROS burst (**Figure 2B**). Phosphorylation of MAPK in *H. vulgare* was also induced in response to the β-1,3/1,4-MLG tetrasaccharide mixture and the β-1,3/1,4-MLG trisaccharide G4G3G, whereas it was almost or completely undetectable upon G4G4G3G or G3G4G treatment (**Figure 2C**). In *A. thaliana* Col-0 plants, only slight generation of ROS could be detected upon treatment with the β-1,3/1,4-MLG tetrasaccharide mixture, which was weaker than chitin or flg22-induced ROS production (**Figure 2D**). The phosphorylation of MAPK6 and MAPK3 was triggered upon application of β-1,3/1,4-MLG tetrasaccharides but was almost undetectable in response to β-1,3/1,4-MLG trisaccharides (**Figure 2E**). The influx of calcium ions was induced in response to all commercially available β-1,3/1,4-MLG tetra- and trisaccharides (**Figure 2F**). Furthermore, analysis of the expression of *WRKY33* and *WRKY53* revealed a transcriptional up-regulation of both genes upon β-1,3/1,4-MLG tetra- and trisaccharide elicitation (**Figures 2G,H**).

Together, these experiments corroborate the ability of β-1,3/1,4-MLG oligosaccharides to act as elicitors of immune responses in the monocot *H. vulgare* and the dicot *A. thaliana*. Notably, the analyzed immune responses were stronger in response to β-1,3/1,4-MLG tetrasaccharides in both *H. vulgare* and *A. thaliana*, possibly indicating that the corresponding receptor has a higher affinity to longer β-1,3/1,4-MLG oligosaccharides.

Known Receptors and co-Receptors Are Not Involved in Perception of β-1,3/1,4-MLG Oligosaccharides

To identify molecular components involved in β-1,3/1,4-MLG oligosaccharide perception and signaling, only the dicot model plant *A. thaliana* was used, as a substantial number of mutants of components of the plant immune system are available. LysM domain containing RLKs have previously been shown to be involved in the perception of carbohydrate-based elicitors (Miya et al., 2007; Shimizu et al., 2010; Willmann et al., 2011; Wan et al., 2012; Cao et al., 2014; Desaki et al., 2018; Mélida et al., 2018; Wanke et al., 2020). The Arabidopsis genome encodes for five LysM-RLKs, namely CERK1/LYK1, and LYK2 to LYK5 (Zhang et al., 2007). CERK1 is involved in the detection of chitin together with LYK4 and LYK5 as well as bacterial peptidoglycan and β-1,3-glucans (Kaku et al., 2006; Miya et al., 2007; Petutschnig et al., 2010; Willmann et al., 2011; Liu et al., 2012; Cao et al., 2014; Erwig et al., 2017; Mélida et al., 2018). The LysM-RLP LYM2 does not play a role in canonical chitin-triggered immune responses, but facilitates the reduction of molecular flux via plasmodesmata in response to chitin (Faulkner et al., 2013; Cheval et al., 2020). To test a potential involvement of CERK1, LYK5, LYK4, and LYM2 in perception of β-1,3/1,4-MLG oligosaccharides, MAPK phosphorylation in *cerk1*-2, *lyk5*-2 *lyk4*-2, *lym2*-1, and *lym2*-4 upon elicitation with lichenase-generated β-1,3/1,4-MLG oligosaccharides was assessed. To generate β-1,3/1,4-MLG oligosaccharides, *H. vulgare* derived β-1,3/1,4-MLG polysaccharide was incubated for 60 min with *B. subtilis* lichenase. The hydrolysis was confirmed via TLC (**Supplementary Figure 3**). The intensity of MAPK phosphorylation was not altered in the tested mutants in comparison to the wild-type (**Figure 3A**) indicating that

perception of β-1,3/1,4-MLG oligosaccharides is independent of CERK1, LYK4, LYK5, and LYM2.

The LRR-RLK FLAGELLIN SENSING 2 (FLS2) and the LRR-RLK EF-TU RECEPTOR (EFR) are required for the perception of the prototypical protein-derived MAMPs flg22 and elf18, respectively (Gómez-Gómez and Boller, 2000; Zipfel et al., 2006). In order to assess whether EFR or FLS2 are involved in β-1,3/1,4-MLG oligosaccharide perception, phosphorylation of MAPK in *fls2c* and *efr-1* in response to enzymatically generated β-1,3/1,4-MLG oligosaccharides (**Supplementary Figure 3**) was tested. Western Blotting revealed the same intensity of MAPK phosphorylation in Col-0, *fls2c*, and *efr-1*, suggesting that neither FLS2 nor EFR play a role in β-1,3/1,4-MLG oligosaccharides perception (**Figure 3B**).

The co-receptors BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (BAK1) and the adaptor kinase SUPPRESSOR OF BRASSINOSTEROID INSENSITIVE 1 (SOBIR1) are required for the activation of early immune responses upon perception of several elicitors (Chinchilla et al., 2006; Heese et al., 2007; Roux et al., 2011; Schwessinger et al., 2011). To address whether BAK1 or SOBIR1 are required for β-1,3/1,4-MLG oligosaccharide perception, MAPK phosphorylation in response to β-1,3/1,4-MLG oligosaccharides was monitored in mutants of SOBIR1 and BAK1. The level of MAPK phosphorylation was similar between the wild-type (Col-0) and the tested mutants, suggesting that neither SOBIR1 nor BAK1 are involved in perception of β-1,3/1,4-MLG oligosaccharides (**Figures 3C,D**). Together, these results demonstrate that the tested prototypical receptors and co-receptors are dispensable for perception of β-1,3/1,4-MLG oligosaccharides and prompt the conclusion that a yet unknown receptor (complex) mediates β-1,3/1,4-MLG oligosaccharide perception and signal transduction.

Arabidopsis Ecotypes Exhibit a Conserved Capacity for β-1,3/1,4-MLG Oligosaccharide Perception

As PTI can vary between different Arabidopsis accessions due to distinct genomic receptor repertoires, pattern-sensitive and pattern-insensitive accessions have previously been used to identify PRRs governing perception via comparative genomics (Gómez-Gómez et al., 1999; Gómez-Gómez and Boller, 2000; Jehle et al., 2013; Zhang et al., 2013).

To identify potential Arabidopsis ecotypes that are insensitive toward β-1,3/1,4-MLG oligosaccharides, the level of MAPK phosphorylation upon application of β-1,3/1,4-MLG oligosaccharides was determined in 112 different accessions. First, parental ecotypes of the Multiparent Advanced Generation Inter-Cross (MAGIC) recombinant inbred lines (Kover et al., 2009) were chosen, because these would have facilitated mapping of potential β-1,3/1,4-MLG oligosaccharide receptors. However, all parental ecotypes responded to β-1,3/1,4-MLG oligosaccharide treatment with an almost invariant MAPK activation pattern (**Figure 4**). Similarly, a collection of 95 additional Arabidopsis accessions that were tested for their ability to perceive β-1,3/1,4-MLG-derived oligosaccharides as MAMP, also showed qualitatively unaltered MAPK phosphorylation capacity in response to enzymatically produced β-1,3/1,4-MLG oligosaccharides (**Supplementary Figure 4**). The fact that more than 100 Arabidopsis accessions were sensitive toward β-1,3/1,4-MLG oligosaccharides suggest that the underlying perception and signaling machinery is highly conserved within the species *Arabidopsis thaliana*.

DISCUSSION

During plant-microbe interactions, microbial and plant CWDEs are secreted into the extracellular space, where they act on their opponents' cell walls and release small oligosaccharides (Bacete et al., 2018). Thus, the carbohydrate-rich cell walls of plants and their pathogens represent a source for potential DAMPs and MAMPs. In the last years, several cell wall-derived DAMPs and MAMPs have been identified (Saijo et al., 2018; Hou et al., 2019; Pontiggia et al., 2020), but it is conceivable that a substantially larger number of cell wall-derived ligands can be perceived by plants. Here we provide evidence that β-1,3/1,4-MLG oligosaccharides activate immune responses in more than 100 accessions of the dicot model plant *A. thaliana* and the monocot crop plant *H. vulgare*.

The β-1,3/1,4-MLG polysaccharide can be found in the cell wall of most members of the *Poales* including *H. vulgare*, but also in evolutionary older plant lineages, e.g., brown algae or horsetail (Fry et al., 2008; Burton and Fincher, 2009; Salmeán et al., 2017). Thus, β-1,3/1,4-MLG oligosaccharides can be classified as DAMPs in monocot species. The cell wall derived OGs are DAMPs that are released upon partial degradation of the pectin component homogalacturonan by fungal polygalacturonases (Ferrari et al., 2013; Benedetti et al., 2015; Pontiggia et al., 2020). Similarly, β-1,3/1,4-MLG oligosaccharides are likely to be released from the abundant monocot β-1,3/1,4-MLG polysaccharide through enzymatic activity of microbial glucanases secreted during monocot plant-microbe interactions.

Genomes of biotrophic, hemibiotrophic and necrotrophic plant pathogens contain a high number of CWDEs of which only a few have been biochemically characterized (Kubicek et al., 2014; Zhao et al., 2014). The genome of the biotrophic barley powdery mildew *Blumeria graminis* f.sp. *hordei* (*Bgh*) was shown to encode for 67 glycoside hydrolases (GHs) that can be classified into 25 different families including e.g., GH5, GH16 or GH17 (Zhao et al., 2014). Enzymes classified as GH5, GH16 or GH17 are predicted to catalyze the hydrolysis of cellulose, hemicelluloses, β-1,3-glucans and β-1,3/1,4-MLGs (Carbohydrate Active Enzymes database[1]; Lombard et al., 2014; Zhao et al., 2014), however, none of the *Bgh* GHs have been biochemically characterized. Notably, transcriptome analysis showed that the expression of one GH16 enzyme (*Bgh01441*) and other CWDEs of barley powdery mildew are upregulated during infection of immunocompromised Arabidopsis mutants, suggesting that they may be involved in degradation of the plant

[1]http://www.cazy.org

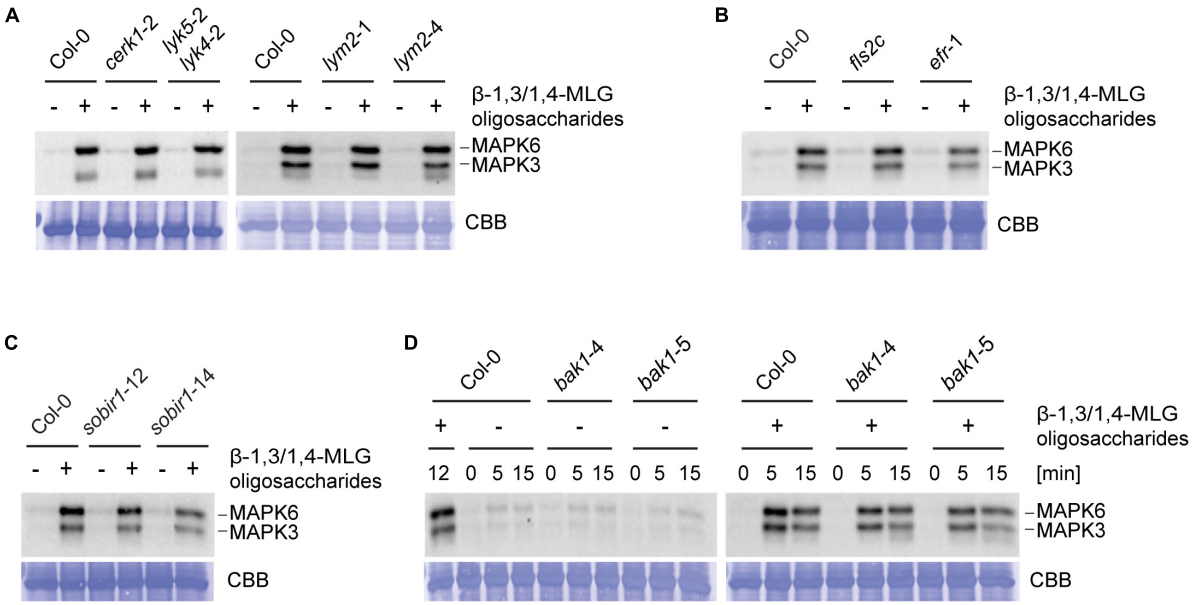

FIGURE 3 | β-1,3/1,4-MLG oligosaccharides induced activation of MAPK is independent of known PTI components. MAPK activation in 14-day old seedlings of **(A)** Col-0, *cerk1*-2, *lyk5*-2 *lyk4*-2, *lym2*-1, and *lym2*-4, **(B)** Col-0, *fls2c*, *efr*-1, **(C)** Col-0, *sobir1*-12, *sobir1*-14 and **(D)** Col-0, *bak1*-4 and *bak1*-5 upon elicitation with enzymatically generated with β-1,3/1,4-MLG oligosaccharides. β-1,3/1,4-MLG oligosaccharides were generated upon incubation of the MLG polymer (10 mg ml^{-1}) with *B. subtilis* lichenase (1 U ml^{-1}) for 60 min (**Supplementary Figure 3**). For seedling treatment, a 1:10 dilution of the hydrolysis products was used. Sodium phosphate buffer (1 mM) served as negative control. **(A–C)** Western Blot shows MAPK phosphorylation upon elicitation with the indicated elicitors for 12 min. **(D)** Western Blot shows phosphorylated MAPK at different time points (0, 5, and 15 min). As loading control, the PVDF membrane was stained with Coomassie Brilliant Blue (CBB). Data show the result from one biological replicate. The experiments were repeated twice with similar results.

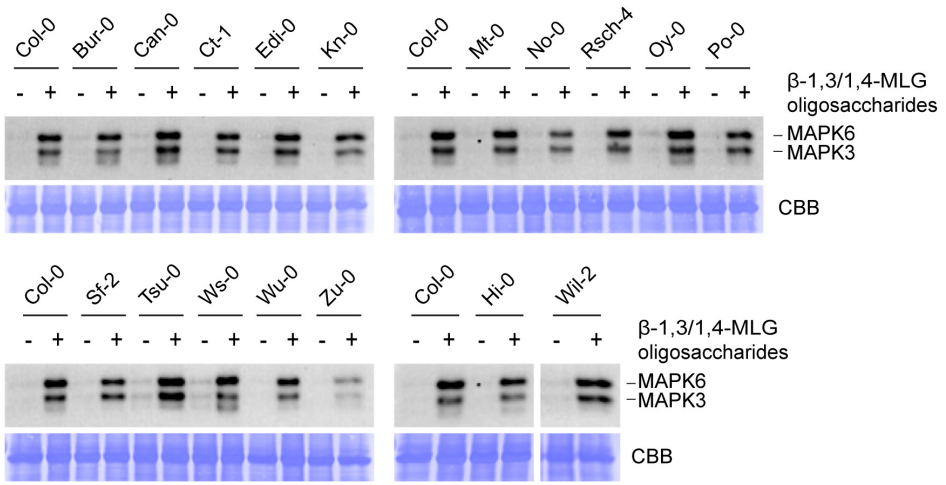

FIGURE 4 | Lichenase-generated β-1,3/1,4-MLG oligosaccharides induce MAPK activation in different Arabidopsis accessions. MAPK activation in 14-day-old seedlings of various ecotypes in response to enzymatically generated β-1,3/1,4-MLG oligosaccharides. β-1,3/1,4-MLG oligosaccharides were generated upon incubation of the MLG polymer (10 mg ml^{-1}) with the *B. subtilis* lichenase (1 U ml^{-1}) for 60 min (**Supplementary Figure 3**). For the treatment, a 1:10 dilution of the hydrolysis products was used. Sodium phosphate buffer (1 mM) served as negative control. As loading control, the PVDF membrane was stained with Coomassie Brilliant Blue (CBB). Data show the result from one biological replicate.

cell wall (Hacquard et al., 2013). The analysis of plant pathogen secretomes and the biochemical characterization of identified CWDEs have the potential to reveal whether CWDEs acting on the β-1,3/1,4-MLG polysaccharide are present in a given monocot plant pathogen species.

In contrast to monocot species, dicot plants such as *A. thaliana* do not contain β-1,3/1,4-MLGs in their cell walls (Zablackis et al., 1995; Burton and Fincher, 2009). However, the experimental data of this work and a recently published study (Rebaque et al., 2021) clearly demonstrate that Arabidopsis plants can detect

β-1,3/1,4-MLG oligosaccharides and react with canonical PTI responses. As β-1,3/1,4-MLG oligosaccharides are not present in Arabidopsis, they are likely to function as a MAMP in this species. Interestingly, β-1,3/1,4-MLG polysaccharides have previously been identified in the plant pathogenic fungus *Rhynchosporium secalis*, the oomycete *Hyaloperonospera arabidopsidis* and the endosymbiotic bacterium *Sinorhizobium meliloti* (Pettolino et al., 2009; Pérez-Mendoza et al., 2015; Rebaque et al., 2021). Microbial cell walls can contain between 50–60% glucans (Bowman and Free, 2006), however, to what extent β-1,3/1,4-MLGs contribute to their chemical structure remains largely elusive. Thus, β-1,3/1,4-MLGs may be more abundant microbial cell wall components than previously thought.

Upon pathogen attack, plant chitinases and β-1,3-glucanases are secreted, act on fungal cell wall components and release immunogenic oligosaccharides (Keen and Yoshikawa, 1983; Ham, 1991; Uknes et al., 1992; Doxey et al., 2007; Balasubramanian et al., 2012; Pusztahelyi, 2018). Similarly, β-1,3;1,4-glucanases may be secreted by host and non-host plants. The Arabidopsis genome harbors more than 400 genes encoding for GHs, of which only a few have been characterized (Carbohydrate Active Enzymes database (see text footnote 1); Lombard et al., 2014). Glucanases that may act on β-1,3/1,4-MLGs can be found in GH families 5, 6, 7, 8, 9, 11, 12, 16, and 17. The Arabidopsis genome harbors 13, 26, 33, and 51 genes encoding for enzymes categorized as GH5, GH9, GH16, and GH17 family members, respectively. Of these, only two GH5 proteins, two GH9 proteins, twelve GH16 proteins and three GH17 proteins have been analyzed (Carbohydrate Active Enzymes database (see text footnote 1); Lombard et al., 2014), but did not exhibit β-1,3;1,4-glucanase activity. However, not yet characterized proteins belonging to these GH families may be able to catalyze the hydrolysis of β-1,3/1,4-MLGs and might be involved in the generation of immunogenic MLG oligosaccharides upon microbial attack.

Besides *A. thaliana*, also the crop plants pepper and tomato can detect β-1,3/1,4-MLG oligosaccharides (Rebaque et al., 2021), which suggests that the responsible perception machinery is evolutionary highly conserved among dicot plants. Our study supports this conclusion and goes beyond in demonstrating an invariable intraspecific conservation in the dicot species Arabidopsis as well as potential evolutionary maintenance in the monocot lineage.

Perception of carbohydrate-based ligands was shown to be mediated by LysM domain containing PRRs (Miya et al., 2007; Petutschnig et al., 2010; Shimizu et al., 2010; Willmann et al., 2011; Wan et al., 2012; Cao et al., 2014; Desaki et al., 2018; Mélida et al., 2018; Xue et al., 2019; Wanke et al., 2020). The LysM RLK CERK1 has been suggested as co-receptor for several carbohydrate-based ligands due to its involvement in the perception of chitin, peptidoglycan and β-1,3-glucans in Arabidopsis (Kaku et al., 2006; Miya et al., 2007; Petutschnig et al., 2010; Willmann et al., 2011; Mélida et al., 2018; Wanke et al., 2020). Moreover, the LysM proteins LYK4, LYK5, and LYM2 are components of chitin perception and closure of plasmodesmata in response to chitin, respectively (Kaku et al., 2006; Miya et al., 2007; Petutschnig et al., 2010; Wan et al., 2012; Faulkner et al., 2013; Cao et al., 2014; Erwig et al., 2017; Xue et al., 2019; Cheval et al., 2020). However, our results indicate that detection of β-1,3/1,4-MLG oligosaccharides does not require the LysM proteins CERK1, LYK4, LYK5, and LYM2 in Arabidopsis. Notably, Arabidopsis CERK1 is also not required for the activation of PTI in response to cellobiose (de Azevedo Souza et al., 2017). Whether or not this also holds true for glucose trimers and cellodextrins, which have recently been shown to have comparatively higher elicitor activities (Locci et al., 2019) remains to be shown. However, our data are in conflict with the observations of Rebaque et al. (2021), who suggest that MLG perception is at least partially dependent on CERK1, LYK4, and LYK5. It is hard to rationalize this discrepancy, but the direct comparison of β-1,3/1,4-MLG oligosaccharide-induced MAPK activation in the wildtype and corresponding mutant genotypes that we conducted on the same membrane revealed no significant difference.

We also tested mutants of the prototypical protein MAMP receptors FLS2 and EFR (Gómez-Gómez and Boller, 2000; Zipfel et al., 2006), as well as the promiscuous co-receptor BAK1 and adaptor kinase SOBIR1, which are involved in the perception of many proteinaceous ligands (Chinchilla et al., 2006; Heese et al., 2007; Roux et al., 2011; Schwessinger et al., 2011). In support of a role in protein-derived MAMP/DAMP receptor complex formation, these proteins are dispensable for β-1,3/1,4-MLG oligosaccharide recognition. The observation that known components of the immune system are not implicated in β-1,3/1,4-MLG oligosaccharide perception implies that yet unknown molecular components mediate immune activation in response to β-1,3/1,4-MLG oligosaccharides. In previous studies, elicitor-insensitive Arabidopsis accessions have been used to identify immune receptors and signaling components (Gómez-Gómez et al., 1999; Gómez-Gómez and Boller, 2000; Jehle et al., 2013; Zhang et al., 2013). However, more than 100 *A. thaliana* accessions tested in this study were sensitive to β-1,3/1,4-MLG oligosaccharides indicating that the sensing and signaling machinery is highly conserved. We look forward to the identification of the molecular components required for β-1,3/1,4-MLG oligosaccharide perception by forward genetics in the future.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

VL, HB, EP, and SB conceived and designed the experiments. SB conducted all experiments, except for HPAEC-PAD, and MALDI-TOF analyses that were performed by GA and NJ. SB, EP, HB, and VL analyzed and discussed the data. SB, EP, and VL 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/articles/10.3389/fpls.2021.682439/full#supplementary-material

Supplementary Figure 1 | Thin Layer Chromatography (TLC) of enzymatic degradation of the *H. vulgare* β-1,3/1,4-MLG polysaccharide. 10 mg ml^{-1} β-1,3/1,4-MLG polysaccharide of *H. vulgare* were dissolved in 100 mM sodium phosphate buffer (pH = 6.5) and incubated with active lichenase (0.025 U ml^{-1}) of *B. subtilis*. Upon 0, 15, 30, 45, 60, 120, and 240 min incubation time, samples were taken and the enzyme was inactivated by incubation for 15 min in boiling water. For TLC, 5 μl of the respective hydrolyzate was applied to the plate. As controls, 5 μl of a mixture of a β-1,3/1,4-MLG tetrasaccharide and trisaccharide (10 mg ml^{-1}) as well as β-1,3/1,4-MLG polysaccharide (10 mg ml^{-1}) were included. The TLC running buffer contained isopropanol:ethylacetate:H_2O in a ratio of 2:2:1. The carbohydrates were visualized by wetting the plate with 10% sulfuric acid in methanol and incubation at 99°C for 30–60 min.

Supplementary Figure 2 | Verification of masses and HPAEC-PAD profiles of commercially available β-1,3/1,4-MLG MLG oligosaccharides. The purity of the purchased β-1,3/1,4-MLG MLG oligosaccharides was tested with MALDI-TOF **(A,C,E,G)** and HPAEC-PAD **(B,D,F,H)**. For MALDI-TOF, 10 μg ml^{-1} of the respective β-1,3/1,4-MLG oligosaccharide was mixed 1:5 with a 2,5-dihydrobenzoic acid MALDI matrix and analyzed. The expected masses of the pure β-1,3/1,4-MLG oligosaccharides as well as the sodium and potassium adducts are indicated. For HPAEC-PAD analysis, 56 or 45 μM of the MLG tetrasaccharide or MLG trisaccharide, respectively, were analyzed.

Supplementary Figure 3 | Thin Layer Chromatography (TLC) of enzymatic degradation of the *H. vulgare* β-1,3/1,4-MLG polysaccharide. 10 mg ml^{-1} β-1,3/1,4-MLG polysaccharide of *H. vulgare* were dissolved in 100 mM Sodium phosphate buffer (pH = 6.5) and incubated with active lichenase (1 U ml^{-1}) of *B. subtilis* for 60 min. The enzyme was inactivated by incubating the hydrolyzate for 15 min in boiling water. For TLC, 5 μl of the hydrolyzate was applied to the plate. The TLC running buffer contained isopropanol:ethylacetate:H_2O in a ratio of 2:2:1. The carbohydrates were visualized by wetting the plate with 10% sulfuric acid in methanol and incubation at 99°C for 30–60 min.

Supplementary Figure 4 | Lichenase-generated β-1,3/1,4-MLG oligosaccharides induce phosphorylation of MAPK in various Arabidopsis accessions. MAPK activation in 14-day-old seedlings of different *A. thaliana* ecotypes in response to β-1,3/1,4-MLG oligosaccharides. β-1,3/1,4-MLG oligosaccharides were generated upon incubation of the β-1,3/1,4-MLG polysaccharide (10 mg ml^{-1}) with the *B. subtilis* lichenase (1 U ml^{-1}) for 60 min (**Supplementary Figure 3**). For the treatment, a 1:10 dilution of the hydrolysis products was used. Sodium phosphate buffer (1 mM) served as negative control. As loading control, the PVDF membrane was stained with Coomassie Brilliant Blue (CBB). Data show the result from one biological replicate.

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Extracellular Fragmented Self-DNA Is Involved in Plant Responses to Biotic Stress

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A growing body of evidence indicates that extracellular fragmented self-DNA (eDNA), by acting as a signaling molecule, triggers inhibitory effects on conspecific plants and functions as a damage-associated molecular pattern (DAMP). To evaluate early and late events in DAMP-dependent responses to eDNA, we extracted, fragmented, and applied the tomato (*Solanum lycopersicum*) eDNA to tomato leaves. Non-sonicated, intact self-DNA (intact DNA) was used as control. Early event analyses included the evaluation of plasma transmembrane potentials (Vm), cytosolic calcium variations ($(Ca^{2+})_{cyt}$), the activity and subcellular localization of both voltage-gated and ligand-gated rectified K^+ channels, and the reactive oxygen species (ROS) subcellular localization and quantification. Late events included RNA-Seq transcriptomic analysis and qPCR validation of gene expression of tomato leaves exposed to tomato eDNA. Application of eDNA induced a concentration-dependent Vm depolarization which was correlated to an increase in $(Ca^{2+})_{cyt}$; this event was associated to the opening of K^+ channels, with particular action on ligand-gated rectified K^+ channels. Both eDNA-dependent $(Ca^{2+})_{cyt}$ and K^+ increases were correlated to ROS production. In contrast, application of intact DNA produced no effects. The plant response to eDNA was the modulation of the expression of genes involved in plant–biotic interactions including pathogenesis-related proteins (PRPs), calcium-dependent protein kinases (CPK1), heat shock transcription factors (Hsf), heat shock proteins (Hsp), receptor-like kinases (RLKs), and ethylene-responsive factors (ERFs). Several genes involved in calcium signaling, ROS scavenging and ion homeostasis were also modulated by application of eDNA. Shared elements among the transcriptional response to eDNA and to biotic stress indicate that eDNA might be a common DAMP that triggers plant responses to pathogens and herbivores, particularly to those that intensive plant cell disruption or cell death. Our results suggest the intriguing hypothesis that some of the plant reactions to pathogens and herbivores might be due to DNA degradation, especially when associated to the plant cell disruption. Fragmented DNA would then become an important and powerful elicitor able to trigger early and late responses to biotic stress.

Keywords: tomato, transmembrane potential, calcium signaling, ROS, ion channel activity, RNA-seq, receptor-like kinase, ethylene-responsive elements

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INTRODUCTION

Although it is not fully understood how it is generated, extracellular fragmented DNA (eDNA) contributes to the species-specific discrimination of self- versus non-self (Duran-Flores and Heil, 2018) and can be used by plants to build resistance against the surrounding environment (Gallucci and Maffei, 2017). One of the most stimulating perspectives in plant crop production is the application of self-eDNA to drive responses similar to the intrinsic DNA damage response. A growing body of evidence indicates that extracellular fragmented self-DNA, by acting as a signaling molecule, might be able to trigger inhibitory effects on conspecific plants and function as a damage-associated molecular pattern (DAMP) (Mazzoleni et al., 2015). To explain eDNA action, two general mechanisms have been proposed: the presence of membrane receptors able to trigger a signal transduction cascade of events or the possibility that fragmented DNA may enter somehow into the cytosol and interfere with some biological processes (Duran-Flores and Heil, 2015). In support of the first hypothesis are results showing that eDNA can trigger very early events, like the membrane depolarization and the cytosolic influx of calcium ions in a dose-dependent manner (Barbero et al., 2016). Moreover, the persistence of a membrane depolarization after the washing out of eDNA suggested that eDNA might interact with either membrane receptors or ligand-gated ion channels (Barbero et al., 2016). However, to date, no plant receptors able to recognize fragments of eDNA with a level of sequence-specificity have been reported.

An open question remains whether eDNA acts directly on the plant cell and provokes growth-inhibition effects (Mazzoleni et al., 2015) or acts as a DAMP and plays a role as an elicitor. In plant roots, DNA is excreted and released to the root cap environment by lytic processes (Driouich et al., 2019), but DNA could also be degraded by infection and disruption of root cap cells (Monticolo et al., 2020). In the latter case, eDNA might be involved in plant responses to biotic stress and could be released along with other elicitors in the extracellular environment (Plancot et al., 2013). Interesting, pathogen's eDNA also plays a role, as recently reported by Serrano-Jamaica et al. (2021), who found that the foliar application of eDNA from the pathogens *Phytophthora capsici*, *Fusarium oxysporum*, and *Rhizoctonia solani* triggers plant defense pathways. Therefore, it appears that both plant and pathogen eDNAs can prompt plant responses both above and belowground.

Although with different strategies and rates, by feeding on plants, some herbivore and pathogens disrupt cell integrity and generate the leakage of ions, the delivery of lytic enzymes from lysosomes, and the degradation of organelles and their content. A common reaction to this devastating event is the alteration of the plasma transmembrane potential (Vm), the production of reactive oxygen species (ROS), and the triggering of calcium signaling, that eventually leads to local and systemic modulation of biotic stress-responding genes (Maffei et al., 2007; Bricchi et al., 2012, 2013; Zebelo and Maffei, 2015). Several elicitors of plant responses to biotic stress have been characterized (Maffei et al., 2012) and specific receptors have been described (Iida et al., 2019); however, since most of the biotic stress causes cell disruption, DNA degradation, and fragmentation cannot be excluded and eDNA might interfere with elicitors and receptors.

The current knowledge on eDNA effects on plants still lacks the demonstration that application of self-eDNA to a plant may trigger both early events (mostly involving the plant cell plasma membrane) and the signal transduction pathway that leads to gene expression. Therefore, the aim of this work is to assess whether plant eDNA can elicit specific plant reactions as found in response to biotic stress. In order to verify this hypothesis, we extracted and fragmented tomato (*Solanum lycopersicum*) DNA and tested its effects on tomato leaves by evaluating early (Vm, calcium, and potassium channel activity, ROS generation) and late events (gene expression by RNA-Seq and qPCR analyses). Here we show that application of eDNA can induce tomato early and late events, with pattern similar to those described for plant responses to biotic stress.

MATERIALS AND METHODS

Plant Material and Sampling

Tomato *S. lycopersicum* L. seeds cv "cuore di bue" (Franchi seed company, Italy) were sown in glass plates with wet filter paper and incubated in a growth chamber (25°C, 16/8 h light/dark, PPFD 100 μmol m^{-2} s^{-1}) for 5 days. Seedlings were then transferred in polyethylene plastic pots (8 cm Ø) containing a mixture of peat, soil (Klasmann-Deilmann, Germany), sand, and vermiculite (Unistara, Italy) and grown in plant growth chambers with a light intensity of 120 μmol m^{-2} s^{-1}. Plants were watered three times a week and fertilized twice a week with a 0.1% solution containing N:P:K (12:10:10). Experiments were conducted with 20- to 30-day-old seedlings by sampling expanded leaves.

For all studies, we used mechanical damage in order to allow eDNA to penetrate the leaf tissues, as reported earlier (Barbero et al., 2016). In particular, for Vm analyses, plant responses were induced on mechanically damaged tomato leaves by tomato eDNA. A pattern wheel was used to simulate a mechanical damage for all microscopic studies. As negative controls, undamaged leaves were used, and in order to compare the effect of eDNA with controls, we defined the timing of wounding at 30 min. That is, the application was performed continuously for 30 min, while mechanical damage was performed once. For molecular studies, plants were exposed to eDNA for 1 h, and then the total RNA was extracted from control and treatments.

DNA Extraction and Sonication

Leaves of tomato were collected and dried in oven at 60°C for 72 h. For DNA extraction, 800 mg of dried material were ground to powder in liquid nitrogen with mortar and pestle. Total DNA was isolated using both cetyl trimethyl ammonium bromide (CTAB) method, according to Wilke's protocol (Wilke, 1997) and a DNeasy Plant Mini Kit as described by the manufacturer (Qiagen, Valencia, CA, United States[1]). Briefly, PVPP (Polyvinylpolypyrrolidone, Sigma, Milan) powder was added to the tissue before grinding. Tissues were homogenized

[1] http://www.qiagen.com/

with 10 ml of extraction buffer (100 mM Tris–HCl, pH 8.0, 1.4 M NaCl, 0.02 mM EDTA, 2% CTAB, and 0.2% β-mercaptoethanol). After centrifugation (13,000 rpm for 10 min), an equal volume of chloroform:isoamyl alcohol (24:1) was added, and samples were centrifuged again (13,000 rpm for 20 min). We repeated this step and, after incubation for 30 min with a 1:100 volume of RNAse, the DNA was precipitated with isopropanol. Then samples were centrifuged at 13,000 rpm for 10 min, and the DNA pellet was washed twice with 76% aqueous ethanol, 0.2 M sodium acetate, and 70% aqueous ethanol subsequently. Finally, the pellet was air-dried and resuspended in PE buffer (5 mM Tris/HCl, pH 8.5).

DNA from tomato leaves was fragmented by sonication using a Bandelin Sonopulse HD2070 (Bandelin, Berlin, Germany) at 90% power with a 1 s pulse for 15 min. Following the manufacturer's recommendation, samples were maintained in a layer of ice during the sonication process (see Barbero et al., 2016 for further details on the protocol). Capillary gel electrophoresis with the Agilent 2100 Bioanalyzer (Agilent Technologies) was used to assess quality and length of sonicated band sizes, according to manufacturer's instructions. DNA extract was spectrophotometrically quantified at 260 nm on a NanoDrop ND 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States) and visually verified on 1.2% agarose gel using Gel Doc EZ System (Bio-rad, CA, United States).

Determination of Transmembrane Potentials

The transmembrane potential (Vm) was determined in leaf segments with glass micropipettes with a tip resistance of 4–10 MΩ and filled with 3 M KCl as previously detailed (Maffei and Bossi, 2006). Leaf segments were settled for 60–120 min in 5 mM Mes-NaOH (pH 6.0). Perfusion of the buffer was obtained by a multichannel Ismatec Reglo (Ismatec SA, Glattbrugg, Switzerland) peristaltic pump (flow rate 1 ml min^{-1}). Based on the topographical and temporal determination of Vm performed previously, the electrode was inserted between 0.5 and 1.5 mm from the leaf edge zone. Vm variations were recorded through a PC digital port with a data logger. eDNA was assayed at 50 and 100 μg ml^{-1}. 0.05 M KCl was used as a control of Vm depolarization, according to Barbero et al. (2016).

Evaluations of Intracellular Calcium Variations by Confocal Laser Scanning Microscopy (CLSM) and Calcium Orange

Calcium Orange dye (stock solution in DMSO, Molecular Probes, Leiden, Netherlands) was diluted in 5 mM MES-Na buffer (pH 6.0) to a final concentration of 5 μM. This solution was applied to tomato leaves attached to the plant, as previously reported (Maffei et al., 2004; Bricchi et al., 2010; Barbero et al., 2016). After 1 h incubation with Calcium Orange, the leaf was mounted on a Leica TCS SP2 (Leica Microsystems Srl, Milan, Italy) multiband confocal laser scanning microscopy stage without separating the leaf from the plant in order to assess the basic fluorescence levels as a control. Then 50 μl of 200 μg ml^{-1} of eDNA was applied and after 30 min the calcium signature was observed. The microscope operates with a Krypton/Argon laser at 543 and 568 nm wavelengths: the first wavelength excites Calcium Orange, resulting in green fluorescence and the second mainly excites chlorophyll, resulting in red fluorescence. All images were obtained with an objective HCX APO 40× in water immersion with an NA of 0.8. Scan speed was set at 400. The microscope pinhole was 0.064 mm and the average size depth of images was between 65 and 70 μm; the average number of section per image was 25 and the pictures were represented as the merging of stacks. Image format was 1024 × 1024 pixels, 8 bits per sample and 1 sample per pixel. At least five plants were used for each experiment and the CLSM analyses were performed on different leaves.

CLSM Localization of Voltage- and Ligand-Gated K^+ Channels Using FluxOR™

Voltage-gated K^+ channels were assayed by using the FluxOR™ potassium ion channel kit from Invitrogen (Molecular Probes). Non-detached tomato leaves were gently placed on a glass slide and incubated in the dark for 1 h with 100 μl of loading buffer (deionized water, FluxOR™ assay buffer, and probenecid) by following the manufacturer's instructions. Plants were treated with 50 μl of 200 μg ml^{-1} of eDNA as above. Just before observation 50 μl of stimulus buffer (deionized water, FluxOR™ chloride-free buffer, K_2SO_4, and Tl_2SO_4) were added by following the manufacturer's instructions. CLSM fluorescence was assayed by a Leica TCS SP2 microscope equipped with an argon laser (excitation wavelength of 488 nm). Fluorescence was visible after about 50 min from treatment. Emissions were recorded using a 520–535 nm bandpass filter as detailed previously (Bricchi et al., 2013).

CLSM Subcellular Localization of H_2O_2 and Active Peroxidases by Using 10-Acetyl-3,7-Dihydroxyphenoxazine (Amplex Red)

Tomato leaves from rooted plants in pot were treated with 50 μl of 200 μg ml^{-1} of eDNA after incubation with the dye 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) as described earlier (Maffei et al., 2006). The Molecular Probes Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (A-22188) was used. The Assay Kit contains a sensitive, one-step assay that uses the Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) to detect hydrogen peroxide (H_2O_2) or peroxidase activity. The Amplex® Red reagent, in combination with horseradish peroxidase (HRP), was used to detect H_2O_2 released from eDNA treated leaves or generated in enzyme-coupled reactions after eDNA application. The reagent was dissolved in MES-Na buffer 50 mM (pH 6.0) containing 0.5 mM calcium sulfate to obtain a 50 μM solution. Leaves were then mounted on a Leica TCS SP2 microscope as described above. Scannings were recorded after 180 min using the HCX PL APO 63×/1.20 W Corr/0.17CS objective. The microscope was operated with a Laser Ar (458 nm/5 mW; 476 nm/5 mW; 488 nm/20 mW; 514 nm/20 mW), a Laser HeNe 543 nm/1.20 mW, and a Laser HeNe 633 nm/10 mW.

RNA-Seq Data Processing and Analysis

To assess the effect of eDNA on tomato gene expression, four plants were treated with 200 ng μl^{-1} eDNA as described above. Four controls were represented by intact DNA. After 1 h treatment, leaves were immediately harvested in liquid nitrogen and stored at −80°C for subsequent analysis.

Total RNA was isolated using TRIzol kit (Invitrogen), according to the manufacturer's protocol. Quantity and quality of the starting RNA were checked by Qubit and Bioanalyzer (Agilent), and libraries were prepared using the TruSeq RNA Sample Prep Kit (Illumina), following the manufacturer's instructions. Sequencing was performed on the Illumina NextSeq 500 platform. After quality controls with FastQC,[2] sequencing reads were aligned to *S. lycopersicum* (tomato) 2.50 genome reference (SL2.50) using TopHat v2.0.13 (Kim et al., 2013). Gene expression levels were quantified with the "HTSeq" framework v0.6.1 (Anders et al., 2015), using the International Tomato Genome Sequencing Project (ITAG) v2.4 gene/transcripts annotation. Differential expression analysis was carried out with the DESeq2 (Love et al., 2014) R/Bioconductor package.

Validation of RNA-Seq Gene Expression by qRT-PCR

Samples as above were used for the qPCR analyses which were run on a QUANTSTUDIO 3 Real-Time System (Thermo Fisher Scientific, Waltham, MA, United States) using SYBR green I with ROX as an internal loading standard. The reaction mixture was 10 μL, comprising 5 μL of 2X Maxima^{TM} SYBR Green qPCR Master Mix (Maxima SYBR Green/ROX qPCR Master Mix 2X, Thermo Fisher Scientific, United States), 0.5 μL of 1:10 diluted cDNA and 100 nM primers (Integrated DNA Technologies, Coralville, IA, United States). Furthermore, non-templates were run as a negative control using only total RNA without reverse transcription to monitor for genomic DNA contamination and the same was done by using water with water. Primers were designed using Primer 3.0 software (Rozen and Skaletsky, 2000) as reported in **Supplementary Table 1**. The thermal conditions for all genes were: 10 min at 95°C, 40 cycles 15 s at 57°C, and 20 s at 72°C. Fluorescence was read following each annealing and extension phase. All runs were followed by a melting curve analysis from 55 to 95°C. The linear range of template concentration to threshold cycle value (Ct value) was determined by preparing a dilution series, using cDNA from three independent RNA extractions analyzed in three technical replicates. Primer efficiencies for all primer pairs were calculated using the standard curve method. All amplification plots were analyzed with the QUANTSTUDIO 3 software to obtain Ct values (Pfaffl, 2001).

The following groups of genes were analyzed: Calcium-related genes: calcium-binding EF hand family protein (*Solyc10g006700*), calmodulin (*Solyc04g058160*), calcium-binding phospholipase D (*Solyc01g091910*). Oxidative stress-related genes: ubiquinol oxidase (*Solyc08g075550*), polyphenol oxidase F, chloroplastic, PPO (*Solyc08g074630*), peroxidase (*Solyc03g025380*), catalase (*Solyc01g100630*). Proton pump-related genes: V-type proton ATPase subunit a (*Solyc11g072530*), proton pump interactor 1 (*Solyc08g068850*), proton pump interactor 1 (*Solyc05g008780*). Defense-related genes: 4-coumarate-CoA ligase-like protein (*Solyc06g035960*), β-1,3-glucanase (*Solyc01g060020*), chymotrypsin inhibitor-2 (*Solyc09g084450*), Kunitz-type protease inhibitor (*Solyc03g098780*), multidrug resistance protein ABC transporter family (*Solyc05g014500*), pathogenesis-related protein 1a (*Solyc01g106620*), pathogenesis-related protein P2 (*Solyc01g097240*), pathogenesis-related protein-1 (*Solyc01g106610*), polygalacturonase (*Solyc12g096730*), sesquiterpene synthase (*Solyc07g052130*), trypsin inhibitor-like protein precursor (*Solyc11g022590*), wound induced protein (*Solyc07g054780*), wound/stress protein lipoxygenase, LH2 PLAT domain-containing protein (*Solyc03g096550*), wound-induced proteinase inhibitor 1 (*Solyc09g084470*). Heat shock proteins (Hsps) and chaperones: heat shock protein Hsp90 (*Solyc07g047790*), heat shock protein (*Solyc03g117630*), heat shock protein 22 Mitochondrial (*Solyc08g078700*), heat shock protein 70 (*Solyc03g082920*), heat shock transcription factor 1 (*Solyc02g079180*), hsc1 heat shock protein 70 kDa (*Solyc06g076020*), hsc70.3 er21 ethylene-responsive heat shock protein cognate 70 (*Solyc04g011440*), hsp40 Chaperone protein (*Solyc11g071830*), hsp90 heat shock protein 90 (*Solyc06g036290*), NEF Heat shock protein 4 (*Solyc07g043560*), SHsfA7 Heat stress transcription factor A3 (*Solyc09g065660*). DNA binding: DNA primase/helicase (*Solyc02g022830*), DNA-directed RNA polymerase (*Solyc02g083350*). Receptor-like genes: receptor-like serine/threonine-protein kinase (*Solyc03g025130*), serine/threonine-protein kinase (*Solyc03g112950*), TIR-NBS-LRR resistance protein Toll-Interleukin receptor (*Solyc00g294230*), TIR-NBS-LRR disease resistance-like protein (*Solyc07g052790*), CC-NBS-LRR, resistance protein (*Solyc10g047320*). Phytohormone-related genes: ACC oxidase (*Solyc09g008560*), auxin-induced SAUR-like protein (*Solyc01g111000*), ethylene-responsive nuclear protein (Solyc02g070040), ethylene-responsive transcription factor 4 (*Solyc12g009240*), ethylene-responsive TF1 pathogenesis-related transcriptional factor (*Solyc03g093550*). Photosynthesis: chloroplastic RuBisCO small subunit 3B (*Solyc02g085950*), RuBisCO activase 1 (*Solyc09g011080*).

Four different reference genes TC194780a, actin 1 (*ACT1*); X14449, elongation factor 1α (*EF1*); DQ205342, β-tubulin (*TUB*), and TC193502a, ubiquitin (*UBI*) (see **Supplementary Table 1** for primers), according to Løvdal and Lillo (2009). The best of the four genes was selected using the NormFinder software (Andersen et al., 2004). The relative expression mRNA levels of each gene were calibrated and normalized with the level of the most stable reference genes, *EF1* and *UBI* [in agreement with (Løvdal and Lillo, 2009)]. For each treatment, three biological replicates and three technical replicates were analyzed.

Statistical Analyses

A stem-and-leaf function of SYSTAT 10 was used to treat Vm data to extract the lower and upper hinge from the Gaussian distribution. After filtering the data, the mean value was calculated along with the SE. At least five samples per

[2]https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

treatment group were used for the statistical analysis of all other experimental data. Overall variations in the abundance of Calcium were assessed on Log-transformed data using the analysis of variance (ANOVA), while Tukey's *post hoc* test was used to account for pairwise differences. Data are expressed as mean values ± SE.

RESULTS

Tomato eDNA Induces a Transmembrane Potential Depolarization in Tomato Leaves

Sonication of DNA yielded fragments of different bp, in line with previous studies (Barbero et al., 2016). In particular, tomato eDNA consisted of fragments in the range between 250 and 1000 bp (data not shown). This eDNA was then used as a treatment.

We first assessed the effect of the application of tomato eDNA on tomato mesophyll cells by measuring variations in the leaf plasma transmembrane potential (Vm). We found that tomato leaves have an average mesophyll cell Vm ranging between 113 and 118 mV. Upon perfusion with non-fragmented tomato self-DNA (intact DNA), tomato Vm did not show any significant changes (**Figure 1**). To evaluate the tomato cell response to a Vm depolarizing agent, we perfused tomato leaves with a 0.05 M KCl solution. The effect was a sudden and constant Vm depolarization, which lowered the Vm at about 80 mV (**Figure 1**), as expected (Wakeel, 2013). We then perfused tomato cells with increasing concentrations of eDNA. The effect was a Vm depolarization directly correlated to the eDNA concentration, with 50 μg eDNA producing a 17 mV depolarization and 100 μg eDNA producing a 28 mV Vm depolarization, with respect to intact eDNA (**Figure 1**). While washing leaves with a fresh buffer returned the Vm value of KCl-induced Vm depolarization to almost initial values, the removal of the eDNA at both concentrations caused a Vm hyperpolarization that never reached the initial values. eDNA backwashed treatments at 50 μg eDNA and 100 μg eDNA reached a value of about −108 and −103 mV, respectively (**Figure 1**).

Application of Tomato eDNA Is Associated With Increased Tomato Cytosolic Calcium Concentration

Membrane depolarization depends on the differential distribution of ions across the plasma membrane (Maffei et al., 2007). To assess the response of tomato plants to self-eDNA on tomato cytosolic calcium (Ca^{2+}_{cyt}), we analyzed both localization and semi-quantitative evaluation of Ca^{2+}_{cyt} by CLSM, using Calcium Orange as a selective calcium indicator (Kanchiswamy et al., 2014). A preliminary dose-dependent analysis allowed to assess that 200 μg ml^{-1} eDNA could induce a significant response (data not shown). **Figure 2** shows the chlorophyll and calcium orange fluorescence as well as the merging of the two signals in controls (where no treatment is applied) and in leaves treated with 200 μg ml^{-1} of either intact DNA or eDNA. The images clearly show that only after eDNA

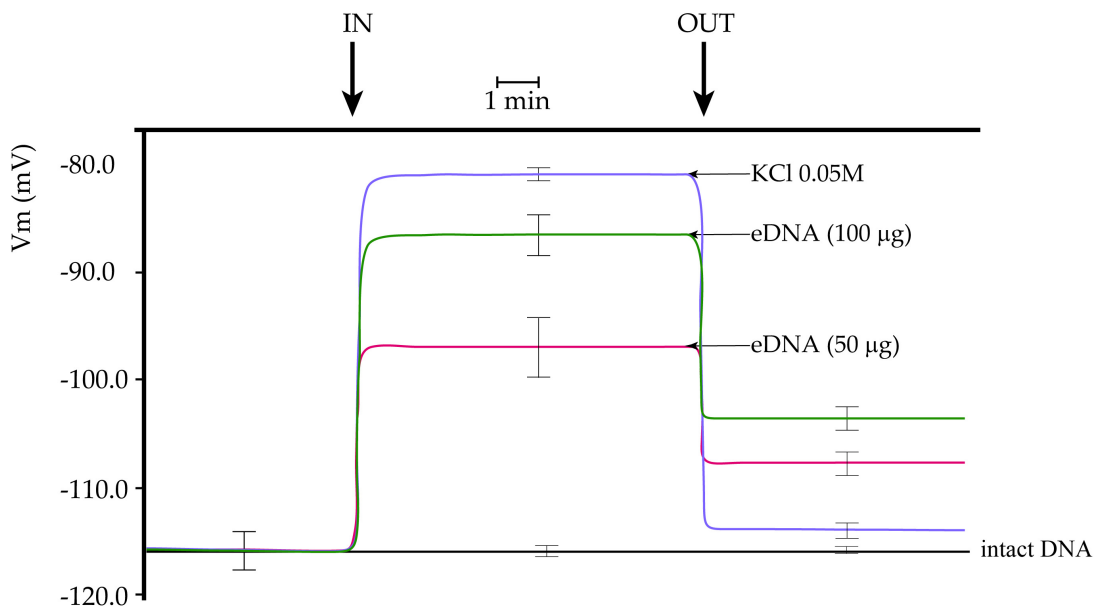

FIGURE 1 | Plasma membrane potential (Vm) depolarization in response to different concentrations of tomato fragmented self-DNA (eDNA) on tomato leaves. No effect on Vm was found after non-fragmented self-DNA (intact DNA) application. 0.05 M KCl caused an expected and almost completely reversible Vm depolarization. Vm depolarization was dependent on eDNA concentration. Washing eDNA treated leaves with fresh buffer was unable to recover the tomato leaf Vm completely. A time scale is indicated. Error bars represent SE (n = 8–10). IN, timepoint when tomato cells were perfused with either KCl, eDNA, or intact DNA; OUT, timepoint when fresh buffer was perfused to wash out molecules.

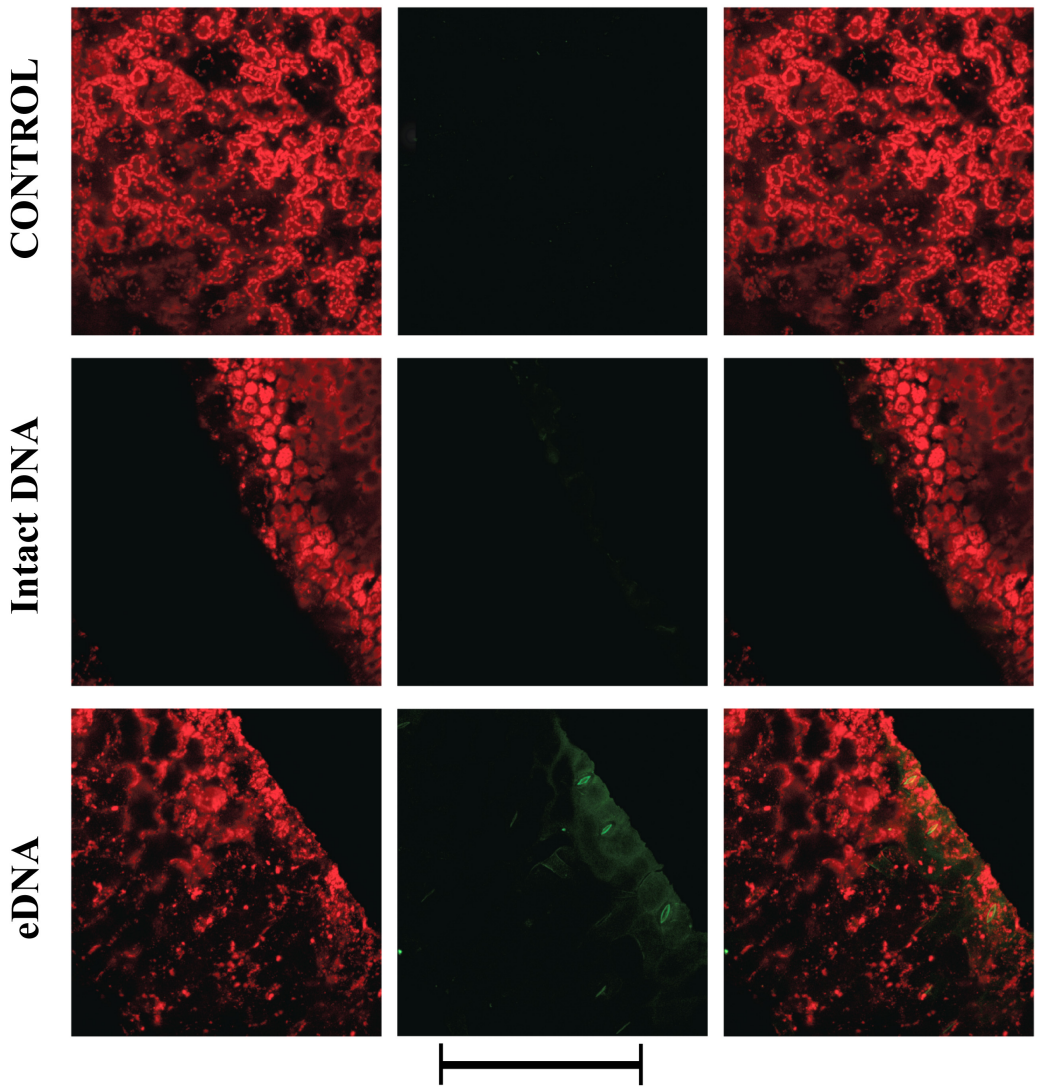

FIGURE 2 | Intracellular calcium variations in tomato leaves in controls (no treatment) and upon treatments with tomato intact DNA and eDNA. False-color image analysis reconstructions from confocal laser-scanning microscope observations, and fluorochemical intracellular Ca^{2+} localization. Fifty microliters of 200 μg ml^{-1} of either eDNA or intact DNA were applied and after 30 min a calcium signature was observed. Pictures represent portions of the tomato leaf blade where the green fluorescence refers to the binding of calcium orange with Ca^{2+}, whereas the chloroplasts are evidenced by a bright red color caused by chlorophyll fluorescence. Scale bar (250 μm) is indicated at the bottom of the figure. Pictures are the results of the merging of 25 individual optical sections.

application a strong fluorescence is associated with the $Ca^{2+}{}_{cyt}$ signature, whereas the signal observed after intact DNA had a similar fluorescence as the Calcium Orange control (**Figure 2**).

Tomato Ligand-Gated and Voltage-Gated Potassium Channels Are Activated by the Application of Tomato eDNA

To gain more insight into the possible causes of Vm depolarization, we tested the activity of both voltage- and ligand-gated K^+ channels using the potassium indicator FluxOR^{TM}. The assay is based on the use of a stimulus buffer containing a low level of thallium ions (Wible et al., 2008). Thallium ions freely flow through open K^+ channels, acting as a surrogate for K^+. When the K^+ channel is stimulated by the presence of tomato eDNA, thallium flows into the cell and binds the FluxOR^{TM} dye, generating a fluorescent signal, proportional to channel activity. The fluorescent indicator measures ion flux in both voltage- and ligand-gated potassium channels. Voltage gated potassium channels are opened by the

co-administration of potassium and thallium in the stimulus buffer. Resting and inward rectifier potassium channels are assayed by adding stimulus buffer with thallium alone. **Figure 3** shows the localization of voltage-gated K^+ channel activity. These channels are activated by variations in the Vm potential. We found that these channels' activity followed in time the calcium signature and could be classified as inwardly rectifying (allowing K^+ influx to the cell) (Cherel, 2004). A strong activity of these channels was found only after treatment with eDNA, whereas the intact DNA showed a signature similar to the control (**Figure 3**).

We also evaluated the localization and activity of ligand-gated K^+ channels. A strong and diffuse fluorescence was detected after treatment with eDNA (**Figure 4**). However, a low level of activity was also detected in both controls and treatment with intact DNA (**Figure 4**).

Tomato Responses to eDNA Are Associated to the Generation of ROS

Having assessed that the Vm depolarization is associated to a K^+ influx and an influx of calcium in the cytosol, we

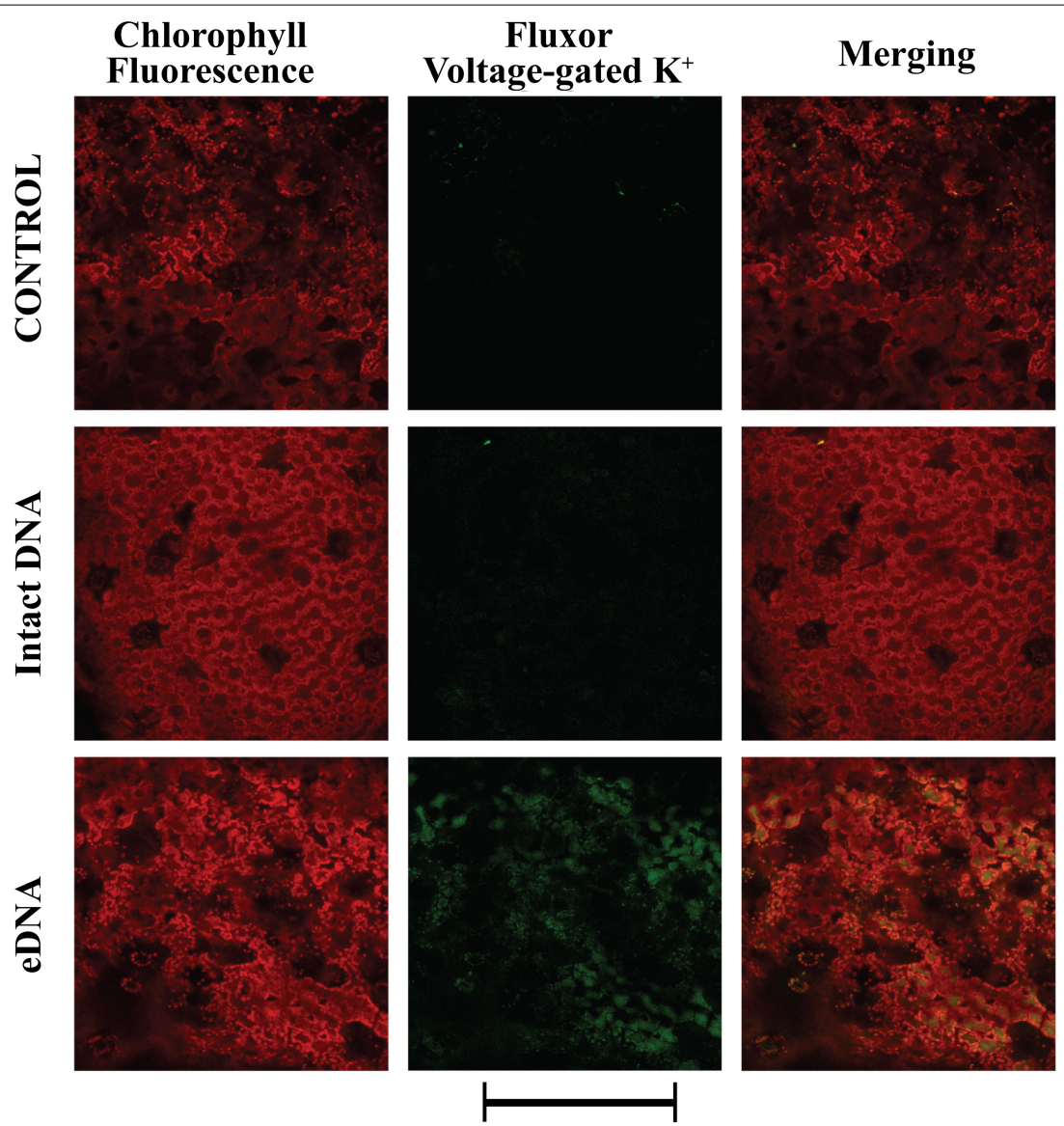

FIGURE 3 | Intracellular potassium voltage-gated variations in tomato leaves in controls (no treatment) and upon treatments with tomato intact DNA and eDNA. False-color image analysis reconstructions from confocal laser-scanning microscope observations and fluorochemical evidence of voltage-gated activity. Fifty microliters of 200 μg ml^{-1} of either eDNA or intact DNA were applied and after 50 min the K^+ signature was observed. Pictures represent portions of the tomato leaf blade where the green fluorescence refers to FluxOR^{TM} associated to voltage-gated K^+ channel activity, whereas the chloroplasts are evidenced by a bright red color caused by chlorophyll fluorescence. Scale bar (250 μm) is indicated at the bottom of the figure. Pictures are the results of the merging of 25 individual optical sections.

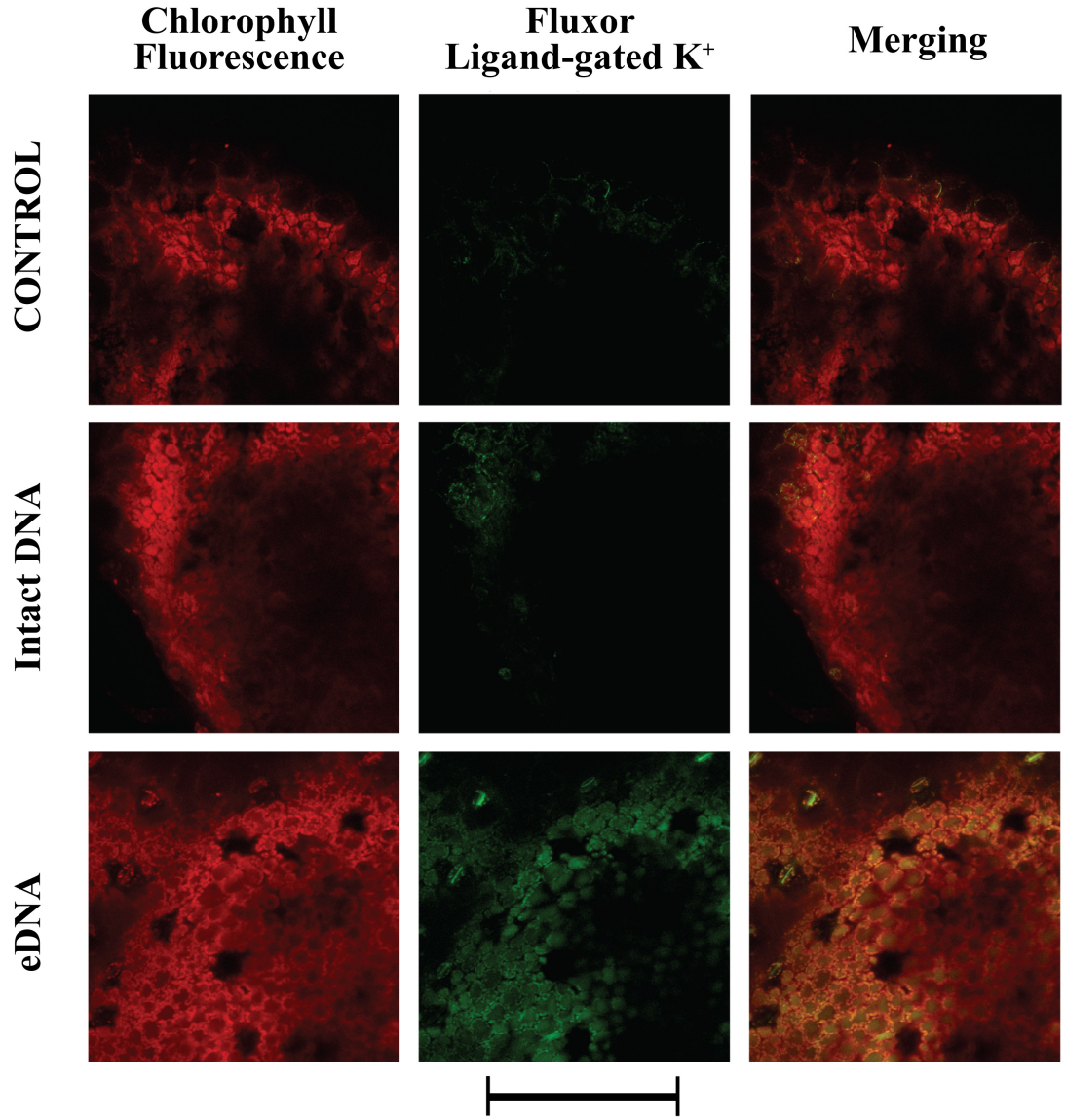

FIGURE 4 | Intracellular potassium ligand-gated variations in tomato leaves in controls (no treatment) and upon treatments with tomato intact DNA and eDNA. False-color image analysis reconstructions from confocal laser-scanning microscope observations, and fluorochemical evidence of ligand-gated K^+ channel activity. Fifty microliters of 200 $\mu g\ ml^{-1}$ of either eDNA or intact DNA were applied and after 50 min the K^+ signature was observed. Pictures represent portions of the tomato leaf blade where the green fluorescence refers to FluxOR™ associated to ligand-gated K^+ channel activity, whereas the chloroplasts are evidenced by a bright red color caused by chlorophyll fluorescence. Scale bar (250 μm) is indicated at the bottom of the figure. Pictures are the results of the merging of 25 individual optical sections.

assessed one of the characteristic responses following these early events: the activity of peroxidases and the production of a typical ROS, hydrogen peroxide (H_2O_2) (Zebelo and Maffei, 2015; Camejo et al., 2016). The Amplex® Red reagent, combined with HRP, was used to detect H_2O_2 released from tomato leaves upon treatment with tomato eDNA. Application of intact DNA prompted a faint fluorescence reaction indicating the activity of peroxidases and the production of H_2O_2 (**Figure 5**). However, a stronger fluorescence, which appeared to be associated mostly with chloroplasts, was observed after applying tomato eDNA (**Figure 5**). Amplex® Red reagent was also used as an ultrasensitive assay for peroxidase activity by using H_2O_2 in excess (data not shown), and the results were the same as with the use of HRP.

The Tomato Response to eDNA Is Associated to the Modulation of Gene Expression

To assess the tomato responses to eDNA, we performed a transcriptomic analysis by RNA-Seq of fully expanded tomato

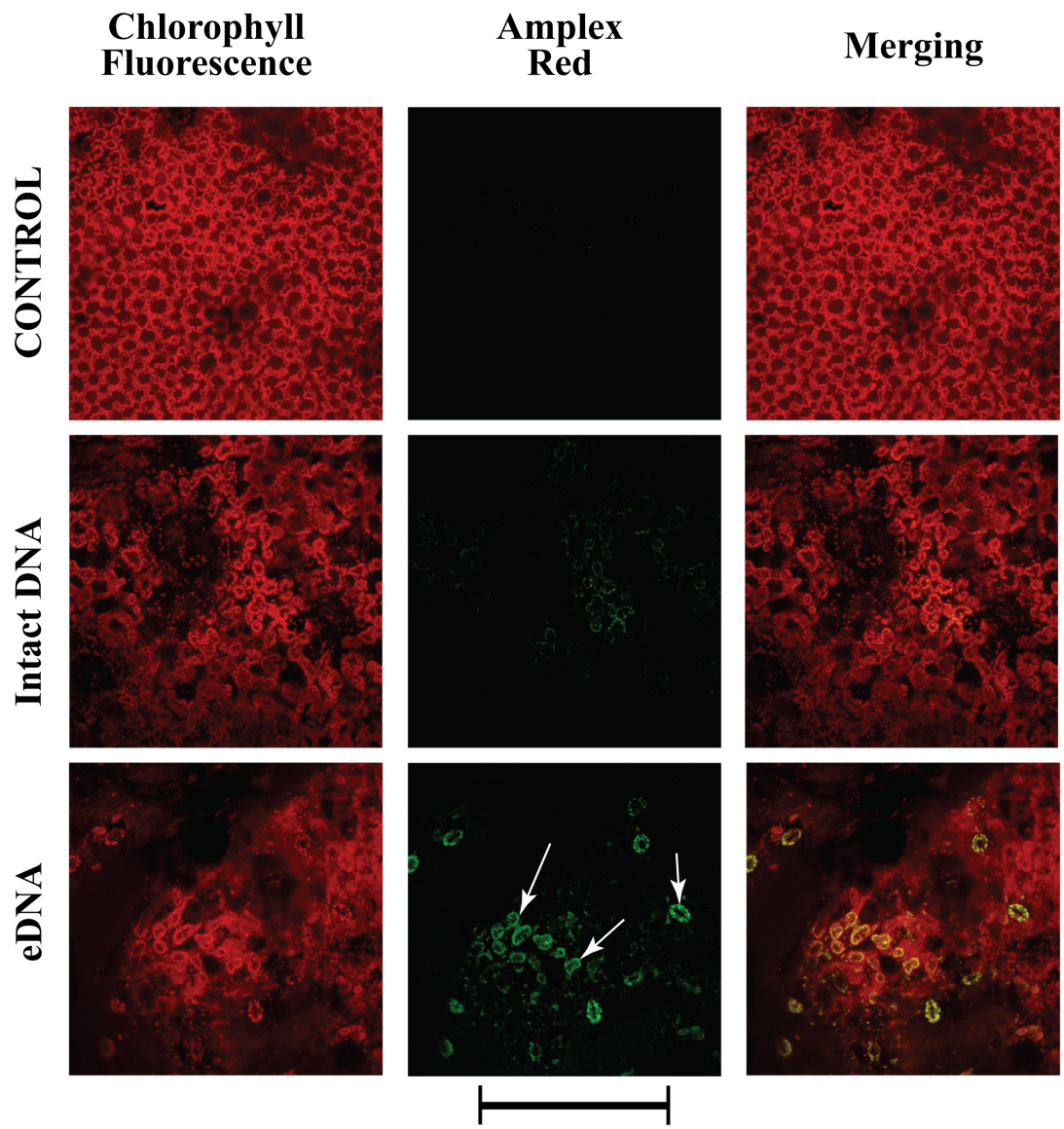

FIGURE 5 | Intracellular variations of H_2O_2 production in tomato leaves in controls (no treatment) and upon treatments with tomato intact DNA and eDNA. The Amplex® Red reagent, in combination with horseradish peroxidase (HRP), was used to detect H_2O_2 released or generated in enzyme-coupled reactions. False-color image analysis reconstructions from confocal laser-scanning microscope observations, fluorochemical H_2O_2, and peroxidase localization. Fifty microliters of 200 μg ml^{-1} of either eDNA or intact DNA were applied and after 180 min the ROS signature was observed in close association with chloroplasts (arrows). Pictures represent portions of the tomato leaf blade where the green fluorescence refers to the binding of Amplex® Red with peroxidase-produced H_2O_2, whereas the chloroplasts are evidenced by a bright red color caused by chlorophyll fluorescence. Scale bar (250 μm) is indicated at the bottom of the figure. Pictures are the results of the merging of 25 individual optical sections.

leaves from 25 day-old plants grown in pots and treated with 200 μg ml^{-1} eDNA. Controls were represented by plants growing in the same conditions (i.e., temperature, gravity, atmospheric pressure, and Photosynthetic Flux Density) and treated with 200 μg ml^{-1} intact DNA. For significant analysis, genes were filtered based on their adjusted (FDR corrected) *P*-values calculated from the bioinformatic analysis. In general, almost all biological replicates analyzed were retained in the analysis and the genes satisfying a corrected *P*-value cut-off of 0.05 and fold change ≥ 2 ranged from 2 to 3% out of the total gene number (**Supplementary Table 2**). A total of 34,725 reads provided 845 DEGs of which 574 were downregulated and 271 upregulated. Several classes of genes were modulated including receptors, among them the TIR-NBS-LRR Toll-Interleukin receptor (*Solyc00g294230*).

We then analyzed the gene ontology (GO) of the biological processes of downregulated and upregulated genes and calculated the fold enrichment (FE) (i.e., the ratio between input data – i.e., the number of down/upregulated genes – versus the number of expected genes for each GO category,

see **Supplementary Table 3**). Most of the downregulated genes (90%) showed a FE > 2, with particular reference among others to myo-inositol biosynthetic and metabolic processes (GO:0010264 and GO:0033517; >100 FE), nitric oxide biosynthetic and metabolic processes (GO:0006809 and GO:0046209; >70 FE), ROS biosynthetic process (GO:1903409; >70 FE), cell wall biosynthetic processes (GO:0031506 and GO:0000032; >47 FE), jasmonic acid biosynthetic process (GO:0009695; >47 FE), and sucrose transport (GO:0015770; >47 FE) (**Supplementary Figure 1**).

Many upregulated genes (47%) showed a GO FE > 2. Among these, oxygen transport (GO:0015671; >66 FE), defense response to Gram-negative bacterium (GO:0050829; >66 FE), lactate biosynthetic process (GO:0019249; >66 FE), adenine biosynthetic, metabolic, and salvage processes (GO:0046084, GO:0046083, GO:0006168; >33 FE), auxin influx (GO:0060919; >33 FE), and several cellular ion homeostasis processes (GO:0030002, GO:0072501, GO:0030320, GO:0030643, GO:0072502, GO:0072505, GO:0055083, GO:0055062, GO:0072506; all > 33 FE as well as GO:0046916, GO:0055076, GO:0006875, GO:0055065, GO:0055082, GO:0019725, GO:0098771, GO:0030003, GO:0050801, GO:0030001; all > 2 FE) (**Supplementary Figure 2**).

We then processed the downregulated and upregulated genes by using Genevestigator (performed on February 2021) to obtain a hierarchical clustering by comparing the genes modulated in response to treatment with eDNA against all available data from the database of *S. lycopersicum*. In the group of genes downregulated after treatment with eDNA, almost all downregulated genes are also downregulated in two clusters of genes (**Supplementary Figure 3**, CL1 and CL2) which are involved in the processes of columella, pericarp, placenta, collenchyma, and parenchyma development. In contrast, several genes downregulated after treatment with eDNA are also downregulated by *Pseudomonas syringae* pv. *tomato* (**Supplementary Figure 3**, CL3). With regards to genes upregulated after treatment with eDNA, the Genevestigator analysis returns the presence of three clusters (**Supplementary Figure 4**, CL1, CL2, and CL3) which show upregulation in *S. lycopersicum* processes involved in epidermic, parenchyma, vascular tissues development (**Supplementary Figure 4**, CL4), and seed development (**Supplementary Figure 4**, CL5).

The Response of Tomato Leaves to Tomato eDNA Is the Modulation of Many Genes That Respond to Biotic Stress

Having assessed the involvement of ion homeostasis and tissue development, two important aspects involved in plant responses to pathogens and herbivores, we focused our attention on the analysis of genes expressed in *S. lycopersicum* responses to biotic stress. The hierarchical clustering of eDNA downregulated genes against the *S. lycopersicum* database of Genevestigator shows a high expression potential for genes involved in responses to *P. syringae* pv. *tomato* (**Supplementary Figure 5**, CL1), nematodes (**Supplementary Figure 5**, CL2), other pathogens (**Supplementary Figure 5**, CL3), and responses to pathogen elicitors (**Supplementary Figure 5**, CL4). The same situation was observed for genes upregulated after treatment with eDNA, with a high expression potential for genes involved in pathogen interactions (**Supplementary Figure 6**, CL1 and CL3), nematodes (**Supplementary Figure 6**, CL2), and pathogen elicitors (**Supplementary Figure 6**, CL4).

Validation of RNA-Seq Gene Expression by qPCR Reveals a Quantitative Modulation of Biotic Stress-Related Genes Upon Treatment With eDNA

The gene expression obtained by RNA-Seq analysis was validated by qPCR. We selected groups of downregulated and upregulated genes related to different categories related to genes coding for proteins involved in channel activity (Vm variations) and to calcium, potassium, and ROS (CLSM analyses) as well as plant responses to biotic stress.

The first group of genes is related to calcium, ROS, and proton pumps (**Figure 6A**). A calcium-binding EF hand family protein (*Solyc10g006700*) and calmodulin (*Solyc04g058160*) were upregulated, whereas the calcium-binding phospholipase D (*Solyc01g091910*) was downregulated by treatment with eDNA. Oxidative stress-related genes included ubiquinol oxidase (*Solyc08g075550*) and catalase (*Solyc01g100630*) that were downregulated by treatment with eDNA, whereas the chloroplastic polyphenol oxidase F (*Solyc08g074630*) and a peroxidase (*Solyc03g025380*) were upregulated. With regards to proton pump-related genes, V-type proton ATPase subunit a (*Solyc11g072530*) and two proton pump interactor 1 (*Solyc08g068850* and *Solyc05g008780*) were downregulated (**Figure 6A**).

Several genes involved in plant defense were regulated by treatment with eDNA (**Figure 6B**). Downregulation was found for 4-coumarate-CoA ligase-like protein (*Solyc06g035960*), a multidrug resistance protein ABC transporter family (*Solyc05g014500*), polygalacturonase (*Solyc12g096730*), and a sesquiterpene synthase (*Solyc07g052130*). On the other hand, β-1,3-glucanase (*Solyc01g060020*), chymotrypsin inhibitor-2 (*Solyc09g084450*), Kunitz-type protease inhibitor (*Solyc03g098780*), pathogenesis-related protein 1a (*Solyc01g106620*), pathogenesis-related protein P2 (*Solyc01g097240*), pathogenesis-related protein-1 (*Solyc01g106610*), a trypsin inhibitor-like protein precursor (*Solyc11g022590*), a wound-induced protein (*Solyc07g054780*), a wound/stress protein lipoxygenase, LH2 PLAT domain-containing protein (*Solyc03g096550*), and a wound-induced proteinase inhibitor 1 (*Solyc09g084470*) were all upregulated. In particular, a strong upregulation was found for chymotrypsin inhibitor-2, a trypsin inhibitor-like protein precursor and a wound/stress protein lipoxygenase (**Figure 6B**).

Among the genes regulated in response to eDNA, several Hsps and chaperones were downregulated with particular reference to the mitochondrial heat shock protein 22 (*Solyc08g078700*) and heat shock transcription factor 1 (*Solyc02g079180*) (**Figure 6C**).

A large number of receptor-like kinases (RLK) was downregulated in response to application of eDNA, including

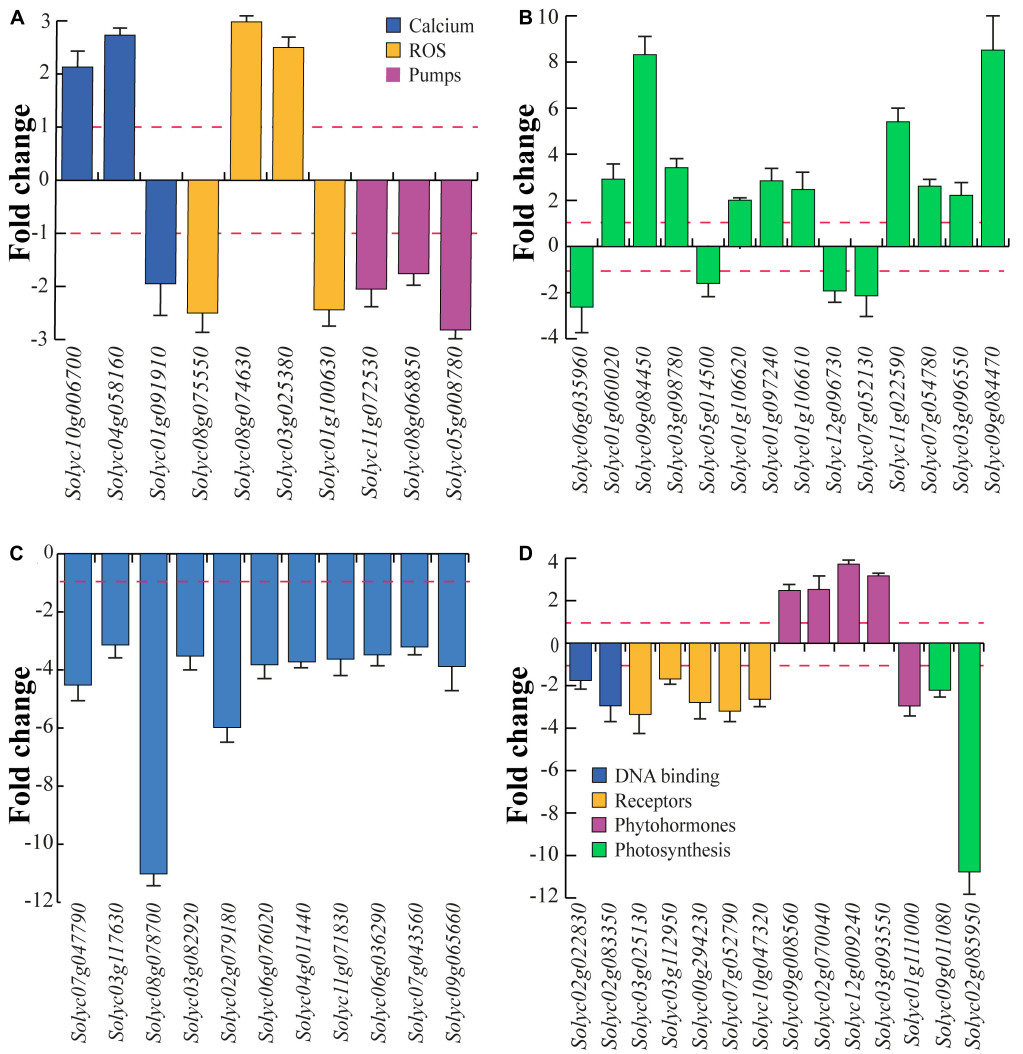

FIGURE 6 | Differential gene expression of tomato selected genes in response to eDNA. The data are expressed as fold change in relation to controls (intact DNA). To emphasize the visualization of data, fold change values below 1 were plotted as –1/value, in order to obtain negative fold change values (indicating downregulation). **(A)** Gene expression of calcium-, ROS-, and proton pump-related genes. **(B)** Genes involved in plant defense. **(C)** Gene expression of heat shock proteins and heat shock factors. **(D)** Gene expression of DNA binding-, receptor like-, phytohormone-, and photosynthesis-related genes. In all figures, the red dotted lines represent the control (intact DNA) level; metric bars indicate standard deviation ($N = 3$).

several serine/threonine-protein kinases, leucine-rich repeat (LRR), tyrosine protein kinases as well as toll-interleukin receptors (**Supplementary Table 2**). Downregulation was confirmed for DNA binding DNA primase/helicase (*Solyc02g022830*) and DNA-directed RNA polymerase (*Solyc02g083350*) as well as for receptor-like serine/threonine-protein kinase (*Solyc03g025130*), serine/threonine-protein kinase (*Solyc03g112950*), TIR-NBS-LRR resistance protein Toll-Interleukin receptor (*Solyc00g294230*), TIR-NBS-LRR disease resistance-like protein (*Solyc07g052790*), and CC-NBS-LRR, resistance protein (*Solyc10g047320*) (**Figure 6D**). Among phytohormones, ethylene was involved in the upregulation of ACC oxidase (*Solyc09g008560*), ethylene-responsive nuclear protein (*Solyc02g070040*), ethylene-responsive transcription factor 4 (*Solyc12g009240*), and ethylene-responsive TF1 pathogenesis-related transcriptional factor (*Solyc03g093550*), whereas an auxin-induced SAUR-like protein (*Solyc01g111000*) was downregulated in response to application of eDNA (**Figure 6D**). Finally, a strong downregulation was found for chloroplastic RuBisCO small subunit 3B (*Solyc02g085950*) followed by a twofold downregulation of RuBisCO activase 1 (*Solyc09g011080*) (**Figure 6D**).

DISCUSSION

In this work, we provide evidence that the application of extracellular fragmented self-eDNA (eDNA) to tomato leaves induces a typical response to biotic stress, supporting the stimulating hypothesis that some plant responses to pathogens

and herbivores might be triggered by the degradation of the plant DNA. Evidence was supported by evaluating both early (Vm variations, calcium and potassium channel activity, and ROS generation) and late events (gene expression).

eDNA Induces Early Tomato Events Which Are Typical of the Biotic Stress Response

In plants, early events occur within seconds to minutes upon biotic/abiotic stress perception (Maffei et al., 2007). One of the early events in plant interactions with the surrounding environment is the variation in the plasma transmembrane potential (Vm) (Zebelo and Maffei, 2015). The application of eDNA caused a consistent increase of $Ca^{2+}{}_{cyt}$ in treated tomato plants, which is a typical response of plants to both pathogens and herbivores (Bricchi et al., 2012; Singh and Pandey, 2020), including tomato (Zebelo et al., 2012). As a consequence, the Vm was depolarized and a possible calcium-dependent Vm depolarization was followed by the activation of potassium channels, as it is typical in plant–insect and plant–pathogen interactions (Amborabe et al., 2008; Bricchi et al., 2013). The increase of both Ca^{2+} and K^+ cellular concentrations are associated with the observed Vm depolarization. Moreover, we found that the tomato response to eDNA was a stronger fluorescence of the ligand-gated K^+ channels, with respect to voltage-gated channels. In plants, K^+ conductance by ligand-gated channels is restricted in the presence of millimolar concentrations of Ca^{2+} (Leng et al., 2002) and this might explain the reason for the higher activity. This ligand-gated fluorescence could also explain the reason why Vm depolarization is not fully recovered after washing eDNA treated leaves with a fresh buffer solution. We hypothesize that eDNA might bind or interfere with the binding activity of K^+ channels.

The generation of ROS is another early event in plant–pathogen (Camejo et al., 2019), plant–herbivore interactions (Zebelo and Maffei, 2015), systemic signaling (Fichman and Mittler, 2020), and plant immune response (Kuzniak and Kopczewski, 2020). ROS act as important signal transduction molecules and may act downstream or upstream of several signal transduction pathways (Foyer and Noctor, 2005). Moreover, increases in the production, accumulation, and signaling of ROS are one of the main causes of programmed cell death (Farooq et al., 2019). We observed that the tomato response to eDNA was a ROS production which was localized mainly in the chloroplasts. Besides being involved in biotic stress-induced Ca^{2+} signals (Nomura et al., 2012), during pathogen and herbivore attack, chloroplasts are important sources of ROS (Maffei et al., 2006; Camejo et al., 2016), and the generation of ROS may interfere with several plant cell functions, including photosynthetic and other metabolic processes (Sierla et al., 2012). Interestingly, the strong chloroplastic-localized ROS production was associated to a strong downregulation of the RuBisCO (*Solyc02g085950*) gene expression. Furthermore, the ROS biotic-induced production is transmitted to chloroplasts via calcium ions that play an important role in regulating nuclear gene expression, making ROS important modulators of the plant immune response (Zabala et al., 2015). Therefore, we can conclude from our results that the tomato response to eDNA involves most of the early events triggered by biotic stress, including the alteration of the membrane potential due to the increased levels of $Ca^{2+}{}_{cyt}$, which causes the opening of voltage-gated K^+ channels and the regulation of ligand-gated K^+ channels; these early events are followed by a ROS production which are mainly localized in the plant cell chloroplasts.

The Tomato Early Responses to eDNA Treatment Are Followed by Modulation of Biotic Stress-Related Genes

Transcriptomic analyses of leaves treated with eDNA reveal a significant modulation of tomato genes. A consistent downregulation was found for most of the processes involved in the plant–biotic response, including the generation of ROS and jasmonate involvement, but other important processes were also affected like the cell wall biosynthetic process and the sucrose transport (see **Supplementary Figure 1**). In contrast, ion homeostasis and responses to pathogens were upregulated (**Supplementary Figure 2**). A deeper search in the Genevestigator database confirmed the correlation between up- and down-regulation of genes in response to eDNA and plants' responses to pathogens, pathogens' elicitors and nematodes (**Supplementary Figures 3–6**).

The validation of RNA-Seq analysis allowed us to focus on specific biotic stress-related genes. The positive correlation between the CLSM calcium signature (**Figure 2**) and the upregulation of both calcium-binding EF-hand family protein member (*Solyc10g006700*) that is homologous to a *S. tuberosum* calcium-binding protein which is involved in resistance to *Phytophthora infestans* (Muktar et al., 2015) and calmodulin (*Solyc04g058160*) (**Figure 6A**) is interesting. EF-hand motifs in the hydrophilic C-terminal domain of tomato have been correlated to $Ca^{2+}{}_{cyt}$ regulation of ROS production (Amicucci et al., 1999), whereas calmodulin, a calcium-binding protein with a helix-loop-helix (EF-hand) motif and one of the key mediators in plant immune responses (Cheval et al., 2013), is required for a successful defense response to pathogens (Zheng et al., 2018) and is upregulated by *Botrytis cinerea* infection (Yu and Du, 2018) a plant pathogen that causes cell disruption and death (Camejo et al., 2016; Oren-Young et al., 2021). On the other hand, the downregulation of the calcium-binding phospholipase D (*Solyc01g091910*), a protein that hydrolyses different membrane phospholipids and that is implicated in plant–pathogen interactions (Zhao, 2015), has also been shown to interfere with ethylene signaling regulation (Dek et al., 2018). The ROS signals work downstream from Ca^{2+} (Farooq et al., 2019) and the $Ca^{2+}{}_{cyt}$ increase was associated with the upregulation of chloroplastic polyphenol oxidase F (*Solyc08g074630*) and a peroxidase (*Solyc03g025380*). Polyphenol oxidase upregulation is involved in tomato resistance to herbivory (Lin et al., 2021) and pathogens (Zhang and Sun, 2021), whereas peroxidase activity was associated to tomato resistance to early blight disease (Alizadeh-Moghaddam et al., 2020). On the opposite, ubiquinol oxidase (*Solyc08g075550*) and catalase (*Solyc01g100630*) were

downregulated in response to eDNA treatment. In plants, the alternative oxidase catalyzes the oxidation of ubiquinol and reduces oxygen avoiding the proton translocation by bypassing some steps in the respiratory pathway (Finnegan et al., 2004). Since the ubiquinol oxidase activity reduces the ATP production, we suppose that the downregulation of this gene might be a plant cell strategy to cope with the altered homeostasis due to eDNA action and provide more ATP for proton pump activity. On the other hand, the catalase downregulation is directly correlated to the increased ROS production, being catalase one of the major ROS scavengers in plants (Fones and Preston, 2012).

The plant cell Vm is maintained by the activity of the proton pump (Falhof et al., 2016). We recently showed that during plant–herbivore interactions, the Vm depolarization is sustained by the strongly reduced effects of insect's oral secretions on the interaction between H^+-ATPase and 14-3-3 proteins, suggesting that one of the leading players in biotic stress-dependent Vm depolarization is the inhibition of the proton pump (Camoni et al., 2018). The V-type proton ATPase (*Solyc11g072530*) interacts with 14-3-3 proteins (Klychnikov et al., 2007) while the tomato proton pump interactors (*Solyc08g068850* and *Solyc05g008780*) are regulated by abiotic stress (Garcia et al., 2011). The downregulation of these genes in response to eDNA treatment was positively correlated with the Vm depolarization caused by application of eDNA (**Figures 1**, **6A**).

The Tomato Response to eDNA Involves the Modulation of the Expression of Genes Involved in Plant Late Responses to Biotic Stress

The signal transduction pathway that uses calcium and ROS as second messengers eventually leads to the regulation of defense response genes. Besides the typical biotic stress-responsive genes like pathogenesis-related proteins (PRPs) (*Solyc01g106620*, *Solyc01g097240*, and *Solyc01g106610*), including β-1,3-glucanase (*Solyc01g060020*), a strong upregulation was also found for a series of proteinase inhibitors (PIs) (*Solyc09g084450*, *Solyc03g098780*, *Solyc11g022590*, and *Solyc09g084470*). PIs play a vital role in defenses against pests and pathogens, especially against herbivores and, in tomato, PI genes have been recently found to mediate the response of tomato to biotic stress by balancing hormone signals (Fan et al., 2020). Another response to tomato to eDNA was the downregulation of 4-coumarate-CoA ligase-like protein (*Solyc06g035960*) which encodes for an enzyme that thioesterifies coumaric acid to coenzyme A (CoA) to form coumaroyl CoA, the precursor of a vast diversity of phenylpropanoids (Alberstein et al., 2012). The function of this gene has also been correlated to its ability to impair membrane functions such as ion transport (Kienow et al., 2008). Another intriguing plant response to eDNA is the downregulation of a polygalacturonase (*Solyc12g096730*) a gene that encodes an enzyme that catalyzes the hydrolysis and disassembly of pectin in plant cell walls (Caffall and Mohnen, 2009). Suppression of the gene can repress the pectin depolymerization and change the postharvest pathogen susceptibility (Ke et al., 2018).

Treating Tomato With eDNA Downregulates Genes Coding for Calcium-Dependent Protein Kinases, Heat Shock Transcription Factors, and Heat Shock Proteins

Many molecular chaperones are stress proteins and many of them were originally identified as heat shock (HS) proteins (Hsp), with particular reference to abiotic stress (Wang et al., 2004). Heat shock transcription factors (Hsfs) family members exert their anti-stress effects by regulating a series of H molecular chaperones, and other functional protein genes (Kovtun et al., 2000) and Hsp expression result from the binding of an Hsf to the HS element (HSE) in the promoter region of Hsp genes (von Koskull-Doring et al., 2007). Treatment with eDNA prompted the downregulation of the heat shock transcription factor 1 (*Solyc02g079180*) and heat stress transcription factor A3 (*Solyc09g065660*), which along with the downregulation of calcium-dependent protein kinases (CPK1, *Solyc03g031670*, **Supplementary Table 2**), was associated to the downregulation of several small and large Hsps. In plants, a correlation between calcium binding activity and Hsf has been demonstrated. For instance, some CPKs phosphorylate Hsfs which promote the transcriptional activation of plant defense genes (Kanchiswamy et al., 2010b) and the impairment of CPK downregulates the expression of several Hsps (Kanchiswamy et al., 2010a). In tomato, Hsfs regulate a wide range of metabolic pathways and have been identified as major players in physiological development in response to stress (Paupiere et al., 2020). Although Hsps play a major role in abiotic stress responses (e.g., to heat) as molecular chaperones, Hsps are involved in protein folding and in avoiding the irreversible aggregation of denatured proteins (Sun et al., 2002). In tomato, the activation of Hsps prevents lipid peroxidation, the generation of excessive reactive radicals and increases the secretion of plant antioxidant enzymes (Khan et al., 2020). Therefore the downregulation of all Hsps in response to eDNA treatment might be associated to the reduced scavenging activity and the increased production of ROS. Downregulation of Hsps, with particular reference to small Hsps, like the strongly downregulated heat shock protein 22 (*Solyc08g078700*), has been observed upon herbivory (Bricchi et al., 2012), confirming their involvement also in biotic stress.

The Tomato Response to eDNA Is the Modulation of Receptor-Like Protein Kinases and Ethylene-Responsive Factors

Our transcriptomic analysis reveals that plants respond to eDNA treatment by modulating several other genes involved in plant responses to biotic stress. In plants, pathogen-associated molecular patterns (PAMPs) and DAMPs are mainly recognized via receptor-like kinases (RLKs) (Duran-Flores and Heil, 2018). RLKs were largely downregulated (**Supplementary Table 2**). RLKs play a role both in abiotic stress (e.g., cold, salt, and drought tolerance) (Ye et al., 2017) and resistance to infection by several pathogens (Bundó and Coca, 2016). In

tomato, RLKs are involved in biotic stress and knockdown of an RLK resulted in increased sensitivity to fungi and reduced resistance against the pathogen *B. cinerea* (Xu et al., 2020). Interestingly, a significant downregulation was found for TIR-NBS-LRR Toll-Interleukin receptor (*Solyc00g294230*) (**Supplementary Table 2**). Emerging evidence suggests that TLR-mediated signal transduction pathways lead to the movement of calcium deposits through calcium channel activity (Liu et al., 2008; Zhang et al., 2013).

Ethylene is a crucial phytohormone involved in plant responses to biotic stress as well as in tomato fruit development (Liu et al., 2021). In tomato, ethylene is induced by the pathogenic fungus *Oidium neolycopersici* (Kissoudis et al., 2016) and by *P. syringae* pv. *tomato* (Moran-Diez et al., 2020), the latter being a pathogen that causes a rapid and localized programmed cell death (PCD) (Moyano et al., 2020). Ethylene biosynthesis involves the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene, a reaction catalyzed by ACC oxidase (Boller et al., 1979). The plant response to eDNA was the upregulation of ACC oxidase (*Solyc09g008560*) and the same modulation was found for several ethylene-responsive transcription factors (*Solyc02g070040*, *Solyc12g009240*, and *Solyc03g093550*) (**Figure 6D** and **Supplementary Table 2**). Ethylene-responsive factors (ERFs) belong to a subfamily of the AP2/ERF superfamily which is involved in tomato response to the pathogens *P. syringae* pv. *tomato* (He et al., 2001) and *Stemphylium lycopersici* (Yang et al., 2021), among others.

CONCLUSION

Plant reactions to biotic stress encompass signal transduction cascades, receptors, and biochemical pathways involved in responding to pathogens and herbivores. Recent reports suggest that the application of fragmented pathogen DNA may have an impact when applied in crop protection strategies to cope with pathogens (Serrano-Jamaica et al., 2021). Here we show that not only pathogen fragmented DNA but also self-eDNA induces plant responses typical of biotic responses to pathogens and herbivores. The early and late responses induced by treatment of tomato leaves with tomato eDNA imply the "recognition of small-sized nucleotide molecules" as suggested by several authors (Duran-Flores and Heil, 2018; Heil and Vega-Munoz, 2019; Monticolo et al., 2020) and the involvement of CPKs, RLKs, ERFs, ion homeostasis (calcium, and potassium involvement) and ROS production demonstrated in this work are strongly consistent with this proposition. Moreover, the ROS production induced by eDNA may trigger further DNA degradation and PCD events, which would reinforce the plant response to eDNA. Our results support the intriguing hypothesis that some of the plant reactions to pathogens and herbivores might be due to the plant cell DNA degradation, particularly when associated to the plant cell disruption. Passive cell disruption by chewing herbivores and pathogen-triggered necrotic cell death might be a realistic scenario for the release of self-DNA fragments as DAMPs. Pathogen-inflicted cell lysis including the degradation of host DNA by pathogen-derived DNAses has been bought forward already in 1993 (Gerhold et al., 1993) and since then supported by diverse follow-up studies (Gerhold et al., 1993; Isaac et al., 2009; Hadwiger and Chang, 2015). Moreover, necrotrophic pathogens use DNAse to digest their host's DNA as a source of nutrients (and thereby liberate a DAMP that triggers a defense response), and recent studies suggest that pathogens or herbivores can use DNAses as an effector that removes a DAMP and thereby allows them to escape from the detection by the plant immune system (Huang et al., 2019; Park et al., 2019). In summary, there is some interesting evidence for a role of eDNA also in non-controlled (i.e., non-apoptotic) cell death due to (necrotrophic) pathogens or a (chewing) herbivore and fragmented DNA would then become an important and powerful elicitor able to trigger early and late responses to biotic stress.

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: European Nucleotide Archive (ENA) under the accessions ERX5409868, ERX5409869, ERX5409870, and ERX5409871.

AUTHOR CONTRIBUTIONS

MM conceived and designed the study and drafted the manuscript. FB, MM, MI, and MG performed the experiments. FB revised it critically. All authors approved the final version of the article.

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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.2021.686121/full#supplementary-material

Supplementary Figure 1 | Gene ontology (GO) of the biological processes of downregulated genes by fold enrichment.

Supplementary Figure 2 | Gene ontology (GO) of the biological processes of upregulated genes by fold enrichment.

Supplementary Figure 3 | Hierarchical clustering by comparing the genes downregulated by treatment of plants with eDNA against the Genevestigator database of *S. lycopersicum*.

Supplementary Figure 4 | Hierarchical clustering by comparing the genes upregulated in response to eDNA against the Genevestigator database of *S. lycopersicum*.

Supplementary Figure 5 | Hierarchical clustering of the expression potential of genes downregulated in response to eDNA against the *S. lycopersicum* database of Genevestigator.

Supplementary Figure 6 | Hierarchical clustering of the expression potential of genes upregulated in response to eDNA against the *S. lycopersicum* database of Genevestigator.

Supplementary Table 1 | Primers used in this work.

Supplementary Table 2 | Tomato genes downregulated and upregulated by application of eDNA.

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