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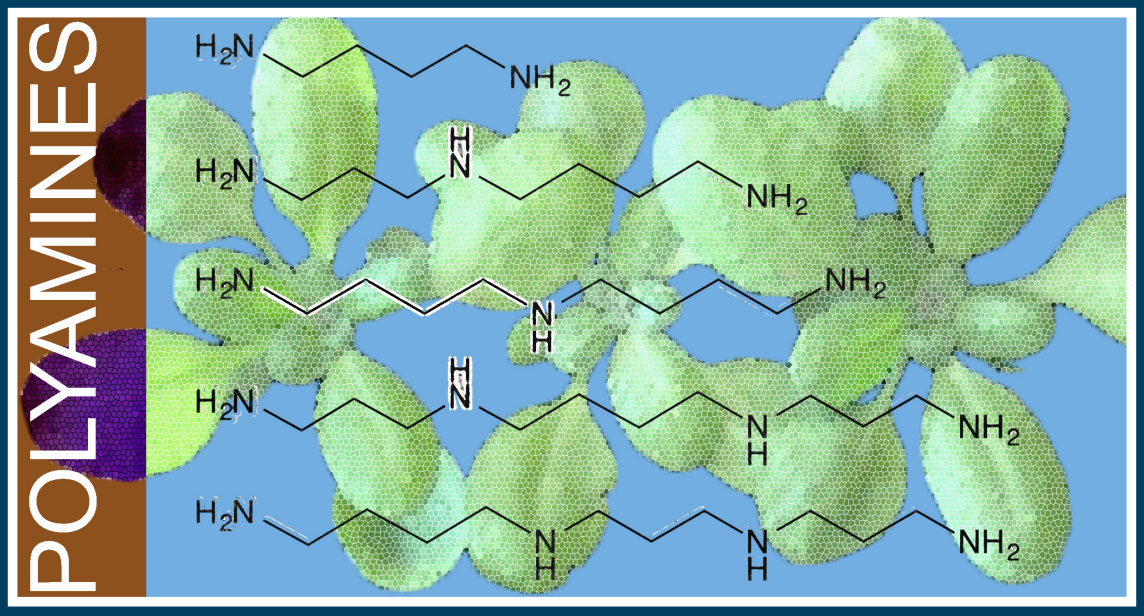

PLANT POLYAMINES IN STRESS AND DEVELOPMENT

Topic Editors
Antonio F. Tiburcio and Rubén Alcázar

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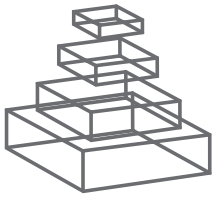

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PLANT POLYAMINES IN STRESS AND DEVELOPMENT

Topic Editors:
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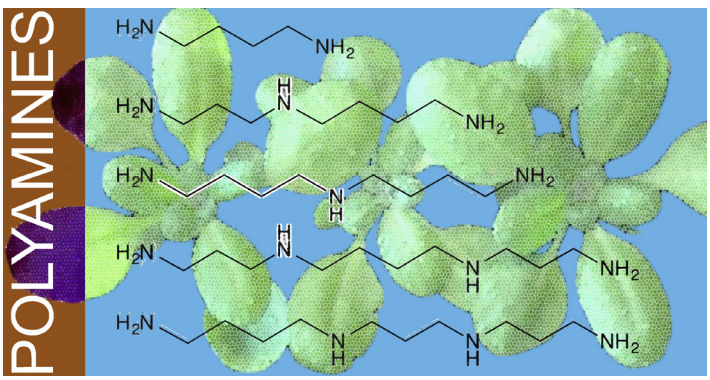

Chemical structure of major polyamines in plants.

Polyamines are small aliphatic polycations which have been involved in key stress and developmental processes in plants. In the recent years, compelling genetic and molecular evidences point to polyamines as essential metabolites required for resistance to drought, freezing, salinity, oxidative stress among other type of abiotic and biotic stresses. In addition to their role as stress-protective compounds, polyamines participate in key developmental processes mediated by specific signaling pathways or in cross-regulation with other plant hormones.

Our Research Topic aims to integrate the multiple stress and developmental regulatory functions of polyamines in plants under a genetic, molecular and evolutionary perspective with special focus on signaling networks, mechanisms of action and metabolism regulation.

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EDITORIAL
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Plant polyamines in stress and development: an emerging area of research in plant sciences

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Keywords: polyamines, putrescine, spermidine, spermine, thermospermine, transglutaminase, stress, ROS

Compelling evidence indicates the participation of polyamines in abiotic and biotic stress responses in plants. Indeed, genetic engineering of polyamine levels in plants has successfully improved biotic and abiotic stress resistance in model plants and crops. We anticipate that many of the current challenges in agriculture to cope with climate change and maintain nutritional quality of fruits and vegetables can be approached by considering the polyamine pathway.

The polyamine field is very dynamic as demonstrated in the large number of monthly publications in all disciplines studying polyamines (including plant sciences, human health, and microbiology). It is composed by a broad spectrum of research laboratories spread around the world, which have provided important contributions into mechanistic processes, present and future practical applications. Still, some areas remain to be explored which makes this a fascinating topic in plant sciences. In this topic, the Editors aimed at establishing a broad perspective of polyamine action in plant stress and development by inviting key researchers in the field. We would like to thank all contributors for joining us in this special topic in Frontiers in Plant Science and we hope that authors have enjoyed the interactions and discussions with editors and reviewers around their excellent works.

This topic contains five reviews, five original research studies and one hypothesis and theory article. Minocha et al. (2014) provides a review update about the complex relationship between polyamines and abiotic stress tolerance with selected examples of polyamine genetic engineering that improve tolerance traits, the concept of stress priming and interactions of polyamines with ROS and other signaling pathways. Do et al. (2014) analyze the polyamine transcriptome and metabolome in rice cultivars differing in salt tolerance, which provides an interesting comparison with potential applications in plant breeding. The interactions between biotic stress and polyamines are reviewed by Jiménez-Bremont et al. (2014) who synthesizes the current knowledge of polyamine metabolism in compatible and incompatible interactions, discusses about the capacity of phytopathogenic microbes of modulating polyamine metabolism for their own benefit, interactions with beneficial microorganisms and practical applications to induce biotic stress tolerance. Marco et al. (2014) reports that overexpression of *SAMDC1* enhances the expression of defense-related genes in *Arabidopsis* and promotes disease resistance against bacterial and oomycete pathogens. Another complementary perspective, Valdés-Santiago and Ruiz-Herrera (2014) provide an original and illustrative view on recent advances about polyamine metabolism in fungi, ranging from mutant characterization to potential mechanisms of action in response to various stresses in selected fungal models. Although free polyamines often capture most of our attention, polyamines are present in free and bound forms resulting from interactions with cellular macromolecules. Some of these interactions occur by covalent linkages with specific proteins in reactions catalyzed by transglutaminases (TGase). Del Duca et al. (2014) provide an original review about the role of TGase on senescence and cell death in various plant models. Interestingly, the role of plant TGase is mediated by a similar molecular mechanism described for apoptosis in animal cells, which opens an interesting field for further exploration in the future. In the context of mechanistic processes, accumulating evidence suggests that polyamines play essential roles in the regulation of plant membrane transport. The review by Pottosin and Shabala (2014) summarizes the effects of polyamines and their catabolites (i.e., ROS) on cation transport across plant membranes, and discuss the implications of these effects for ion homeostasis, signal-transduction, and adaptive responses of plants to environmental stimuli. The regulation of ROS homeostasis by the polyamine back-conversion pathway catalyzed by polyamine oxidase 3 (PAO3) has been investigated by Andronis et al. (2014) in an original article. From a developmental perspective, Tong et al. (2014) provide evidence for the modulation of auxin signaling by thermospermine, which sheds light into polyamine mechanisms of action on plant development. In ripening apple fruit, Deyman et al. (2014) report the interaction of polyamines with products of polyamine catabolism (i.e., GABA). Traditionally, polyamines are described as organic polycations, when in fact they are bases that can be found in a charged or uncharged form. Although uncharged forms represent less than 0.1% of the total polyamine pool, Ioannidis and Kotzabasis (2014) propose that the physiological role of uncharged polyamines could be crucial in chemiosmosis. The authors explain the theory behind polyamine pumping and ion trapping in acidic compartments (i.e., the lumen of chloroplast) and how this regulatory process could improve either photochemical efficiency and the synthesis of ATP or fine tune antenna regulation and make plants more tolerant to stress.

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REVIEW ARTICLE
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Polyamines and abiotic stress in plants: a complex relationship[1]

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The physiological relationship between abiotic stress in plants and polyamines was reported more than 40 years ago. Ever since there has been a debate as to whether increased polyamines protect plants against abiotic stress (e.g., due to their ability to deal with oxidative radicals) or cause damage to them (perhaps due to hydrogen peroxide produced by their catabolism). The observation that cellular polyamines are typically elevated in plants under both short-term as well as long-term abiotic stress conditions is consistent with the possibility of their dual effects, i.e., being protectors from as well as perpetrators of stress damage to the cells. The observed increase in tolerance of plants to abiotic stress when their cellular contents are elevated by either exogenous treatment with polyamines or through genetic engineering with genes encoding polyamine biosynthetic enzymes is indicative of a protective role for them. However, through their catabolic production of hydrogen peroxide and acrolein, both strong oxidizers, they can potentially be the cause of cellular harm during stress. In fact, somewhat enigmatic but strong positive relationship between abiotic stress and foliar polyamines has been proposed as a potential biochemical marker of persistent environmental stress in forest trees in which phenotypic symptoms of stress are not yet visible. Such markers may help forewarn forest managers to undertake amelioration strategies before the appearance of visual symptoms of stress and damage at which stage it is often too late for implementing strategies for stress remediation and reversal of damage. This review provides a comprehensive and critical evaluation of the published literature on interactions between abiotic stress and polyamines in plants, and examines the experimental strategies used to understand the functional significance of this relationship with the aim of improving plant productivity, especially under conditions of abiotic stress.

Keywords: arginine, biochemical markers, gamma-aminobutyric acid, glutamate, ornithine, proline, reactive oxygen species, stress priming

INTRODUCTION

Polyamines (PAs) are small, positively charged, organic molecules that are ubiquitous in all living organisms. The three common PAs in plants are putrescine (Put), spermidine (Spd) and Spm, with some plants also having thermospermine (tSpm) in place of or in addition to Spm. The simplicity of their structure, their universal distribution in all cellular compartments, and presumed involvement in physiological activities ranging from structural stabilization of key macromolecules to cellular membranes make them an attractive group of metabolites to assign a multitude of biological functions. Their accumulation in large amounts in the cell could presumably sequester extra nitrogen (N) thus reducing ammonia toxicity and also balance the total N distribution into multiple pathways. It is not surprising that fluctuations in their cellular contents are often related to varied responses of plants to different forms of stress and to different phases of growth activity. As much as their cellular functions are diverse, and sometimes contradictory, so are their roles in plant stress. They have been deemed important in preparing the plant for stress tolerance and to directly aid in ameliorating the causes of stress, and at the same time, their own catabolic products are responsible for causing stress damage. Several aspects of the relationship between PAs and abiotic stress in plants and their seemingly contradictory roles in the process have been reviewed over the years (Galston and Sawhney, 1990; Alcázar et al., 2006a, 2010, 2011a; Kusano et al., 2007; Liu et al., 2007; Bachrach, 2010; Alet et al., 2011; Hussain et al., 2011; Shi and Chan, 2014).

ABIOTIC STRESS IN PLANTS—ASSESSMENT OF THE SITUATION

Before delving into the specific roles of PAs in plant stress responses, a few details are important to consider regarding the phenomenon of "abiotic stress." The first and the foremost is

[1]This is Scientific Contribution Number 2553 from the New Hampshire Agricultural Experiment Station

the lack of a precise definition of this term. Each plant constantly faces a changing microenvironment from the moment it starts its growth, be it from a seed or a vegetative cutting. On a daily basis, these changes occur from sunrise to sunset (e.g., light, temperature, changes in CO_2 and O_2), and with every cell division, cell enlargement and differentiation activity within the organism. Over its lifetime, there are significant changes in the growth environment; some caused by weather events (like rain or drought), and others part of seasonal changes in temperature and day length. For perennials, there still are the longer-term climatic changes that are relevant to their life. Despite difficulties of precisely defining stress, thousands of experimental studies have involved a variety of stress treatments (mostly short term, i.e., minutes to hours and days) and analysis of the physiological, biochemical and molecular responses of plants to such treatments when they were growing under otherwise "normal" conditions—thus in most cases significant deviation from status quo may be considered stressful.

It is well known that a particular environmental change may be stressful for one species but not for another living under the same conditions. In fact, even within the same species differences exist for response to the same climatic conditions because of genotypic differences among individuals and/or variations in the soil microclimate. A plant's response(s) may involve avoidance of the imposed stress or short-term adaptation to it with the ability to revert back to the original growth and metabolic state. This is in contrast to the evolutionary adaptation (e.g., halophytes, xerophytes, thermophiles) and the long-term physiological adaptations, e.g., those in shade loving plants vs. those that grow better in full sun, and plants requiring large quantities of fertilizer vs. those that can thrive on marginal lands. In most cases the genetics and physiology of a plant allow it to live in a wide range of environmental conditions (as defined by the climate) while in others the range of acceptable environments may be rather narrow. The developmental stage of the plant also plays a significant role in its response to changing environment.

Abiotic stress exposure in plants can be divided into three arbitrary stages: stress perception, stress response and stress outcome (**Figure 1**). Depending on the nature of stress, its perception can be localized to a specific group of cells, tissues and organs or it could be widespread. Additionally, stress could arise suddenly or slowly. For example, exposure of roots to a heavy metal in fertilizer or saline water or to flooding is likely to be different from that if the plant started its life in the presence of these stressors. On the other hand, drought due to lack of programmed irrigation and/or excessive transpiration, or a gradual increase in

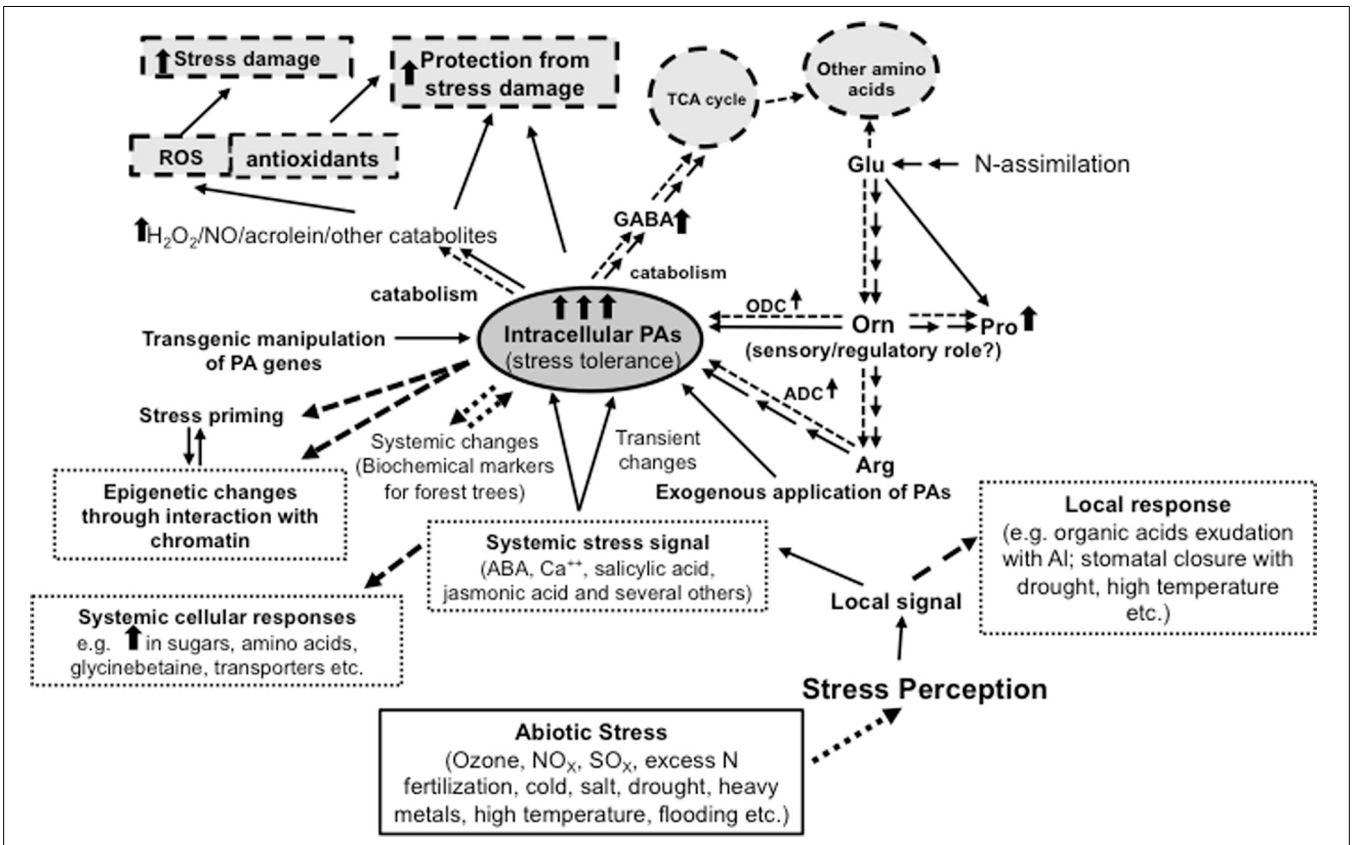

FIGURE 1 | Diagrammatic representation of the complexity of interactions between polyamines and abiotic stress response in plants. The Figure also shows a central role of ornithine in the metabolic interaction of polyamines with glutamate, proline, arginine and γ-aminobutyric acid. While multiple arrows indicate multiple steps, the dotted arrows indicate increased flux/positive regulatory role. Thick upright arrows indicate increase in concentrations or effect.

ozone concentration in the air, are examples of slow exposure to stress. In the latter instances, the precise organ or tissue perceiving stress is difficult to determine. Therefore, the perception of sudden vs. gradual exposure to stressors can be physiologically quite different and must involve different sensing mechanisms. Likewise, whilst the initial exposure to stress may be limited to a certain plant organ (e.g., roots in the case of salt or heavy metal), yet the response is often systemic. In cases when the tolerance mechanism includes stress avoidance (e.g., exclusion of toxic or harmful chemicals including heavy metals) by interfering with uptake mechanisms, the response is generally limited to the same tissues and/or organs that perceive the stress signal. Yet again, even when the responding tissues are the same as the perceiving tissues, e.g., secretion of organic acids in the presence of Al (Kochian et al., 2004; Yu et al., 2012), the metabolism of the entire organ/plant may be affected with broad tissue-specificity. Hence, explanation of the effects of stress on plant metabolic changes like those in PAs must take into account the experimental conditions being used.

The transmission of the stress signal also involves a multitude of mechanisms; some of which are common for different types of stress. For example, drought, flooding, salt, heavy metals, ozone, and sometimes heat or cold all show a common set of physiological responses, which involve regulatory metabolites like abscisic acid (ABA), salicylic acid and jasmonate or methyl jasmonate (MeJa). Frequently, these modulators of stress may affect metabolites that are common for tolerance and/or amelioration of a variety of stresses (e.g., γ-aminobutyric acid - GABA, proline - Pro, glycinebetaine) or they may be specialized (e.g., phytochelatins in response to heavy metals). Polyamines, in combination with Pro and GABA belong to the former group with almost universal involvement in a variety of stress responses.

POLYAMINES AND ABIOTIC STRESS IN PLANTS

The history of PAs and their roles in stress tolerance in plants goes back to almost four decades (Hoffman and Samish, 1971; Murty et al., 1971). The issues related to PA functions in stress are especially difficult to study because of their ubiquitous presence and absolute necessity for cell survival, and their presence in relatively large (millimolar) quantities. One of the most confounding problems relating to the role of PAs in abiotic stress is the lack of our understanding of the mechanisms underlying their function(s). The above arguments are consistent with the recent portrayal of PAs by Hussain et al. (2011) as "mysterious modulator of stress response in plants," perhaps because their roles span a large spectrum of cellular activities but their mechanisms of action are rather poorly understood. The authors cite numerous studies in which overall PA metabolism is increased in response to a variety of abiotic stresses - chemical or physical. Several publications (Alcázar et al., 2006a; Takahashi and Kakehi, 2010; Alet et al., 2011; Hussain et al., 2011; Gupta et al., 2013; Shi and Chan, 2014) have elegantly summarized the various likely roles of PAs in tolerance and/or amelioration of stress in plants. These include: (i) serving as compatible solutes along with Pro, glycinebetaine and GABA; (ii) interactions with macromolecules like DNA, RNA, transcriptional and translational complexes, and cellular and organellar membranes to stabilize them; (iii) role in directly scavenging oxygen and hydroxyl radicals and promoting the production of antioxidant enzymes and metabolites; (iv) acting as signal molecules in the ABA-regulated stress response pathway and through the production of H_2O_2; (v) regulators of several ion channels; and, finally (vi) participation in programmed cell death. To this list can be added their role in metabolic regulation of ammonia toxicity, nitric oxide (NO) production, and balancing organic N metabolism in the cell (Nihlgård, 1985; Moschou et al., 2012; Guo et al., 2014).

The facts that PAs are often present in large quantities and their biosynthesis uses Glu, a key amino acid for N assimilation, as the starting material, it can be envisioned that large changes in their biosynthesis and catabolism (e.g., >5–10-fold) could cause major homeostatic shifts in cellular metabolism. Therefore, under conditions of stress, PAs could perform these functions better when changes in their metabolism are transient and within narrower limits, thus avoiding catastrophic perturbations in the overall cellular homeostasis of C and N (Minocha et al., 2000; Bhatnagar et al., 2001; Bauer et al., 2004; Majumdar et al., 2013). However, in perennial trees exposed to persistent environmental stress from air pollutants and resulting changes in soil chemistry, the altered metabolic homeostasis may stabilize enhanced PA levels in a way that they can be used as biochemical markers of stress (Minocha et al., 2000, 2010) In these situations their role could be more prophylactic in preventing stress damage rather than short-term protection. For more details, see Section Polyamines as Metabolic Markers of Long-Term Environmental Stress in Forest Trees.

There are four types of studies that make a strong case in favor of the importance of PAs in plant stress response (Galston and Sawhney, 1990; Alcázar et al., 2006a, 2010; Kusano et al., 2007; Liu et al., 2007; Bachrach, 2010; Alet et al., 2011; Hussain et al., 2011; Shi and Chan, 2014). These include: (i) up-regulation of PA biosynthesis in plants via transgene expression generally increases their tolerance to a variety of stresses; (ii) increased PA accumulation in plants under stress conditions is accompanied by increase in the activity of PA biosynthetic enzymes and the expression of their genes; (iii) mutants of PA biosynthetic genes generally have less tolerance of abiotic stress; (iv) while exogenous supply of PAs makes the plants tolerant to stress, inhibition of their biosynthesis makes them more prone to stress damage. Some highlights of the recent studies in these areas are summarized here:

TRANSGENICS AND STRESS TOLERANCE

In reviewing the literature on the improvement of stress tolerance in transgenic plants over-expressing a homologous or a heterologous gene encoding a PA biosynthetic enzyme, a few conclusions stand out (for key points of the major studies and references, see **Tables 1**, **2**):

(1) Every one of the PA biosynthetic enzyme genes has been expressed as a transgene in several plant species; in most cases a constitutive promoter controls the transgene expression.
(2) Experiments with transgenics have typically involved short-term treatments with stress followed in many cases by removal of the treatment to study recovery from stress. Only in a few cases have the plants been brought to maturity and analyzed for total biomass or yield of the desired product

Table 1 | Genetic manipulation of *ODC, ADC,* and *SAMDC* genes and enhanced tolerance to abiotic stress in transgenic plants.

Plant species	Promoter::Transgene	Stress application (short or long term)	Increase in enzyme activity	Increase in Put	Increase in Spd	Increase in Spm	Outcome	Citation
Nicotiana tabacum var. xanthi	35S::Mouse *ODC*	NaCl (200 mM; up to 4 week from germination or 15 day old seedlings subjected to 300 mM NaCl for 4 week)	Very high (mouse ODC; native ODC or ADC activity was lower in the transgenics)	2–3-fold	2–3-fold	NS	Greater tolerance to salt stress	Kumria and Rajam, 2002
Oryza sativa	ABA-inducible:: *Avena sativa ADC*	NaCl (150 mM; 2-day in 10-day old seedlings)	3–4-fold	1.7–2.2-fold	NA	NA	Increased tolerance to salinity stress	Roy and Wu, 2001
Oryza sativa	*35S::Datura stramonium ADC*	Drought (60-day old plants; 6 day in 20% PEG followed re-watering for 3 day)	NA	1.5–4-fold	NS	NS	High tolerance to drought	Capell et al., 2004
Solanum melongena	*35S::Avena sativa ADC*	Salinity (150–200 mM NaCl; 8–10 day), drought (7.5–10% PEG; 8–10 day), low temperature (6–8°C; 10 day), high temperature (45°C for 3 h), cadmium (0.5–2 mM for 1 month) in 8–10 day old seedlings	3–4-fold (ADC, DAO), ∼2-fold (ODC)	3–7-fold	3–5-fold	∼2-fold	Enhanced tolerance to multiple stresses	Prabhavathi and Rajam, 2007
Arabidopsis thaliana	35S::*Arabidopsis thaliana ADC2*	Drought (4 week-old plants for 14 day followed by 7 day recovery)	NA	2–12-fold	NS	NS	Increased tolerance to drought	Alcázar et al., 2010
Arabidopsis thaliana	35S: *:Arabidopsis thaliana ADC1*	Low temperature [3 week-old plants for ∼9 day at 4-(−11)°C followed by a 2 week recovery]	NA	∼3–5-fold	NS	∼(−) 1.3–1.9-fold	Greater tolerance to low temperature	Tiburcio et al., 2011
Arabidopsis thaliana	*pRD29A::Avena sativa ADC*	PEG (11-day seedlings for 13 h), low temperature (3-week old plants for 10 day)	∼10–17-fold (low temp)	∼3–5-fold (low temp)	NS	NS	Greater resistance to dehydration and low temperature stress	Alet et al., 2011
Arabidopsis thaliana adc1-1 mutant	35S: *Poncirus trifoliate ADC*	High osmoticum, drought, and low temperature (up to 14-day from germination, 1–18 day in 3–4 week-old plants)	NA	∼2-fold	NS	NS	Enhanced resistance to high osmoticum, dehydration, long-term drought, and low temperature stresses	Wang et al., 2011
Lotus tenuis	*pRD29A::Avena sativa ADC*	Drought (6–8 week-old plants exposed to soil water potential of −2 MPa)	∼2.2-fold (drought)	∼3-fold (drought)	NS	NS	Increased tolerance to drought	Espasandin et al., 2014

(Continued)

Table 1 | Continued

Plant species	Promoter::Transgene	Stress application (short or long term)	Increase in enzyme activity	Increase in Put	Increase in Spd	Increase in Spm	Outcome	Citation
Oryza sativa	*ABA inducible :Tritordeum SAMDC*	NaCl (150 mM; 11 day-old seedlings for 2 day)	NA	1.3-fold (salt)	2.4-fold (salt)	2.8-fold (salt)	Enhanced salt tolerance	Roy and Wu, 2002
Nicotiana tabacum var. xanthi	*35S::Homo sapiens SAMDC*	NaCl (250 mM), PEG (20%) up to 2 months from sowing	∼1.3–5-fold (overall SAMDC), ∼2-fold (DAO)	∼2.4–2.7-fold	∼1.4–2.4-fold	∼1.4-fold	Greater tolerance to salt and drought	Waie and Rajam, 2003
Nicotiana tabacum	35S::*Dianthus caryophyl/us SAMDC*	Salt (NaCl; 0–400 mM from sowing through 8 week) Low temperature (5 week-old plants for 24 h at O°G)	2-fold	NS	2.1-fold	1.7-fold	Increased tolerance to oxidative, salt, low temperature, and acid stresses	Wi et al., 2006
Lycopersicon esculentum Mill.	35S:: *Saccharomyces cerevisiae SAMDC*	High temperature [35 day old plants for 4 day at 38°C/30°C (d/n) followed by 3 day recovery period]	NA	NS	∼1.4-fold	∼1.4-fold	Higher tolerance to high temperature	Cheng et al., 2009
Oryza sativa L. subsp. Japonica cv. EYI105	*Ubi::Datura stramonium SAMDC*	Osmoticum (PEG; 60 day-old plants for 6 day followed by 20 day recovery period)	NA	NS	1.5–2-fold	NS	Greater tolerance to high osmoticum induced drought and better recovery	Peremarti et al., 2009
Nicotiana tabacum	35S:: *Malus domestica SAMDC2*	Low temperature (4°C; 5 day-old seedings for 0, 6, 120 h, and 30 day), PEG (20%; 5 day old seedings for 6 h), NACl (150 mM and 250 mM; 15-day old seedings for 48 h, and 60 day)	NA	1.1–1.5-fold	1.2–1.6-fold	1.7–2.2-fold	Enhanced tolerance to low temperature, high osmoticum, and NaCl	Zhao et al., 2010
Arabidopsis thaliana	35S:: *Capsicum annuum SAMDC*	Drought (2 week old plants for 6 h or 3 week-old plants for 11 day followed by 3 day recovery)	1.4–1.6-fold (total SAMDC)	NS	∼1.8-fold	∼1.7-fold	Increased drought tolerance	Wi et al., 2014

Fold increases of PAs in transgenic plants are from the basal level unless otherwise stated (NA, not available; NS, not significant).

Table 2 | Genetic manipulation of *aminopropyl transferase* genes and enhanced tolerance to abiotic stress in the transgenic plants.

Plant species	Promoter::Transgene	Stress application (short or long term)	Increase in enzyme activity	Increase in Put	Increase in Spd	Increase in Spm	Outcome	Citation
Arabidopsis thaliana	*35S::Cucurbita ficifofia SF'DS*	Low temperature (25 day-old plants at −5°C for 40 h followed by 5 day recovery), salinity (75 mM NaCl for 45 day *in-vitro*), high osmoticum (250 mM sorbitol for 70 day *in-vitro*), drought (3 week-old plants for 15 day), oxidative stress (leaf discs at 0.5–5 μM for 14 h)	5–6-fold (SPDS)	NS	1.3–2-fold	1.6–1.8-fold	Increased tolerance to low temperature, salinity, hyperosmosis, drought, and paraquat toxicity	Kasukabe et al., 2004
Ipomoea batatus	*35S::Cucurbita ficifolia SPDS*	Salt (NaCl; 114 day from planting), Low temperature (10–30°C for 6 h), high temperature (42–47°C for 5 min)	NA	1.5-fold	2-fold	NS	Enhanced tolerance to salt, drought, extreme temperatures, and oxidative stress	Kasukabe et al., 2006
Pyrus communis L. "Ballad"	*35S::Malus sylvestris* var. *domestica SPDS*	Salt (250 mM NaCl for 10 day), high osmoticum (300 mM mannitol for 10 day), heavy metals (500 μM $CuSO_4$ for 15 day; 30 μM $AlCl_3$, 50 μM $CdCl_2$, 500 μM $PbCl_2$, and 500 μM $ZnCl_2$ for 21–30 day *in-vitro*)	NA	1.1–1.6-fold	1.3–1.9-fold	0.6–1.7-fold	Greater tolerance to salt, high osmoticum, and heavy metals	Wen et al., 2008, 2009, 2010
Lycopersicon esculentum	*35S::Malus sylvestris* var. *domestica SPDS1*	Salt (100 or 250 mM NaCl; 4 week-old plants for 60–65 day)	NA	NS	∼1.5–1.6-fold	NS	Enhanced tolerance to salt stress	Neily et al., 2011
Arabidopsis thaliana	*35S::Arabidopsis thaliana SPMS*	High temperature (7–15 day-old seedlings for 0.5–2.5 h at 42–45°C)	NA	1.6-fold	(−)4–6-fold	4.4–4.7-fold	Enhanced tolerance to thermal stress	Sagor et al., 2013

Fold increases of PAs in the transgenic plants are above the basal level unless otherwise stated (NA, not available; NS, not significant).

(seeds, fruit, or leaf biomass) or its quality (e.g., nutritional properties).

(3) Measurements of stress response have included visual symptoms of water loss or wilting, changes in fresh weight, dry weight, ion release, gene expression, and analysis of enzyme activities and cellular metabolite, etc.

(4) Transgenic manipulations of *ADC* or *ODC* in plants have resulted in a significant increase in Put content (typically 3–10-fold) with relatively smaller (<2-fold) changes in Spd and Spm. Transgenic manipulation of *SPDS*, *SPMS*, and *SAMDC* causes smaller (compared to Put) increase of Spd and Spm contents (∼2–3 fold).

(5) The fold increase in Put content often varies with the plant species, homologous or heterologous gene sources, nature of the promoter, developmental stage of the plant, and the tissues analyzed.

ENZYME ACTIVITY AND GENE EXPRESSION OF POLYAMINE BIOSYNTHETIC ENZYMES

An increase in cellular PAs in the initial stages of stress treatment is often accompanied by increased activity of Put biosynthetic enzymes like ADC and ODC, but generally not those involved in the biosynthesis of higher PAs, i.e., SAMDC, SPDS, and SPMS (Majumdar et al., 2013). This observation is consistent with the specificity of response being limited often to changes in Put in most cases, and also that the cellular contents of higher PAs often change only within a narrow range (Minocha et al., 1997, 2000, 2010; Bhatnagar et al., 2002; Majumdar et al., 2013). In some cases, where two or more copies of a gene encoding the same enzyme are present, often a general increase in gene expression for all copies is observed (Urano et al., 2004, 2009; Hu et al., 2005; Do et al., 2013; Guo et al., 2014).

The detailed functional enrichment analyses have been reported for differential gene expression in high Put-producing transgenic *A. thaliana* over-expressing a homologous *ADC2* gene using microarrays (Alcázar et al., 2005; Marco et al., 2011a,b). The results showed that the direct target of increased Put accumulation included genes responsive to salt, heavy metals, cold, and oxidative stresses, besides those involved in basic cellular processes, e.g., translation and ribosome structure. Several genes associated with IAA biosynthesis, transport and auxin-related transcription factors, ABA biosynthesis and ABA-related transcription factors, and other signal transduction-related genes were also significantly up regulated in the transgenic plants. On the other hand, Spm over-producing transgenic *A. thaliana* (with a homologous *SAMDC1* or *SPMS* transgene) positively affected defense-related (biotic/abiotic stresses) genes, signaling pathway genes (e.g., mitogen activated protein kinases - MAPKs), and genes associated with ABA-, JA-, and SA- biosynthesis related enzymes. The commonalities of up-regulated stress-related gene expression in Put and Spm-overproducers (e.g., Ca^{++} signaling-related genes and ABA biosynthetic genes) suggest overlapping functions of Put and Spm (by interacting with ABA or modulating Ca^{++} homeostasis), which are common to tolerance against drought, salt or low temperature.

In a comprehensive study of two rice cultivars (*Oryza sativa* L. ssp. *indica* and *japonica*) kept under 18 days of drought stress, Do et al. (2013) noted up-regulation of several genes and related metabolites involved in PA biosynthesis via Orn/Arg pathways. Of the 21 genes associated with the Orn/Arg pathway, 11 co-localized with the drought-related QTL regions. Although Put was the predominant PA under normal conditions, Spm became dominant upon exposure to drought indicating Put to Spm conversion, similar to what Alcázar et al. (2011b) had observed in *A. thaliana*. There was also an increase in the expression of selected paralogs of *SAMDC*, *SPDS*, and *SPMS* genes. However, in a comparison of high and low Put cell lines of *Populus nigra x maximowiczii* growing in suspension cultures, Page et al. (2007) found no major differences in the expression of most of the genes (q-PCR) of the Glu-Orn-Arg/Pro/Put pathway, indicating that increased flux of Glu→Orn (in high Put cells) was not transcriptionally regulated.

Recently the role of endogenously produced Put affecting the expression of drought responsive gene 9-cis-epoxycarotenoid dioxygenase (NCED) - can enzyme that controls ABA biosynthesis under stress, was studied in lotus (*Lotus tenuis*), using a heterologous oat (*Avena sativa*) *ADC* gene under the control of a drought/ABA inducible promoter *RD29A* (Espasandin et al., 2014). Drought increased the expression of oat *ADC* by ∼100-fold, total ADC activity by ∼2-fold and Put by ∼3.6-fold, with only minor changes in Spd and Spm. The non-transgenic plants showed relatively smaller changes of these parameters upon exposure to drought. Higher Put contents in the transgenic plants significantly increased (∼3-fold) the expression of *NCED* as compared to the wild type plants, suggesting the possibility of transcriptional regulation of ABA synthesis by Put.

MUTANTS OF POLYAMINE BIOSYNTHETIC GENES AND STRESS

Mutants for almost all of the key PA biosynthetic genes have been tested for their stress tolerance properties. Since PAs are an absolute requirement for growth in all organisms, and most PA biosynthetic genes are present in at least two copies in plants, knockouts for single gene mutants are often the only feasible approach to study their involvement in stress. Based on extensive analysis of Arabidopsis mutants, no single gene of the pathway has been found to be absolutely essential or to play a specific role in stress response. The mutant studies further showed a reduction in seed development in cases where more than one gene was affected (Imai et al., 2004; Urano et al., 2005; Ge et al., 2006), hence maintenance of homozygous double mutants of the two gene copies encoding the same enzyme has not been possible.

An Arabidopsis double knockout (*acl5/spms*) compromised for tSpm and Spm biosynthesis showed hypersensitivity to NaCl and KCl but not to $MgCl_2$ (Yamaguchi et al., 2006; Alet et al., 2011). Altered tSpm and Spm levels in the mutant were shown to impair Ca^{++} homeostasis thereby affecting their overall monovalent: bivalent charge ratio leading to a differential response to salts. In addition to salt and drought stresses, Spm also plays a significant role in heat tolerance as shown by hypersensitivity of a T-DNA insertion mutant of *SPMS* in Arabidopsis exposed to higher temperature. The hypersensitivity was overcome either by exogenous supply of Spm (and tSpm) or by increasing endogenous Spm by constitutive over-expression of a homologous *SPMS* (Sagor et al., 2013).

An Arabidopsis single mutant of *ADC* (*adc1* or *adc2*) with significantly reduced Put content showed increased sensitivity to low temperature (Cuevas et al., 2008). The *adc* mutants showed reduced expression of *NCED3* (ABA synthesis). Complementation and reciprocal complementation with ABA and Put, respectively improved low temperature tolerance of the mutants.

A potential role of ABA (Christmann et al., 2005) in the induction of genes that encode PA biosynthetic enzymes was demonstrated independently (Urano et al., 2004, 2009; Alcázar et al., 2006b). This interaction was further confirmed by studies with ABA-deficient mutants (in which increase in ADC under stress was not seen), analysis of the promoter regions of several PA biosynthetic enzyme genes that have ABRE-like motifs, and the direct effects of applied ABA on Put production.

ABIOTIC STRESS AND EXOGENOUS SUPPLY OF POLYAMINES

Besides transgenic up-regulation of cellular PAs, exogenous application of PAs also shows similar results, i.e., increased stress tolerance, while chemical inhibition of their biosynthesis exhibits increase in damage from stress. Protection from exogenous PAs could presumably come from their direct interactions with the membranes, reduction of oxidant activity, serving as compatible osmolites or their ionic properties (Hu et al., 2005; Ndayiragije and Lutts, 2006; Wang et al., 2007; Afzal et al., 2009). With respect to the use of exogenous PAs and/or the inhibitors of PA biosynthesis, most studies have been restricted to *in vitro* use of callus, leaf explants or young plants, and, with a few exceptions, that have involved short-term stress treatments. The following recent reports are examples of the effects of exogenously supplied PAs on stress response in plants:

- Foliar spray of 0.1 mM Put in wheat (*Triticum aestivum* L.) at the time of anthesis and prior to application of drought stress, significantly increased photosynthetic attributes, increased contents of Pro, total amino acids and soluble sugars, improved water status, reduced membrane damage, and significantly increased total grain yield as compared to the control plants (Gupta et al., 2012).
- Using an *in vitro* system of detached tobacco leaf discs, Kotakis et al. (2014) found that pre-treatment with 1 mM Put 1 h prior to polyethylene glycol (25%) induced osmotic stress, prevented significant water loss and maintained maximum photosystem II photochemical efficiency (F_v/F_m).
- Foliar application of 2.5 mM Put or Arg increased tolerance to high temperature (35 ± 2°C for 4–8 h) in 30–35 day old wheat (*T. aestivum* cv. Giza 168) plants. At 5 days after spray, the plants had higher amounts of endogenous Put, Spd and total amino acids, and lower amounts of ammonium and ethylene. Total yield at 155 days in the Put-treated plants was higher vs. the controls (Hassanein et al., 2013).
- Exogenous application of Spd (for 7 days) at early booting stage of rice (*Oryza sativa* L. ssp. *indica*) prior to treatment with NaCl (that continued till maturity) significantly increased grain yield, Ca^{++} content in the grains, and a higher K^+/Na^+ ratio as compared to the non-treated control plants (Saleethong et al., 2013).
- Exogenous Spd added at the same time as NaCl increased cellular contents of Spd, Spm and Pro in *Panax ginseng* seedlings by activating antioxidant-based defense system, thereby reducing H_2O_2 and superoxide molecules (Parvin et al., 2014).
- Similar effects of exogenous PAs on ameliorating NaCl stress in 5-month old sour orange (*Citrus aurantium* L.) plants were seen by Tanou et al. (2014). They suggested that it was due to reprogramming the oxidative status of cells by increased expression of genes producing antioxidant enzymes. Proteomic studies reveal reduced protein carbonylation and tyrosine nitration, and increased protein *S*-nitrosylation by PAs.
- In a detailed study with Bermuda grass (*Cynodon dactylon*) Shi et al. (2013) found that exogenous PAs, while mitigating drought and salt stresses, significantly increased the abundance of antioxidant enzymes and several other stress-related proteins. These results are consistent with what Zhao et al. (2010) had earlier reported in the same species, where water deficit significantly affected proteins associated with photosynthesis and antioxidant-mediated defense pathways. These results reinforce the role of intracellular Put positively affecting photosynthetic machinery with enhanced capabilities of transgenic plants for biomass accumulation reviewed by Sobieszczuk-Nowicka and Legocka (2014).

REACTIVE OXYGEN SPECIES AND POLYAMINE CATABOLISM

A multifaceted interaction of PAs with Reactive Oxygen Species (ROS) and antioxidants is perhaps among the most complex and apparently contradictory physiological and biochemical interactions in plants. A functional association between ROS and abiotic stress has been known from the time of their discovery since ROS are capable of causing widespread damage to a variety of cellular metabolites as well as macromolecules (Pottosin et al., 2014 and references therein). Some of the overlapping responses of plants to drought, salinity and other abiotic stresses are presumably related to maintaining a healthy water status in the cells, which requires the removal of ROS and related free radicals involving oxygen. Thus, increase in ROS production in stress tolerant plants is often accompanied by increased biosynthesis of antioxidants and associated antioxidant enzymes to ameliorate the ROS from cellular environment.

Numerous studies have emphasized the complexity of interaction between PAs and the ROS, especially when plants are under stress (Bhattacharjee, 2005; Gill and Tuteja, 2010; Velarde-Buendía et al., 2012; Pottosin et al., 2014). Typically when cellular PA contents are up, their catabolism also increases, the levels of H_2O_2 increase, and various ROS as well as the antioxidant systems (enzymes and metabolites) are also up, hence their roles in preventing damage from stress are beneficial as well as deleterious. The role of PAs in augmenting antioxidant based defense systems to impart tolerance against heavy metals, UV and other stresses that are potent inducers of superoxide molecules causing oxidative damage to the living cells have been reported in several studies (Bouchereau et al., 1999; Groppa et al., 2007; Thangavel et al., 2007; Mapelli et al., 2008; Rakitin et al., 2009; Jantaro et al., 2011; Pothipongsa et al., 2012; Chmielowska-Bąk et al.,

2013; Mandal et al., 2013; Scoccianti et al., 2013). This is consistent with the diverse roles of PAs including the fact that an increase in cellular PA titers contributes to both sides of the ROS-antioxidant equation under conditions of stress. While on one side the ROS participate in abiotic (and biotic) stresses as parts of signal transduction pathways to induce protective responses (Moschou et al., 2012), on the other side they also directly cause membrane damage, chlorophyll destruction and oxidation of several important metabolites in the cell. Likewise PAs have been implicated in several protective responses in the cell (Bouchereau et al., 1999; Zepeda-Jazo et al., 2011; Pothipongsa et al., 2012; Tanou et al., 2012, 2014), including the protection of membranes and other macromolecules, which are the targets of ROS damage. Furthermore, PA catabolism can contribute directly to cell damage, interestingly via the production of H_2O_2 and acrolein as observed in tobacco cells (Kakehi et al., 2008; Mano, 2012; Takano et al., 2012) as well as mammalian systems (Sakata et al., 2003; Yoda et al., 2006; Mohapatra et al., 2009; Saiki et al., 2009; Yoshida et al., 2009). Yet the same source of H_2O_2 (i.e., PA catabolism) is needed for lignin production in the apoplast adjacent to the plasma membrane (Moschou et al., 2008).

A major interaction between PAs and ROS presumably occurs at the level of plasma membrane where PAs (due to their strong positive charge) can effectively block cation channels (Williams, 1997; Dobrovinskaya et al., 1999a,b; Zepeda-Jazo et al., 2008; Bose et al., 2011; Zepeda-Jazo et al., 2011). Their specificity for selectively blocking outward Na^+ channels (vs. the K^+ channels) in the tonoplast membrane apparently helps the vacuole to contain Na^+within it, thus changing the effective K^+/Na^+ ratio in the cytoplasm under conditions of stress. Other interactions of PAs with the ion channels have been discussed in several recent reviews (Del Rio and Puppo, 2009; Demidchik and Maathuis, 2010; Pottosin et al., 2012, 2014).

POLYAMINES AND STRESS MEMORY/PRIMING

Bruce et al. (2007) have elegantly described the importance of evolutionary and long-term adaptation to environmental stress in plants. They postulate that plants' responses to short-term exposure to stress are governed by a combination of their innate ability (genetic and evolutionary) as well as previous events in the life of the individual plant, i.e., exposure to stress during early development, which they term as "priming." While the evolutionary basis of adaptation to various abiotic and biotic stresses is obvious from the distribution of plants in different macro- and micro-ecosystems, there are numerous examples of the effects of priming on physiological responses of plants to repeated exposure to several different types of stresses (Jisha et al., 2013 and references therein). These include tolerance to salt, transient exposure to drought, flooding, high and low temperature, and ozone. In other cases of growth under conditions of steady stress (e.g., salt or heavy metals in the soil), priming usually occurs during seed germination, which may have longer-lasting effects on the growth of a plant, even though the overall growth rate may be slower. Adaptations of different species or different ecotypes of the same species to different environments are excellent examples of a combined role of priming and genetic selection within a continuum of climatic/environmental conditions. It was suggested that priming due to early exposure of plants to various forms of abiotic stress might involve epigenetic changes that are stable over the life of a plant.

Epigenetic effects of the changing environmental on gene expression are widely accepted; however, the mechanism of such epigenetic adaptations is not well understood. It is now known that epigenetic changes mostly occur at the level of chromatin, and involve sequence-specific DNA methylation, histone acetylation and methylation, and other similar modifications. While most of the epigenetic changes are stable within the life of an organism, others are reversible through exposure to certain growth and development regulators, and still others appear to be transmitted to the next generation through sexual reproduction (Sano, 2010; Shao et al., 2014; Sharma, 2014). Control of totipotency by plant hormones in cell and tissue culture and stem cell research in animals are excellent additional examples of the role of various external chemical and physical factors in controlling epigenetic changes that regulate cell fate.

As discussed above in relation to the cellular functions of PAs, one could envision a critical role for them in affecting epigenetic changes related to priming for stress. It can be argued that increased PA accumulation in response to short-term stress affects the epigenetic modifications of DNA and histones because of their ability to interact with chromatin (Pasini et al., 2014 and references therein). Important aspects of this premise would include: (i) a fundamental role for PAs in epigenetic changes that normally occur in the life of an organism through specific interactions with DNA prior to or during methylation (Krichevsky et al., 2007; Sharma et al., 2012), and (ii) the enhancement of specific epigenetic changes occurring under conditions of priming for stress (cold or salt treatment of seeds during germination to develop tolerance, desiccation of seeds and buds during dormancy, etc.).

POLYAMINES, PROLINE, NITRIC OXIDE, ARG AND γ-AMINOBUTYRIC ACID—THE ORNITHINE CONNECTION

It is interesting to note that changes in cellular contents of PAs and Pro often seem to occur in a coordinated manner rather than the two moving in opposite directions even though their biosynthesis shares a common precursor, i.e., Glu (Delauney and Verma, 1993; Aziz et al., 1998; Mattioli et al., 2009; Mohapatra et al., 2010; Verslues and Sharma, 2010). When PA biosynthesis is increased - either in response to abiotic stress or through genetic manipulation of ODC or ADC - (Wen et al., 2008; Majumdar et al., 2013), it obviously must cause an increase in the flux of Glu into Orn and Arg, depending upon the route of Put biosynthesis (ODC or ADC). In this regard, we (Page et al., 2007) have shown that the increased flux of Glu to Orn and Arg is apparently regulated at the biochemical level without involving major changes in the expression of genes encoding various enzymes of this pathway. Also, under both situations Pro content increases. However, it is not always clear as to what pathway is involved in increased Pro biosynthesis, i.e., directly from Glu by Δ^1-pyrroline-5-carboxylate synthetase (P5CS) or from Orn by Orn aminotransferase (OAT). A third metabolite whose cellular content seems to follow the same pattern as Put and Pro is GABA, which, like Pro, is also synthesized by two alternate pathways—from Glu by Glu decarboxylase (GAD)

and from Put by diamine oxidase (DAO). An additional product of the Glu/Pro/Arg/Put/GABA pathway is nitric oxide (NO), whose role in various developmental and physiological processes in plants has recently drawn serious attention (Tun et al., 2006; Mur et al., 2013; Tanou et al., 2014); the production of NO also increases under abiotic stress conditions (Wimalasekera et al., 2011). Consequently, increased flux of Glu through this set of reactions must cause either a major loss of cellular Glu (which is not tolerable if the cells must continue other metabolic functions involving Glu; the minimum being the biosynthesis of proteins and other amino acids) or a coordinated enhancement of its biosynthesis via increased N assimilation (or protein degradation, which could happen under conditions of stress). It can thus be argued that these interactive pathways may involve a common signal and/or a common signaling mechanism (**Figure 1**). The possibility of Orn being involved in such a monitoring and/or signaling pathway for the biosynthesis of all of these metabolites (i.e., Pro, Put, GABA, and perhaps also Arg and NO - Orn is an intermediate for all) has been suggested (Majumdar et al., 2013). They proposed the existence of a mechanism to monitor cellular Orn, and through as yet unknown signaling pathway, increase the flux of Glu into the said pathway, without concomitant effects on the production of Arg. Of course, in the case of abiotic stress, the role of ABA, salicylic acid, jasmonic acid, and other signaling molecules must also be considered.

Ornithine is a non-protein amino acid that is synthesized from Glu (major metabolic entry point of inorganic N in plants) via several steps (Slocum, 2005). It is a metabolic intermediate rather than a terminal product of the PA-amino acid biosynthetic pathways, and occupies a pivotal position contributing to the production of PAs, Arg, and Pro. Augmentation of intracellular Orn titers by manipulation of genes related to Orn biosynthesis has been shown to increase stress tolerance in plants (Kalamaki et al., 2009a,b). Transgenic Arabidopsis plants constitutively overexpressing a tomato *N*-acetyl-L-Glu synthase gene (*SlNAGS1*) showed up to 9-fold increase in foliar Orn, which was accompanied by a small but significant increase (10–29%) in citrulline and a decrease (∼20%) in Arg levels. Transgenic lines showed increased germination % and higher root tolerance index when grown in media containing 250 mM NaCl; there also was greater tolerance to salt or drought in mature plants as indicated by their bigger leaf size, and higher growth and chlorophyll contents under stress situations. Additionally, the transgenic plants showed better recovery after stress withdrawal.

Besides increasing intracellular Orn titers through genetic manipulation, exogenous application of Orn has also been shown to alleviate abiotic stresses (Ghahremani et al., 2014). In tobacco cells subjected to NaCl stress, application of exogenous Orn significantly increased the activity of antioxidant enzymes, e.g., catalase (325%), peroxidase (270%) and superoxide dismutase (374%), concomitant with significant increases in Put/Spd and significant decrease in H_2O_2 vs. the control cells. Similar stress ameliorating properties of Orn were observed in tobacco cells exposed to high osmoticum (polyethylene glycol); interestingly though, a differential role of D-Orn and L-Orn was observed where the former was more effective under salinity and the latter under drought conditions (Ghahremani et al., 2014).

Correlation between cellular reserves of Orn either as physiological responses of plants to seasonal changes or exogenous application of Orn and tolerance to extreme conditions are also evident from other studies. In leafy spurge (*Euphorbia esula* L.), a perennial weed, a significant increase in free amino acids and soluble protein were observed as an overwintering strategy (Cyr and Bewley, 1989). Cellular Orn increased by 6- to 8-fold in the roots during peak winter compared to the summer months. In detached leaves of cashew (*Anacardium occidentale* L.) plants, exogenous Orn (but not Glu) along with salt stress showed a 2–3-fold increase in Pro contents, suggesting Orn as an effective precursor for Pro accumulation (Da Rocha et al., 2012).

POLYAMINES AS METABOLIC MARKERS OF LONG-TERM ENVIRONMENTAL STRESS IN FOREST TREES

Whereas the topics of correlations between changes in PAs and the response of plants to short term applications of stress have received generous treatment in the literature, the feasibility of using PAs as potential metabolic markers/indicators of environmental stress in plants before the appearance of visual symptoms (e.g., easily measurable growth effects) has received only limited attention. This application is quite relevant to monitoring the health status of perennials in commercial plantations as well as in managed and unmanaged natural forests. Several studies on analysis of foliar metabolites and soluble inorganic ions in mature forest trees have shown a strong positive correlation between PAs (particularly Put) and chronic effects of environmental stress from acid precipitation or excessive N fertilization of soils. The results suggest a potential for developing guidelines to include such biochemical analysis in forest management practices for stress amelioration.

NEED FOR MONITORING THE IMPACT OF ENVIRONMENTAL STRESS ON FOREST TREES

Oxides of sulfur (S) and N, emitted into the environment from industrial processes (e.g., fuel combustion and transportation) react with water to form strong inorganic acids, which make up the major component of acidic deposition (*a.k.a.* "acid rain"). These acids solubilize Ca^{++} from its bound form in the soil, enabling the plant to absorb it easily, thus initially the increase in mobile Ca^{++} may help trees to grow better. However, the acidity also mobilizes aluminum (Al), which does two things: (i) it blocks the uptake of Ca^{++} by roots and (ii) it binds very tightly to soil particles, thereby displacing Ca^{+} from the soil, which eventually leaches from the watershed to surface water bodies (e. g. lakes). The net result is a serious accumulation of soluble N in the inland water bodies (from high N inputs directly from acid rain as well as from the agricultural land and forest runoffs), thus causing algal blooms and harm to other biota. Consequently, some forest soils have become depleted of Ca^{++} to the point where select tree species have developed Ca^{++} deficiency; this has happened in the US (Lawrence et al., 1995; Bailey et al., 1996; Likens et al., 1998; Kobe et al., 2002; Huntington, 2005), Europe (Thimonier et al., 2000; Jönsson et al., 2003) and Asia (Nykvist, 2000). At the same time, N deposition has also reached a level that has either caused or will cause significant harm to the functions and structure of forests (Van Breemen and Van Dijk, 1988;

Nykvist, 2000; Galloway et al., 2004; Pardo et al., 2011). The phenomenon of environmental N deposition exceeding its biological demand in forested watersheds is referred to in the literature as "N saturation" (Aber et al., 1989).

Identification of biochemical and physiological markers (e.g., organic metabolites and inorganic ions) in plants whose concentrations change in response to a single or multiple stressor(s) in a predictable (and stable) manner can be useful in monitoring the status of stress response and recovery due to stress ameliorating treatments. Only a handful of metabolic markers linked to specific functions have been identified in plants growing under chronic stress conditions. Phytochelatins are one example of such markers for heavy metal exposure. To be of practical value in assessing community/forest health, the biochemical markers should have the following characteristics: (a) the cost to develop and test them should be reasonable, (b) they should be relevant to ecosystem function that is under study, (c) they should be sensitive and dose responsive so that change in their cellular content should be higher in magnitude relative to "normal" background fluctuations, (d) they should maintain a longer-term new homeostatic level under persistent stress conditions such as those found in forests, (e) their concentration should revert to normal range when the stress inducer is removed from the environment, and (f) they should have broad applicability over temporal and spatial ranges (Gârban et al., 2005); http://www.esd.ornl.gov/programs/bioindicators/typesandcharacteristics.htm; http://www.fda.gov/ohrms/dockets/ac/01/briefing/3798b1_04_holt/sld005.htm. The PAs seem to have many of these characteristics; therefore, we have proposed to use them as biochemical markers of N saturation stress and Ca depletion/Al accumulation in the Northeastern US forests based on analysis of easily accessible foliage.

POLYAMINES AS BIOCHEMICAL MARKERS FOR STRESS

The signs of environmental stress in trees often develop slowly, i.e., in comparison with those from insect or disease damage. For example, visual symptoms of drought stress or nutrient deficiencies may take several years to appear. Unfortunately, once the symptoms of stress and damage become apparent it is often too late to stop or reverse the decline in forest productivity. As mentioned above, it is often difficult to diagnose the source of stress on trees because multiple factors work together (e.g., Al toxicity, N saturation, nutrient deficiencies, ice or wind storm etc.) to cause the decline in forest productivity over a period of many years. Moreover, different tree species have different tolerance limits for each type of stress. Thus, a comprehensive analysis of the growing environment in an ecosystem (soil, water, animal and plant physiology, and above- and below-ground biomass, including microbes) by multidisciplinary teams that include physiologists, ecologists, pathologists, microbiologists, biogeochemists, and hydrologists must be carried out to assess the complete situation. The joint data collected concurrently would help us to develop links between tree function and environmental disturbances, and risk assessment and stress remediation strategies for forest trees prior to the onset of obvious decline. In this regard, based on studies spanning over multiple years in several ecosystems, including some National Science Foundation (NSF) funded Long-Term Ecological Research (LTER) Sites (www.lternet.edu/lter-sites) within US, we have found PAs (especially Put), along with some of the related metabolites (e.g., GABA and Pro) and inorganic ions to be reliable markers of plant health under conditions of environmental stress or soil nutrient deficiency in forest trees. Their contents in plant tissues like foliage in forest trees show strong correlations with a variety of abiotic stress conditions before the damage due to these conditions is visible.

In our attempts to test the utility of these metabolites as biochemical markers for abiotic stress, our studies have involved multiple sites in the Northeastern US that were either chronically impacted by environmental acid precipitation due to NOx and SOx or experimentally fertilized with N (NH_4NO_3) to simulate the effects of chronic N addition to the soil (Minocha et al., 1997, 2000; Bauer et al., 2004). Fertilization with Ca was also conducted to study the amelioration of Ca^{++} deficiency symptoms (Wargo et al., 2002; Juice et al., 2006; Minocha et al., 2010). One such study involved six red spruce (*Picea rubens* Sarg.) stands in three NE US states, which had suffered long-term exposure to acidic deposition from industrial sources, and where the soil solution pH in the organic soil horizon (Oa) was <4.0. The presence of a large number of dead and dying trees at some of the sites indicated that they were apparently under some form of environmental stress. Analysis of soil chemistry and PAs in needles from apparently healthy trees over a 2-year period revealed: (i) a strong positive correlation between Ca^{++} and Mg^{++} in the needles and in the Oa horizon of the soil; and (ii) that needles from trees growing on relatively Ca^{++}-poor soils with a high exchangeable Al concentration in the soil solution had significantly higher concentrations of Put than those growing on Ca^{++}-rich soils with a low exchangeable Al concentration (Minocha et al., 1997). The magnitude of change in Put was several-fold higher than for Spd or Spm. Putrescine concentration in 1 year-old needles always positively correlated with exchangeable Al ($r^2 = 0.73$, $p \leq 0.05$) and soil solution Al: Ca^{++} ratios ($r^2 = 0.91$, $p \leq 0.01$) of the Oa soil horizon. The study revealed that foliar Put concentration could be used as a reliable biochemical marker for early detection of stress due to soil Ca^{++}-deficiency in natural forests before the appearance of any visual symptoms of stress damage.

In another study at the Delaware River Basin NY, USA along a N deposition gradient (from aerial NOx), foliar Put concentration in sugar maple (*Acer saccharum*) increased with the elevation which was accompanied by rise in ambient level of N deposition. Accompanying changes in soil chemistry included decrease in soil Ca^{++} with increasing N (Ross et al., 2004). Additional evidence for the importance of Ca^{++} in controlling foliar Put came from an experimental addition of Ca^{++} to a large watershed (11.7 ha) at Hubbard Brook Experimental Forest NH, USA. In this case, amelioration of soil Ca^{++} improved the overall health of sugar maple trees and increased seedling regeneration, which was accompanied by a concomitant decrease in foliar Put content. The data further revealed that changes in Put were species-specific in that increased Put indicated stress effect only in species that showed Ca^{++} deficiency in regard to the ambient levels of Ca^{++} in the soil. For example, sugar maple, which is known to be more sensitive to Ca^{++} deficiency showed bigger changes in Put relative to yellow birch (*Betula alleghaniensis*) that was less sensitive to

existing soil Ca^{++} levels (Minocha et al., 2010). An earlier study at an un-glaciated forest at the Allegheny Plateau, PA, USA aimed at identifying the factors contributing to sugar maple decline involved a one-time treatment with Ca^{++} to a Ca^{++}-depleted stand, which also showed improved tree vigor and growth (Wargo et al., 2002). It is surmised that increased foliar Put could possibly substitute for some of the functions of Ca^{++} within the cells as suggested by Minocha et al. (1997).

Long-term (>10 years) studies on chronic N fertilization [NH_4NO_3 and $(NH_4)_2SO_4$] at Harvard Forest MA, USA (HF) and Bear Brook Watershed ME, USA (BBWM), where N fertilization began in 1989, have shown that N-amended pine (*Pinus strobus*) and hardwood (red maple - *Acer rubrum* and red oak - *Quercus rubra*) stands at HF and mixed-wood (red spruce - *Picea rubens*, American beech - *Fagus grandifolia* and sugar maple) stand at BBWM contained higher concentrations of PAs and amino acids in their foliage (Minocha et al., 2000; Bauer et al., 2004) and R. Minocha et al. unpublished data. The changes at both sites were also species-specific and depended on land use history of the sites that had affected their soil N status. There also was a concomitant increase in foliar N from the uptake of ammonia and changes in foliar base elements that were associated with soil base cation losses and nitrate leaching (Aber et al., 2003; Fernandez et al., 2003; Aber and Magill, 2004; Elvir et al., 2005, 2006; Fernandez and Norton, 2010). Data collected over 15 years revealed that N saturation in the pine stand had increased the death of trees at HF. However, in the hardwood stand within half a mile of the pine stand, while maple trees did not survive the chronic N deposition and drought stress of 1998, oak showed no visual symptoms of stress until 2002 (Magill et al., 2000, 2004). Changes in the aboveground tree productivity of both stands was accompanied by changes in the belowground (i.e., soil) fungal and microbial biomass at the HF site (Frey et al., 2004; Wallenstein et al., 2006). In both soil horizons (organic and mineral) at the N-amended hardwood stand, significant rearrangements in bacterial community structure were observed after 20 years of annual N treatment (Turlapati et al., 2013). Biochemical analysis of foliage revealed that Put concentrations were reflective of N storage in most species, and in some, the increase was additive from the response to Ca^{++} deficiency caused by soil nutrient leaching.

In addition to using Put as a biochemical marker of stress, we also found it to be a useful marker to follow the recovery of forest trees from catastrophic stress-causing events like fires, ice damage and silvicultural thinning practices at different sites within the Northeastern and Western regions of the US (Minocha et al., 2013) and R. Minocha et al. unpublished data. Studies were also conducted to demonstrate that PAs played a role in providing cold tolerance to red spruce (Schaberg et al., 2011). These studies further showed that Put content reverted back to normal homeostatic levels upon amelioration of the stress factor(s). The possible diversion of excess N into the production of N-rich metabolites such as PAs and amino acids (Pro, Arg, GABA, and Glu) as reported for pine stand at HF has also been seen at other N treated sites, e.g., a bog population of three ericaceous shrubs (*Vaccinium myrtilloides*, *Ledum groenlandicum*, and *Chamaedaphne calyculata*) at Ottawa, Canada (Bubier et al., 2011) indicating that N flux through this part of the pathway works in a coordinated manner. Our studies are consistent with the results of several other groups who have reported coordinated changes in Put and Arg concentrations in the foliage of forest trees growing under environmental stress conditions (Dohmen et al., 1990; Santerre et al., 1990; Ericsson et al., 1993, 1995; Näsholm et al., 1994, 1998).

CONCLUSIONS AND FUTURE PROSPECTS

As much as it is apparent that plants with high PA contents (due to exogenous supply or endogenous production via genetic manipulation) can tolerate short term exposure to a multitude of stress factors, only a handful of studies on the survival and yield (fresh or dry biomass of usable product) in these plants under prolonged stress conditions or repeated exposure to the same stress, have been reported. Most importantly, no viable plant variety has yet been created or selected based upon genetic modification of PAs either via breeding or via transgene expression, which could be evaluated in comparison with other varieties showing similar characteristics. It must also be pointed out that a similar situation exists with respect to a plethora of other genetic manipulation approaches that have been shown to be effective in imparting short-term stress tolerance in various plant species. It is expected that the advanced high through-put techniques of genomics, transcriptomics and proteomics, coupled with better techniques of monitoring the live plants under stress and their metabolic status (the metabolome), would provide a better holistic picture of the consequences of up-regulation or down-regulation of genes likely to be involved in stress tolerance in relation to metabolites like PAs. The ability to identify unique or common regulatory nodes of metabolic pathways, and the cross-talk among the different pathways that are affected by genetic manipulation of PA metabolism, will provide us effective targets to genetically engineer plants that are tolerant to different abiotic stresses. The best-case scenario for such genetic manipulation will be that PA metabolism can be controlled in a transient and cell/tissue/organ specific manner in response to the earliest perception of stress exposure before the stress reaches its peak to cause damage. This would generate plants, which will produce additional PAs to protect themselves from stress only when needed without significant alterations in PA and amino acids metabolism under normal growth conditions.

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Polyamines control of cation transport across plant membranes: implications for ion homeostasis and abiotic stress signaling

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Polyamines are unique polycationic metabolites, controlling a variety of vital functions in plants, including growth and stress responses. Over the last two decades a bulk of data was accumulated providing explicit evidence that polyamines play an essential role in regulating plant membrane transport. The most straightforward example is a blockage of the two major vacuolar cation channels, namely slow (SV) and fast (FV) activating ones, by the micromolar concentrations of polyamines. This effect is direct and fully reversible, with a potency descending in a sequence $Spm^{4+} > Spd^{3+} > Put^{2+}$. On the contrary, effects of polyamines on the plasma membrane (PM) cation and K^+-selective channels are hardly dependent on polyamine species, display a relatively low affinity, and are likely to be indirect. Polyamines also affect vacuolar and PM H^+ pumps and Ca^{2+} pump of the PM. On the other hand, catabolization of polyamines generates H_2O_2 and other reactive oxygen species (ROS), including hydroxyl radicals. Export of polyamines to the apoplast and their oxidation there by available amine oxidases results in the induction of a novel ion conductance and confers Ca^{2+} influx across the PM. This mechanism, initially established for plant responses to pathogen attack (including a hypersensitive response), has been recently shown to mediate plant responses to a variety of abiotic stresses. In this review we summarize the effects of polyamines and their catabolites on cation transport in plants and discuss the implications of these effects for ion homeostasis, signaling, and plant adaptive responses to environment.

Keywords: cytosolic calcium, ion channels, ion pumps, plasma membrane, polyamines, reactive oxygen species, stress, vacuole

INTRODUCTION

Polyamines (PAs) are acknowledged regulators of plant growth, development, and stress responses. In the model plant *Arabidopsis*, changes in the expression of different enzymes of the PAs biosynthesis and respective levels of individual PAs are stress-specific, and these changes mediate stress tolerance (Alcázar et al., 2010); same may be true also for other plants (see below). Polyamines at physiological pH are polycations, bearing from 2 (putrescine, Put) to 4 (spermine, Spm or thersmospermine, tSpm) positive charges. Thus, PAs can stabilize membranes or nucleic acids, binding to their negative surfaces (Galston and Sawhney, 1990; Kusano et al., 2008). They can also act as a source of reactive oxygen species (ROS) but also as ROS scavengers and activators of key of antioxidant enzymes (Kusano et al., 2008; Moschou et al., 2008; Pottosin et al., 2014a). In animal cells PAs affect a variety of plasma membrane (PM) cation channels, acting primarily as pore blockers but in some cases also affecting the channel gating and/or regulation by extra- and intracellular ligands (Drouin and Hermann, 1994; Lopatin et al., 1994; Williams, 1997; Lu and Ding, 1999; Huang and Moczydlowski, 2001; Xie et al., 2005; Ahern et al., 2006). PAs effects on vacuolar channels in plants were revealed and partly reviewed (Pottosin and Muñiz, 2002), but the accumulated experimental evidence for the PAs effects on the plant PM ion channels and pumps was not properly discussed until now. This review is aimed to fill this gap and provide a comprehensive overview on our current knowledge of PA control over cation transport across plant membranes, and its implications for ion homeostasis, signaling, and plant adaptive responses to environment.

STRESS-INDUCED CHANGES IN POLYAMINE CONTENT AND THEIR ROLE IN PLANT ADAPTIVE RESPONSES TO ENVIRONMENT

Polyamine levels are strongly modulated by literally every known abiotic factor (see **Table 1**, for selected examples), often reaching the millimolar level (such as for putrescine; Galston and Sawhney, 1990; Alcázar et al., 2006) under stress conditions. The current consensus is that, rather than being merely collateral effect of stress-induced metabolic changes, these changes are beneficial to plant performance upon stress (Alcázar et al., 2010; Gupta et al., 2013) and therefore represent an important component of plant adaptive mechanisms. Three major lines of evidence support this claim, which can be illustrated for salinity and drought stresses—two key abiotic stresses affecting agricultural crop production

Table 1 | Stress-induced changes in the level of free polyamines in plants.

Species	Stress	Put	Spd	Spm	References
Pine	Drought	Up	Up	Up	De Diego et al., 2013
Pepper	Drought	Up[a]	Up[a]	Up[a]	Sziderics et al., 2010
Rice	Drought	Down	Up	Up	Yang et al., 2007
Wheat	Osmotic	Up	Up	Up	Grzesiak et al., 2013
Potato	Osmotic	Down	Down	Down	Li et al., 2005
Bromus	Osmotic	Down	Down	Down	Gicquiaud et al., 2002
Cucumber	Flooding	Up	Up	Up	Shi et al., 2008
Citrus	Flooding	Up	Up[b]	Up[b]	Arbona et al., 2008
Tobacco	Flooding	Up	Up	Steady	Hurng et al., 1994
Rice	Anoxia	Up	Steady or up	Steady or up	Reggiani et al., 1989
Arabidopsis	Heat	Up	Up	Up	Sagor et al., 2013
Tobacco	Heat	Up	Up	Up	Cvikrova et al., 2012
Wheat	Heat	Down	Up	Up	Goyal and Asthir, 2010
Wheat	Cold	Up	Up	Up	Kovacs et al., 2010
Maize	Cold	Up	Steady	Down	Nemeth et al., 2002
Wheat	CO_2	Down	Up	Up	Högy et al., 2010
Common sage	UV	Up	Up	Up	Radyukina et al., 2010
Scots pine	K^+ deficit	Up	Steady	Steady	Sarjala, 1996
Arabidopsis	K^+ deficit	Up	Steady	Steady	Watson and Malmberg, 1996

[a] *In leaves but not root tissues;* [b] *in sensitive genotype only.*

around the world. First, externally applied PAs ameliorate stress symptoms. At salt stress, the beneficial effects are due to improved ionic (K^+/Na^+) relations (Zhu et al., 2006; Ndayiragije and Lutts, 2007; Roychoudhury et al., 2011; Sharma et al., 2013b) and increased antioxidant activity, both enzymatic (Ozturk and Demir, 2003; Tang and Newton, 2005; Chai et al., 2010) and non-enzymatic, e.g., via proline accumulation (Su and Bai, 2008; Sharma et al., 2013b). Similarly to salinity, PAs improved drought tolerance due to the activation of antioxidant systems, both enzymatic (Kubiś, 2008) and non-enzymatic (such as proline, anthocyanins, and soluble phenolics; Farooq et al., 2009). This reduces the amount of ROS produced (Farooq et al., 2010) and stabilizes membrane structures (Kubiś, 2006). Under natural conditions, PA may also play a beneficial role in mycorrhizal development, contributing to improved plant water status and water use efficiency (Wu et al., 2010). Second, in many cases accumulation of PAs positively correlates with salt (Chattopadhayay et al., 2002; Liu et al., 2006; Mutlu and Bozcuk, 2007) and drought (van der Mescht et al., 1998; Liu et al., 2005), resistance. Third, and maybe the strongest line of evidence came from the experiments with the gain- or loss-of-the-function mutants. Whereas overexpression of enzymes of the PA biosynthesis like arginine decarboxylase, ADC (Roy and Wu, 2001), S-adenosylmethionine synthetase, SAMDC (Waie and Rajam, 2003; Qi et al., 2010), and spermidine synthase, SPDS (Neily et al., 2011) resulted in improved salt tolerance, the loss-of-function mutant of PA biosynthesis genes resulted in reduced stress tolerance in several species (Fariduddin et al., 2013). In Arabidopsis, loss-of-the-function mutants in the synthesis of Spm and thermospermine accumulated more Na^+ and performed worse than wild type in survival experiments (Alet et al., 2012). Similarly, the introduction of SAMDC gene led to increased polyamine biosynthesis and increased drought tolerance in tobacco (Waie and Rajam, 2003) and rice (Li and Chen, 2000; Peremarti et al., 2009). Over-expression of ADC in Arabidopsis resulted in a transgenic line with enhanced resistance to high osmoticum, dehydration, and long-term drought (Wang et al., 2011). Transgenic Arabidopsis plants displayed a reduced transpiration rate and stomata conductance, hence, a lesser water loss (Alcázar et al., 2010). Conversely, Arabidopsis mutant plants not capable of producing Spm were hypersensitive to drought (Yamaguchi et al., 2007).

CONTROVERSIES AND INCONSISTENCIES

While a large body of evidence exists suggesting that changes in PA level and plant adaptive responses to environment are positively correlated, negative, or no correlation were reported as well. No correlation between PA content and drought tolerance was found among contrasting rice cultivars (Do et al., 2013) or even higher PAs levels were reported for drought-sensitive chickpea and beans varieties compared with their tolerant counterparts (Juhasz et al., 1997; Nayyar et al., 2005). Although PAs treated rice plants showed improved K^+/Na^+ ratio in shoots, PAs did not protect them against salt. Exogenously applied putrescine (Put) induced a decrease in the shoot water content in the presence of NaCl, while spermidine (Spd) and spermine (Spm) were detrimental for cell membrane stability (Ndayiragije and Lutts, 2006). Low-PA lines of maize appeared to be tolerant to salt stress while high-PA lines were more sensitive (Zacchini et al., 1997). Negative correlation between changes in Put content and salinity stress tolerance was reported in experiments comparing different plants (Zapata et al., 2007). Within six selected species, the most tolerant ones accumulate less Put compared with salt-sensitive ones (Zapata et al., 2008). Thus, it appear the concept "the more PA the better for stress tolerance" does not always held.

To support the above statement, we have tabulated some selected evidence for NaCl-induced changes in the level of free PA in root and leaf tissues of various species (**Table 2**). Of 23 papers cited, eight reported an increase in the level of all three PA; seven have reported a significant reduction in all PA levels; and eight showed mixed results (e.g., an increase in the level of one specific PA accompanied by the concurrent decrease in the level of another PA). Obviously, aspects such as time- and concentration-dependence of PA synthesis and metabolism, its tissue- and organelle-specificity, and inter-conversion between various types and forms of PA, all should be not ignored.

Plant adaptive responses to environment are closely and ultimately related to their ability to control intracellular ion homeostasis and regulate ion transport across cellular membrane (Shabala, 2012). Different tissues show different patterns of ion accumulation, with dicots and monocots sometimes displaying contrasting patterns for a distribution of a certain ion (e.g., Na^+) between different tissues. Understanding of the relative ion accumulation and tissue-specific expression of ion channels and transporters has just started to emerge (Karley et al., 2000; Volkov et al., 2003; Conn and Gilliham, 2010; Gilliham et al., 2011). Plant membranes host hundreds of transport proteins that comprises of ∼5% of the entire Arabidopsis genome (Mäser et al., 2001). Some of them are known to be strongly affected by PAs, and PAs can also exert contrasting effects on the same individual ion transporter via diverse mechanisms of action (see below). Thus, the causal role of PA in plant adaptive responses to environment may be established only in the strict context of the tissue- and organelle-specificity.

Table 2 | Selected examples of the changes in the level of free polyamines induced by salt stress in plant root and leaf tissues.

Species	Put	Spd	Spm	References
Cucumber	Up	Up	Up	Fan et al., 2013
Bean	Down	Down	Down	Shevyakova et al., 2013
Pea	Up	Up	Up	Piterkova et al., 2012
Plantago	Down	Down	Down	Radyukina et al., 2009
Mesembryanthemum	Up	Up	Up	Shevyakova et al., 2006
Mesembryanthemum	Down	Down	Up	Stetsenko et al., 2009
Chickpea	Down	Down	Down	Nayyar et al., 2005
Maize	Up	Up	Up	Rodríguez et al., 2009
Apple	Down	Steady	Down	Liu et al., 2008
Grape	Up	Up	Up	Upreti and Murti, 2010
Bromus	Down	Down	Down	Gicquiaud et al., 2002
Tomato	Up	Up	Up	Botella et al., 2000
Tomato	Down	Down	Down	Aziz et al., 1999
Barley	Up	Up	Up	Zhao et al., 2003
Jojoba	Down	Down	Up	Roussos and Pontikis, 2007
Ginseng	Down	Up	Up	Parvin et al., 2012
Wheat	Down	Up	Up	Reggiani et al., 1994
Lupin	Up	Up	Steady	Legocka and Kluk, 2005
Sunflower	Down	Down	Up	Mutlu and Bozcuk, 2007
Soybean	Down	Down	Steady	Xing et al., 2007
Quinoa	Down	Steady	Up	Ruiz-Carrasco et al., 2011
Sunflower	Down	Down	Up	Mutlu and Bozcuk, 2005
Lettuce	Down	Up	Up	Zapata et al., 2003

POLYAMINES EFFECTS ON THE VACUOLAR CATION TRANSPORT

Slow (SV) and fast (FV) vacuolar channels are non-selective cation channels that are ubiquitously and abundantly expressed in higher plant vacuoles (Hedrich et al., 1988; Pottosin and Muñiz, 2002; Hedrich and Marten, 2011; Pottosin and Dobrovinskaya, 2014). SV channels are encoded by the two-pore cation (TPC1) gene (Peiter et al., 2005), whereas the molecular identity of FV channels is still elusive. Both channels conduct a variety of small monovalent cations with a little preference, but SV channels also conduct alkali earth cations like Ca^{2+} and Mg^{2+} (Amodeo et al., 1994; Brüggemann et al., 1999a; Pottosin et al., 2001; Pottosin and Dobrovinskaya, 2014). SV and FV channels only weakly differentiate between K^+ and Na^+; this also holds for the case of halophyte plants (Bonales-Alatorre et al., 2013). SV channels are activated by the increase in the cytosolic Ca^{2+}, and, with a lower affinity, by Mg^{2+} (Hedrich and Neher, 1987; Ward and Schroeder, 1994; Pottosin et al., 1997; Carpaneto et al., 2001). At the same time, FV channels are inhibited by the micromolar cytosolic Ca^{2+} and Mg^{2+} (Tikhonova et al., 1997; Brüggemann et al., 1999b; Pei et al., 1999). Therefore, one may propose that the contribution of FV and SV currents into the overall tonoplast cation conductance, among other factors, maybe regulated by the cytosolic Ca^{2+}. In this model, FV channels are more active at the resting Ca^{2+} levels, whereas SV channels require a very substantial cytosolic Ca^{2+} increase for their activation.

In animal cells, several K^+ and cation channels, sensitive to Mg^{2+}, are also sensitive to PAs (Williams, 1997). Similarly, Mg^{2+}-sensitive FV channels were efficiently blocked by micromolar concentrations of Spm and Spd, and by millimolar concentrations of Put (Brüggemann et al., 1998; Dobrovinskaya et al., 1999a). The blockage occurred instantaneously, was dose- but not voltage-dependent, and fully reversible (see **Figure 1** and **Table 3**, for a quantitative description).

SV channels possess a wide pore, with about 0.7 nm diameter in the narrowest constriction (Pottosin and Schönknecht, 2007). Therefore, it is not surprising that PAs act as permeable pore blockers (Dobrovinskaya et al., 1999a,b). This blockage of SV channels is qualitatively very similar to PA blockage of sodium- (Huang and Moczydlowski, 2001), cyclic nucleotide-gated- (Guo and Lu, 2000), and ryanodine- or acetylcholine-receptor channels (Uehara et al., 1996; Haghighi and Cooper, 1998) in animal cells. The block of SV channels was voltage-dependent, with the affinity increasing at increased positive potential at the side of the PAs application. At higher potentials, however, a relief of the block due to a "punchthrough" phenomenon was observed, when PAs were forced to cross the entire channel pore by a strong electric field. Comparison of the voltage-dependence of the block from cytosolic and vacuolar sides displayed several interesting features. First, it appears that PAs reached a common docking point from either membrane side. Second, electric and physical distance from the pore entrance to this stop point was approximately three times longer from the cytosolic side as compared to that from the vacuolar side. Consequently, the cytosolic part of the pore can adopt single Spm or two Put molecules at a time, whereas the vacuolar part can contain a single blocking molecule at a time, regardless of whether it is Spm, Put or a quaternary ammonium ion

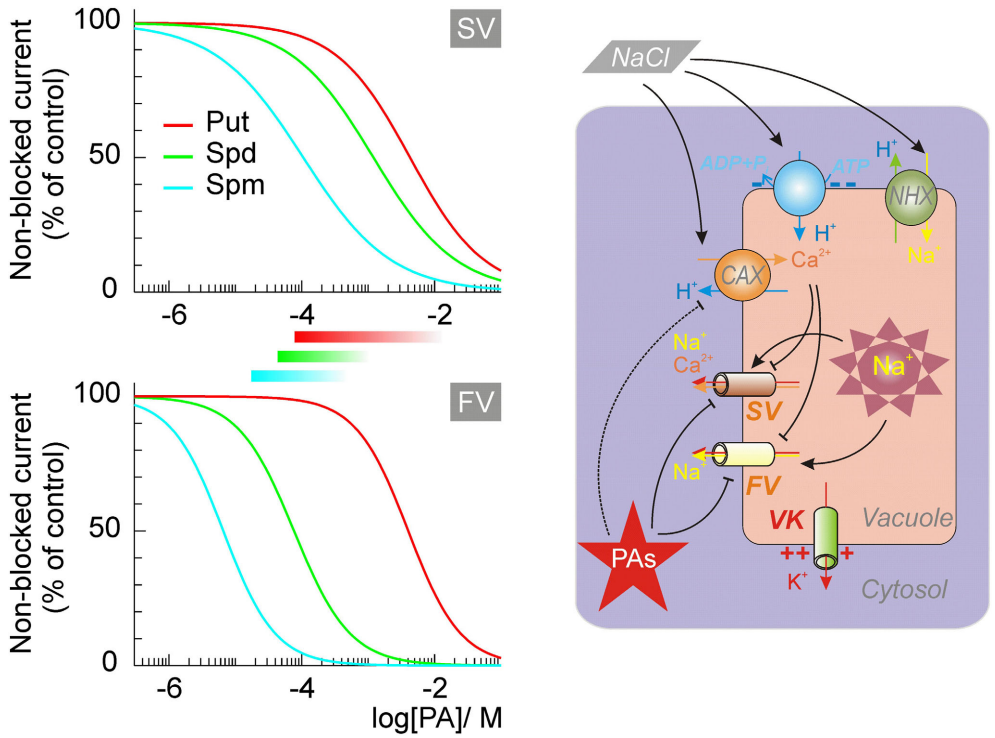

FIGURE 1 | The dose-dependence of the vacuolar cation channels' block by intracellular polyamines and its implications for the salt stress resistance. Dose dependence for SV and FV channels at physiologically attainable (zero) tonoplast potential are drawn, using the values of blocking parameters from Brüggemann et al. (1998) and Dobrovinskaya et al. (1999a,b). Approximate ranges for intracellular PAs in plant cells are indicated by bars. At high salinity, efficient vacuolar Na^+ sequestration is critical for the salt tolerance. This requires the increased Na^+/H^+ antiport activity and a decrease of Na^+ leaks through non-selective FV and SV cation channels. The block by PAs would abolish the FV-mediated current, and strongly suppress the SV current. Continuous operation of the VK, weakly sensitive to PAs, acts as a shunt conductance for the electrogenic H^+-pump, which fuels the active Na^+ uptake, and contributes to the recuperation of the salt-induced cytosolic K^+ loss. Salt stress stimulates expression of the cation-H^+ antiporters, which may reduce the FV and SV activity via the increase of the luminal Ca^{2+}. Over-expression of CAXs is also caused by the inhibition of the Spm^{4+} synthesis. Thus, PAs and vacuolar Ca^{2+} may act as alternative regulators of vacuolar cation channels.

(Dobrovinskaya et al., 1999b). At physiological (~0 mV) tonoplast potential Spm and Spd block was approximately 10-fold weaker than that of the FV channel, but the affinity of both channels for Put was fairly comparable (**Figure 1**, **Table 3**).

Tonoplast also possesses highly K^+-selective channels (VK), belonging to so called two-pore K^+ channels family, TPK (Gobert et al., 2007; Isayenkov et al., 2010). These channels are very abundant in guard cells, where they play an important role in mediation of the vacuolar K^+ release (Ward and Schroeder, 1994). TPK channels may be also found in other tissues (Pottosin et al., 2003; Gobert et al., 2007). TPK is only weakly sensitive to Spm and Spd (K_d ~1 mM) and practically insensitive to the Put (Hamamoto et al., 2008). Thus, accumulation of PAs during salt stress would primarily inhibit the activity of non-selective cation channels, increasing the overall tonoplast K^+/Na^+ selectivity and assisting the efficient vacuolar Na^+ sequestration (**Figure 1**).

Effects of PAs on other vacuolar ion transporters are less explored. *Arabidopsis* mutant, lacking the Spm- and tSpm-synthases, consequently has zero Spm and tSpm levels. This mutant shows generally non-altered phenotype under normal growing conditions, except a reduced stem growth (dwarfism) due to the lack of tSpm (Imai et al., 2004). Yet it was hypersensitive to high NaCl and KCl, but not to the equivalent osmotic stress or high $MgCl_2$ (Yamaguchi et al., 2006). These mutants also have shown symptoms of the Ca^{2+}-deficiency, similar to plants overexpressing vacuolar cation/H^+ exchangers (CAX). Indeed, transgenic Spm-deficient plants have shown overexpression of several vacuolar CAXs (**Figure 1**) but same levels of expression of components of the SOS signaling cascade, responsible for the vacuolar Na^+ sequestration. Causal relations between Spm, CAX-expression, and Ca^{2+} signaling during salt stress remain to be elucidated. Interestingly, whereas Put and Spd but not Spm were essential for the normal growth of *Arabidopsis*, in the case of growth under stress conditions it was just the other way around (Kusano et al., 2008). High ratio of polyamines to diamines positively correlated with a higher activity of vacuolar H^+-ATPase and PPase as well as with a higher level of phospholipids and lower level of galactolipids in the tonoplast under salt stress (Sun et al., 2002; Liu et al., 2006). In addition to the interaction of PAs with tonoplast phospholipids, binding of PAs to the tonoplast correlated with a higher activity of the V-type H^+-ATPase and vacuolar Na^+/H^+ exchanger, conferring salt tolerance (Zhao and Qin, 2004). Already mentioned tSpm appears to have specific roles in the stem elongation, preventing premature cell death of

Table 3 | Summary of polyamine effects on plant ion channels and pumps.

Channel or pump	Mechanism of the PA action	References
VACUOLAR CHANNELS		
SV (TPC1): slow vacuolar (two-pore cation) Ca^{2+}-permeable channel	Direct, reversible. Voltage-dependent block from either membrane side Spm (50 μM) > Spd (500 μM) > Put (3 mM)*	Dobrovinskaya et al., 1999a,b
FV: fast vacuolar monovalent cation channel	Direct, reversible. Voltage-independent block from the cytosolic side Spm (6 μM) > Spd (80 μM) >> Put (4 mM)*	Brüggemann et al., 1998; Dobrovinskaya et al., 1999a
VK (TPK1): vacuolar K^+ (two-pore K^+)	Direct (?) Voltage-independent, cytosolic side Spm ~ Spd (~1 mM) > >Put **	Hamamoto et al., 2008
PLASMA MEMBRANE CHANNELS		
KIRC: inward rectifying K^+ channel -Guard cells (KAT1)	Indirect, cytosolic side, V-independent Spm ~ Spd ~ Put (0.5–1 mM)**	Liu et al., 2000
-Roots (AKT1)	Indirect, extracellular side, V-independent Spm ~ Spd (~1.5 mM) > Put**	Zhao et al., 2007; Zepeda Jazo, 2010
KORC (GORK): outward rectifying K^+ channel	Indirect, extracellular side, V-independent Spm ~ Put (~1 mM)**	Zepeda Jazo, 2010
VI-NSCC: voltage-independent cation channel -roots	Extracellular side, V-independent Spm ~ Spd (~0.4 mM) > Put**	Zhao et al., 2007
-leaves	Extracellular side (indirect?) Spm ~ Put (~0.4 mM)**	Shabala et al., 2007a,b
ROSIC: weakly voltage-dependent, OH•-induced non-selective conductance	Extracellular PAs act as cofactors for ROSIC activation by OH• Spm ~ Spd ~ Put (1 mM)	Zepeda-Jazo et al., 2011; Pottosin et al., 2012; Velarde-Buendía et al., 2012
PLASMA MEMBRANE P-TYPE ATPASES		
ACA: autoinhibited Ca^{2+}-ATPAse	Rapid activation of Ca^{2+}-pumping Spm ~ Put (0.1–1 mM)	Bose et al., 2011; Zepeda-Jazo et al., 2011; Pottosin et al., 2012; Velarde-Buendía et al., 2012
	Long-term potentiation	Sudha and Ravishankar, 2003
AHA: autoinhibited H^+-ATPAse	Rapid activation (coupled to Ca^{2+} pump) Put (1 mM) Rapid activation and/or inhibition (0.1 or mM Spm)	Velarde-Buendía, 2013
	Inhibition of the H^+ pumping; Spm > Spd ~ Put (~1 mM)	Pandolfi et al., 2010
	Activation Spm ~ Spd ~ Put (~1 mM)**	Reggiani et al., 1992; Garufi et al., 2007
	Activation via 14-3-3 proteins binding (Spm only, ~0.1 mM)**	
	Long-term suppression, lower expression	Janicka-Russak et al., 2010

*Numbers in brackets are apparent K_d from the data, presented in **Figure 1**.*

***Numbers in brackets are approximated concentrations of PAs, producing the half-effect.*

When not marked, simply tested experimental concentrations are given.

developing xylem elements (Kakehi et al., 2008; Takahashi and Kakehi, 2010). On the contrary, the maturation of the xylem elements is achieved via PAs exodus to the apoplast and catabolization therein; released H_2O_2 caused coordinated stiffening of cell walls as well as programmed cell death (PCD) of xylem elements, due to the induction of a Ca^{2+}-permeable conductance in their PM (Tisi et al., 2011; see also the section devoted to PAs and ROS cross-talks below).

At the same time, inhibitory effects of PAs on the vacuolar H^+-pump activity were also reported (Tang and Newton, 2005; Janicka-Russak et al., 2010). Interestingly, the steady state H^+ pumping was decreased by PAs, but the V-H^+-ATPase activity was not significantly affected (Janicka-Russak et al., 2010). This may imply that PAs act as uncouplers. In addition, transport of uncharged PAs across the membrane and their protonation–deprotonation reactions may affect the pH buffering capacity in an acidic compartment and eventually affect the H^+-ATPase or ATP-synthase activity, as it was shown for the F-type H^+-ATPase in thylakoids (Ioannidis et al., 2006).

MODULATION OF PLASMA MEMBRANE POTASSIUM AND NON-SELECTIVE CHANNELS BY POLYAMINES

In animal cells, PAs cause a strongly voltage-dependent block of the inward-rectifying (Kir) K^+ channels from the intracellular side; in fact, blockage by PAs is the main cause of the channel inward rectification (Lopatin et al., 1994; Kurata et al., 2010). Inward-rectifying K^+ channels (KIRC) in plants are not related to Kir animal channels and belong to the *Shaker* family, which in animals encode only depolarization-activated K^+ channels (see Sharma et al., 2013a, for a review). It is not surprising, therefore,

that the mechanisms of action of PAs on KIRC may differ from those on Kir. Liu et al. (2000) found that Spm, Spd, and Put, with a little preference, have inhibited KIRC in the guard cell membrane of *Vicia faba* (**Table 3**). The same work also reported that these PA were also efficient in inhibiting the major component of inward K^+ current, encoded by KAT1 channel, in a heterologic system. The effect of PAs was voltage-independent and showed the same dose-dependence as inhibition of stomata movements. *In planta* measurements revealed that under drought conditions Spd level increased to levels above 1 mM, whereas Put and Spm levels were lower and practically unchanged. This data was interpreted as the evidence for Spd-induced stomata closure to reduce water loss under stress conditions. Importantly, Spd was only efficient from the interior of the guard cell. Yet, when Spd was added at the cytosolic side of small excised membrane patches, no effect on the single channel activity was observed (Liu et al., 2000). Thus, Spd effect on the KIRC was most likely *indirect* and mediated by some unknown intracellular factor or signaling pathway. On contrary, KIRC in barley roots was only affected by PAs from the *extracellular* side (Zhao et al., 2007). In addition to KIRC, the outward-rectifying K^+ channel (KORC) was inhibited indiscriminately by Put or Spm (**Table 3**). These channels are widely present in root cortex and epidermis and encoded (in Arabidopsis) by the GORK gene (Mäser et al., 2001). It should be noted that GORK channel in *Vicia faba* guard cells was unaffected by PAs (Liu et al., 2000). Taken together with a great variability of the PA effects on KORC (e.g., an order of magnitude difference between samples; an occasional but not compulsory reversibility of inhibition) observed in our experiments, it is plausible to suggest that PAs effects on plant *Shaker* K^+ channels are indirect and can be mediated by different factors, present in the apoplast and/or in the cytosol.

In addition to K^+ channels, plants express a variety of non-selective cation currents in the PM (see Demidchik and Maathuis, 2007, for a review). The most common voltage-independent non-selective cation current (VI-NSCC) is almost equally permeable for K^+ and Na^+, as well as to divalent cations (Ca^{2+}). This current is instantaneous and only weakly voltage-dependent (Demidchik and Tester, 2002). In addition to instantaneous currents, mixed non-selective currents with instantaneous and time-dependent outward-rectifying components can be frequently recorded. The time-dependent but not the instantaneous components were sensitive to (inhibited by) external Na^+. Thus, it was proposed that VI-NSCC in roots and leaves are major mediators of the toxic Na^+ influx (Shabala et al., 2006). Na^+-permeable VI-NSCC in roots (Zhao et al., 2007) and leaves (Shabala et al., 2007a,b) were inhibited by externally applied PAs (**Table 3**). Effect of PAs on the VI-NSCC, albeit reversible, developed slowly (several minutes). There are two possible explanations: (1) PAs acted from the cytosolic side, and their uptake into the cell required a substantial time or (2) PAs effect on the VI-NSCC was indirect. As Zhao et al. (2007) did not found any significant effect of PAs from the cytosolic side on the VI-NSCC, the second possibility seems to be more plausible. Relatively high active concentrations of external PAs raise the question of the physiological significance of their effects on the PM ion channels. However, available data imply that more than half of tissue PAs is associated with the apoplast (Pistocchi et al., 1988 and references therein). Together with high (up to millimolar) levels of PAs, reached at stress conditions, is justifies a relatively high threshold for the PAs effect on the PM channels.

One of the important determinants of the salt sensitivity mechanism is K^+ loss from plant tissues, caused by the PM depolarization due to the influx of Na^+ (Shabala and Cuin, 2008). In barley the Na^+-induced K^+ efflux is a main cause of the salt sensitivity and a better control of the membrane potential against the depolarization challenge under salinity is crucial for the tolerance (Chen et al., 2007). In pea mesophyll, externally applied PAs not only inhibited the VI-NSCC, but reduced the salt-induced membrane depolarization and associated loss of K^+ (Shabala et al., 2007a,b). Generalizing this idea, Zepeda-Jazo et al. (2008) proposed a simple model, where PAs inhibition of any NSCC active at depolarized potentials will reduce the membrane depolarization and the loss of K^+ via GORK and NSCC. GORK inhibition by PAs can further reduce the K^+ efflux. A prediction of this hypothesis for the NaCl-induced K^+ efflux was tested on maize and *Arabidopsis* roots. Indeed, PAs could ameliorate NaCl-induced K^+ efflux in some cases. But, depending on the root zone, growing conditions and PA species, the effect of PAs could be null or even resulted in a strong potentiation of K^+ efflux (Pandolfi et al., 2010). Obviously, PAs or their catabolites, can cause not only inhibition but also an activation of some cation currents in the PM (see below).

Extracellular application of PAs *per se* induced the membrane depolarization (Di Tomaso et al., 1989; Fromm et al., 1997; Ozawa et al., 2010; Pottosin et al., 2014b), thus, potentially affecting *any* electrogenic transport across the PM and generating driving force for the K^+ efflux. Our recent pharmacological analysis of the Spm-induced depolarization in barley roots suggested that it was mainly caused by the uptake of PAs via a specific route, not shared with inorganic ions (Pottosin et al., 2014b). Recent advances in the characterization of PA-uptake transporters in plants (Fujita et al., 2012; Mulangi et al., 2012a,b) can provide important clues for the mechanisms of PAs uptake and its impact on the electrogenesis at the plant PM.

INTERPLAY BETWEEN POLYAMINES AND ROS IN THE CONTROL OF PASSIVE CONDUCTANCE AND PUMPING ATPases OF THE PLASMA MEMBRANE

PAs are well-known ROS scavengers and activators of the antioxidant enzymes (Ha et al., 1998; Das and Misra, 2004; Tang and Newton, 2005; Kubiś, 2008; Gill and Tuteja, 2010). At the same time, PAs catabolization generates H_2O_2, which can be further converted to different ROS, including the most aggressive one, the hydroxyl radical (•OH). Export of intracellular PAs to the apoplast and their oxidation therein by available diamine (DAO) and/or polyamine (PAO) oxidase to generate H_2O_2 (**Figure 2**) is a common signaling pathway segment, which can be found in a variety of plant adaptive and developmental responses. Depending on the strength of the ROS signal generated by the PAs oxidation, very opposite scenarios—e.g., survival vs. PCD—may be realized (see Moschou and Roubelakis-Angelakis, 2014; Pottosin et al., 2014a, for a recent review).

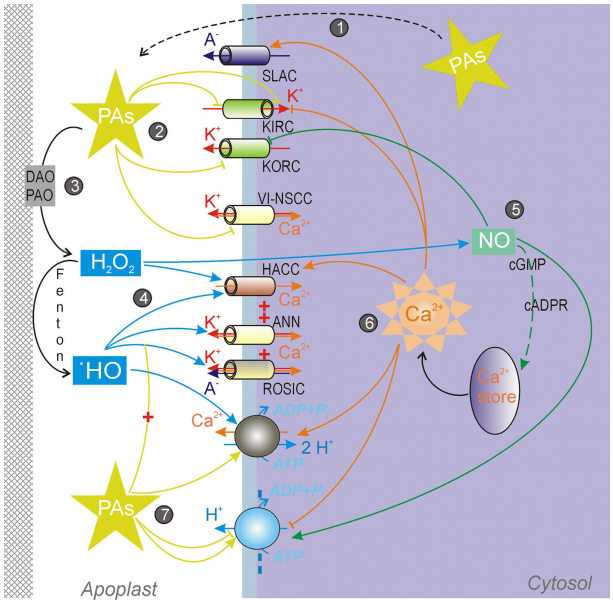

FIGURE 2 | Regulation of ion transport across the plasma membrane by polyamines and their catabolites. (1) PAs are exported from the cytosol to the apoplast against the electrochemical gradient. **(2)** PAs inhibit K^+ (inward-rectifying, KIRC and outward rectifying, KORC) and non-selective voltage-independent cation (VI-NSCC) channels. **(3)** PAs oxidation by diamine (DAO) and/or polyamine (PAO) oxidases generates H_2O_2 in the apoplastic space. There H_2O_2 can be converted to •OH by the single electron reduction catalyzed by transient valency metal ions. **(4)** H_2O_2 and •OH activate a variety of non-selective Ca^{2+}-permeable channels, including hyperpolarization-activated Ca^{2+} influx channel (HACC), annexin-formed channel (ANN), and non-selective voltage-independent conductance (ROSIC). **(5)** H_2O_2, released during PAs catabolization, causes a rapid NO generation. In its turn, NO inhibits KORC by a direct nitrosylation and in1duces the intracellular Ca^{2+} release via a pathway involving cGMP and cyclic adenosine ribose (cADPR). **(6)** Ca^{2+}-sensitive network. Several PM channels, including slow anion channel (SLAC) and pumps, are regulated by cytosolic Ca^{2+}. **(7)** PAs potentiate the ROSIC activation, activate the PM Ca^{2+}-ATPase and alter the activity of the PM H^+-ATPase.

Studies on transgenic *Arabidopsis* plants, overexpressing enzymes of the Put and Spm biosynthesis, revealed cross-talks between PAs and expression of Ca^{2+}-signaling genes, implying a role of PAs in the Ca^{2+} homeostasis and signaling (Marco et al., 2011). A possible link may be via PAs catabolization and ROS-induced Ca^{2+} fluxes. ROS regulate a variety of ion conductances in the PM. Both H_2O_2 and •OH activate hyperpolarization-activated Ca^{2+} influx currents (HACC) in roots and leaves (Pei et al., 2000; Demidchik et al., 2003, 2007). It appears that properties of HACC, activated by H_2O_2 and •OH, are slightly different, despite sharing some characteristics with constitutive HACC. These channels are usually active at non-physiologically large negative potentials (Demidchik and Maathuis, 2007). The presence of the distinct HACC populations, differently responding to H_2O_2 and •OH, is manifested by the fact that in the root mature zone HACC are responding only to •OH, whereas in the distal elongation zone both H_2O_2 and •OH could induce the Ca^{2+} influx (Demidchik et al., 2007). •OH can activate a variety of conductances, which not only mediate Ca^{2+} influx, but also cation (K^+) efflux (**Figure 2**). One of such conductances is mediated by annexin1. It displays both outward and inward rectifying time-dependent components, mediating K^+ efflux and Ca^{2+} influx, respectively (Laohavisit et al., 2012). On the other hand, Demidchik et al. (2010) provided the evidence for the •OH-activation of GORK channels, mediating TEA-sensitive K^+ efflux in *Arabidopsis* roots. However, in pea roots •OH generation caused a rapid cessation of the constitutive GORK (Zepeda-Jazo et al., 2011). Instead, a weakly voltage-dependent instantaneous current, permeable to Ca^{2+}, TEA^+, K^+, and even small anions (Cl^-) developed and reached a steady state after 30 min from the initiation of the •OH treatment. Time-dependent depolarization-activated currents developed much later (after 1 h), and were not studied in detail in this plant model. The instantaneous current was termed ROSIC (for ROS-induced conductance) and mediated by tiny (~1 pS conductance) channels (Pottosin et al., 2014a). Both •OH-induced K^+ efflux in intact pea roots and ROSIC were sensitive to a variety of cation and anion channels blockers (Zepeda-Jazo et al., 2011). ROSIC activation induced membrane depolarization but caused a massive K^+ efflux so that an efflux of anions likely occurred in parallel (Pottosin et al., 2014a,b). A very similar •OH-induced conductance was reported for barley roots (Velarde-Buendía et al., 2012). PAs unexpectedly stimulated ROSIC and •OH-induced K^+ efflux in intact roots, although by themselves they were incapable to induce any K^+ efflux (Zepeda-Jazo et al., 2011; Velarde-Buendía et al., 2012). In pea such potentiation by PAs was restricted to the mature root zone and not observed in the elongation zone (Pottosin et al., 2012). This is consistent with the idea of the presence of distinct ROS-activated channels populations in these zones. Even more strikingly, ROSIC potentiation by PAs was much larger in salt-sensitive barley than in a salt tolerant variety (Velarde-Buendía et al., 2012), again corroborating the idea on the crucial role of the K^+ retention for the salt tolerance (Chen et al., 2007). Importantly, potentiation of the ROSIC by PAs could be demonstrated in isolated root protoplasts, perfused by an artificial intracellular solution, and containing no amine oxidases. Thus, one has to presume that PAs but not their catabolites acted in this case, and that PAs effects on the ROSIC were likely membrane-delimited.

Externally applied PAs also caused a rapid NO generation, which is likely mediated by PAO and DAO with a generation of H_2O_2 upstream to the NO (Tun et al., 2006; Wimalasekera et al., 2011). NO caused the inhibition of GORK due to a direct protein nitrosylation (Sokolovski and Blatt, 2004). NO also caused stomata closure, via a pathway mediated by cGMP and cADPR, and leading to a Ca^{2+} release from an intracellulare store (**Figure 2**; Neill et al., 2002). This Ca^{2+} release causes the inhibition of KIRC and activation of slow anion channels (SLAC), reducing K^+ uptake and stimulating the anion efflux, respectively; NO did not have any direct effect on these channels (García-Mata et al., 2003). Prolonged (4 days) exposure to NO caused also up to three-fold activation of the PM H^+-ATPase (Zandonadi et al., 2010).

Activation of the PM Ca^{2+} permeability by ROS and resulting increases of the intracellular free Ca^{2+} could modulate a variety of ion transport processes across the PM (**Figure 2**). It also exerts a positive feedback regulation on the ROS production by the PM

membrane NADPH-oxidase (Takeda et al., 2008). However, ROS and PAs also affected the Ca^{2+} efflux system, namely the PM Ca^{2+}-ATPase. It was shown that both •OH and PAs activated eosine-sensitive Ca^{2+} pumping in intact roots (Bose et al., 2011; Zepeda-Jazo et al., 2011; Velarde-Buendía et al., 2012; Velarde-Buendía, 2013). In pea roots the threshold for •OH activation of the Ca^{2+} pump was at least by an order of magnitude lower as compared for that for the ROSIC (Zepeda-Jazo et al., 2011). Ca^{2+} efflux induced by •OH in pea roots was transient but could be potentiated by PAs Spm> Spd >Put (Zepeda-Jazo et al., 2011; Pottosin et al., 2012). In the case of Spm, a long-lasting steady state Ca^{2+} efflux was observed, arguing for non-additive effects of •OH and PAs. No such behavior was found in barley roots, where •OH- and PA-effects on Ca^{2+} efflux were roughly additive (Velarde-Buendía et al., 2012). Velarde-Buendía (2013) demonstrated that in pea roots PAs affected H^+ fluxes in a differential manner. Whereas Spm caused a net H^+ influx, Put caused a prolonged vanadate-sensitive H^+ efflux, caused by the H^+-ATPase activation. Spm-induced H^+ influx was consistent with the 1 Ca^{2+}: 2 H^+ exchange mechanism, demonstrated previously (Beffagna et al., 2000). The activation of the H^+-ATPase by Put appeared to be indirect and coupled to the Ca^{2+}-pump activation (Velarde-Buendía, 2013; Pottosin et al., 2014b). As the PM H^+-ATPase is inhibited by cytosolic Ca^{2+} (Kinoshita et al., 1995; Brault et al., 2004), it is logical to propose that a cross-talk between the two pumps is mediated by the intracellular Ca^{2+} changes. Yet a supposed decrease of the intracellular Ca^{2+} by PAs needs to be demonstrated directly in future experiments.

H^+-ATPase operates as a powerhouse, controlling the electric potential difference and the active ion exchange across the PM (Palmgren, 2001). Existing data on immediate effects of PAs on the H^+-ATPase pumping activity are controversial, with both activation (in rice, Reggiani et al., 1992 and wheat, Liu et al., 2005) and inhibition (in maize, Pandolfi et al., 2010) effects reported. Garufi et al. (2007) described a very specific mechanism of the H^+-ATPase activation by intracellular Spm, but not by Spd or Put. Spm promoted the binding of 14-3-3 proteins to the unphosphorylated H^+-ATPase, thus increasing its hydrolytic activity. Long-term treatment with PAs appears to increase the activity of the PM Ca^{2+}-ATPase (Sudha and Ravishankar, 2003) and reduced the magnitude of changes in the H^+-ATPase activity, induced by the salt stress (Sun et al., 2002; Roy et al., 2005; Tang and Newton, 2005). One day incubation with PAs caused a decrease in transcripts of one of the H^+-ATPase isoforms, resulting in substantially decreased H^+ pumping across the PM (Janicka-Russak et al., 2010).

IMPLICATIONS FOR STRESS RESPONSES: CURRENT STAND AND PERSPECTIVE

Global climate change is expected to increase the frequency and severity of drought and flooding events in many regions world-wide (Setter and Waters, 2003; Voesenek and Sasidharan, 2013), severely affecting the crop production. On a global scale, the overall loss in food and fiber production due to abiotic stresses is estimated at US$120 billion p.a. and predicted to increase (http://www.fao.org/docrep/008/y5800e/Y5800E06.htm). Also, global availability of good quality water is also becoming a limiting and increasingly expensive resource, and the cost of irrigation-induced salinity is estimated to exceed US$11 billion p.a. (Shabala, 2013). Thus, understanding the role of PAs in plant adaptive responses to drought, salinity and flooding may be instrumental in breeding crops with improved tolerance to these stresses to overcome the above losses.

DROUGHT STRESS

Multiple mechanisms contribute to plant adaptive responses to drought. The major ones include (Hu and Schmidhalter, 2005; Shabala and Pottosin, 2014): better stomata control and reduced transpiration under drought conditions; efficient osmotic adjustment, and maintenance of the turgor pressure; prevention of the drought-induced accumulation of ROS; improved water use efficiency and control of a long-distance water transport in plants; maintaining optimal energy status; and improved leaf photochemistry via maintenance of the intracellular ionic homeostasis and charge balance. For obvious reasons, each of these mechanisms is intrinsically dependent on membrane transport activity and implies efficient regulation of ion channels and transporters under stress conditions. Indeed, stomatal opening and closure are strongly dependent on the rapid movement of K^+ into and out of the guard cell (Blatt, 2000). As commented above, the ability of PAs to inhibit KIRC in the guard cell membrane of *Vicia faba* (Liu et al., 2000) may be essential to prevent stomatal opening under drought conditions, thus reducing transpirational water losses. Consistent with this notion, Arabidopsis *gork-1* mutant plants lacking functional GORK channels showed much poorer performance under drought stress, due to their inability to close stomata (Hosy et al., 2003). These findings are in a good agreement with the fact that PAs inhibit opening and induce closure of stomata (Liu et al., 2000; Shi et al., 2010). Importantly, KIRC inhibition by the Spd in *Vicia* guard cells occurred only from the cytosolic side (Liu et al., 2000) highlighting the importance of PA compartmentation.

Drought stress also results in a rapid increase in the level of ROS species in plant tissues (Miller et al., 2010). Polyamines may play a dual role in the process. First, PA may play a critical role in drought stress signaling to confer adaptive responses. According to suggested models, drought-induced increase in ABA content may promote PAs accumulation and exodus into the apoplast, where they were oxidized by the apoplastic amine oxidases, producing H_2O_2 to be used in the signaling cascade (Toumi et al., 2010). On the other hand, PAs are known to significantly enhance activity of both enzymatic (Shi et al., 2010; Wang et al., 2011; Radhakrishnan and Lee, 2013) and non-enzymatic (Högy et al., 2010; Asthir et al., 2012) antioxidants. Therefore, the PA control over the balance between ROS production and scavenging may "shape" H_2O_2 signal, conferring differential stress responses between species and genotypes. Again, tissue- and organelle-specificity of PA accumulation is absolutely essential for this process. Also, given the fact that different PAs may have a different potency for H_2O_2 production and ROS scavenging, it is not the absolute quantity but a balance between various PAs that may be critical to determine the cell fate. Consistent with this notion, An et al. (2012) recently showed that the extent of membrane damage

by drought in maize was dependent on Spd+Spm/Put ratio in plant tissues.

Another important signaling component potentially related to changes in PAs levels is the stress-induced Ca^{2+} signature. Stress- or stimuli-induced elevations in cytosolic free Ca^{2+}, $[Ca^{2+}]_{cyt}$, vary in their magnitude, frequency, and shape. These depend on the severity of the stress as well the type of stress experienced, thus creating a unique stress-specific calcium "signature" that is then decoded by signal transduction networks (Bose et al., 2011). The drought stress is not an exception, and transient elevations in cytosolic free Ca^{2+} were reported in response to both hyperosmotic (Ng et al., 2001) and ABA (McAinsh et al., 1997) treatments. As prolonged $[Ca^{2+}]_{cyt}$ elevation is detrimental to normal cell metabolism, the basal $[Ca^{2+}]_{cyt}$ levels must be restored after the signaling process has been completed. This implies involvement of active Ca^{2+} efflux systems, such as PM and endomembrane Ca^{2+}-ATPase pumps and exchangers (Bose et al., 2011). Meanwhile, Ca^{2+} efflux was shown to be induced by •OH and potentiated by PAs in a sequence Spm > Spd > Put (Zepeda-Jazo et al., 2011; Pottosin et al., 2012). Thus, the interplay between tissue-specific ROS and PA production and accumulation may confer the shape of stress-specific Ca^{2+} signatures via the modulation of the Ca^{2+}-ATPase activity.

SALINITY STRESS

Plant salinity stress tolerance is a polygenic trait conferred by a large number of sub-traits; each of these is ultimately related to the regulation of membrane-transport activity and ionic homeostasis. The major traits include (Shabala and Munns, 2012): osmotic adjustment; Na^+ exclusion from uptake by roots; intracellular Na^+ sequestration; K^+ retention in the cytosol; tissue-specific Na^+ sequestration; control of the xylem ion loading; Na^+ removal from the shoot; and oxidative stress tolerance. Many if not all these traits may be causally related to, and controlled by, salinity-induced changes in PA levels in various cells compartments.

Rapid osmotic adjustment is absolutely critical to maintain cell turgor and support expansion growth of roots and shoots under saline conditions. Accumulation of K^+ plays a pivotal role in this process, contributing on average to between 35 and 50% of the cell osmotic potential in crops (Shabala and Pottosin, 2014). At the same time, high intracellular K^+ concentrations are required to determine the cell fate and its transition to the PCD. The loss of potassium has been shown to play a primary role in cell shrinkage, caspase activation, and nuclease activity during apoptosis (one of the forms of PCD) in both mammalian (Hughes and Cidlowski, 1999) and plant (Shabala et al., 2007a,b) systems. In this context, the observation that outward-rectifying K^+ channels in root epidermis were inhibited by Put or Spm (**Table 3**) may be considered as an essential trait enabling K^+ retention in the root and thus contributing to both osmotic adjustment and cell fate determination under saline conditions.

Both changes in the expression levels and activation of existing proteins involved in K^+ transport and sequestration are essential for maintenance in cytosolic K^+ homeostasis under saline conditions. For example, AtCHX17, a member of the CPA2 family of transporters was found to be strongly induced by salinity (Kreps et al., 2002; Cellier et al., 2004), to compensate for NaCl-induced K^+ exodus from the cytosol resulting from salt-induced depolarization of the PM. However, as the transport capacity of high-affinity K^+ transporters is about 3 orders of magnitude lower compared with channels (Shabala and Pottosin, 2014), cytosolic K^+ homeostasis is seriously compromised. Thus, post-translational regulation and modulation of activities of existing channels or transporters by various factors and second messengers (including PAs) is more significant. More details on factors controlling K^+ transport under stress conditions are available in Shabala and Pottosin (2014).

Efficient vacuolar sequestration of the cytotoxic Na^+ is another prominent mechanism conferring salinity tolerance in plants. This sequestration is achieved by mean of the tonoplast Na^+/H^+ antiporters fueled by the vacuolar H^+-ATPase and H^+-PPase pumps (Hasegawa, 2013; Shabala, 2013). In addition, toxic Na^+ ions must be prevented from leaking back into cytosol. Thus, to avoid energy consuming futile Na^+ cycling between cytosol and vacuole and to achieve efficient vacuolar sequestration of toxic Na^+, passive tonoplast Na^+ conductance has to be kept at absolute minimum. This implies a strict and efficient control over Na^+-permeable tonoplast SV and FV channels (Bonales-Alatorre et al., 2013). Each of these channels is PA-sensitive and can be blocked by the physiologically relevant concentrations of PAs (see **Figure 1**). Therefore, salinity stress-induced elevation in PA levels may be essential to enable efficient vacuolar Na^+ sequestration. Consistent with this notion, salt stress inhibited the activity of polyamine synthesizing enzymes (L-arginine decarboxylase and L-ornithine decarboxylase) in glycophyte species of *Vigna radiata* but not in halophyte *P. undulate* (Friedman et al., 1989), thus potentially enabling efficient control over vacuolar Na^+ sequestration in the latter (naturally salt tolerant) species.

Reducing net Na^+ accumulation in the cytosol by controlling the rate of its transport across the PM may be another way of improving plant performance under saline conditions. Non-selective cation channels (NSCC) are considered to be a major pathway of Na^+ uptake into the cell (Demidchik and Maathuis, 2007), and physiologically relevant concentrations of PAs were efficient in inhibiting NSCC-mediated Na^+ currents in leaf (Shabala et al., 2007a,b) and root (Zhao et al., 2007; Zepeda-Jazo et al., 2008) tissues.

FLOODING STRESS

Two major factors affect plant growth and performance in flooded soils: reduced oxygen availability leading to a sharp decline in ATP production, and elemental toxicity originating from the changes in the soil redox potential (Shabala, 2011). Acclimation to flooded conditions requires significant metabolic alterations in living cells. This includes reduced energy consumption, activation of pathways that generate ATP without oxidative phosphorylation, and increased chaperone activity to deal with increased ROS production (Bailey-Serres and Voesenek, 2010; Voesenek and Sasidharan, 2013). Polyamines seem to be instrumental in this metabolic adaptation. Accumulation of Put in flooded roots was shown to be able to stimulate the PM ATPase activity (Bertani et al., 1997), conferring beneficial effects to cell ionic homeostasis and nutrient acquisition. Superoxide radical

and H_2O_2 contents were also reduced in flooding-stressed onion plants after Put pre-treatment (Yiu et al., 2009). Jia et al. (2010) showed that application of exogenous Spd to hypoxic cucumber roots or conversion of Put to Spd and Spm enhanced the aerobic respiration but inhibited the fermentation metabolism in roots, leading to an increase in ATP content and alleviation of the stress symptoms.

A massive increase in the amount of available Mn and Fe in the soil solution is observed within a few days of onset of waterlogging, often to above toxic levels (Marschner, 1995; Zeng et al., 2013), due to the changes in the soil redox potential. Being a transition metal, Fe is highly redox active and, in the presence of H_2O_2, can mediate production of the hydroxyl radical through the Fenton reaction (Rodrigo-Moreno et al., 2013). This may cause lipid peroxidation and damage to key cellular structures as well as result in a massive K^+ exodus from the cytosol mediated by •OH-activated K^+-selective outward rectifying (Demidchik et al., 2010) and non-selective K^+ permeable (Zepeda-Jazo et al., 2011) channels. Both these processes are detrimental to cell metabolism and plant performance under stress conditions. Importantly, a regulatory role of PAs in plant adaptation to flooding seems to be closely related to intracellular K^+ homeostasis. In the absence of K^+, anoxia led to a decrease in Put, Spd, and Spm levels. The presence of K^+ ions during the anaerobic treatment abolished the negative effect of anoxia on polyamine titers and slightly increased them (Reggiani et al., 1993).

OUTLOOK

A lesson learned from studies of the PAs effects on plant ion channels is that, contrary to their animal counterparts, a direct pore blockage mechanism is uncommon. A notable exception to that observation is a high affinity block of vacuolar non-selective cation channels of FV and SV types. Because these channels are ubiquitously expressed in plant tissues the model shown in **Figure 1** describing the impact of PAs on vacuolar cation transport, can be considered as a general one. Validation of this working model can be demonstrated by genetic manipulation of SV, FV and VK channels by silencing, or site directed mutations, affecting channels' sensitivity to PAs. While the molecular identities of SV and VK channels are known, that of the FV channel remains cryptic. Completing of this lacking information will allow the manipulation of the overall tonoplast cation transport and eventually will help to understand the role of PAs in its control in plant responses to abiotic stresses.

In most cases, the action spectrum of PAs depends on the side of the membrane/ compartment, whether it is vacuolar lumen, cytosol or apoplast. Unfortunately, PAs compartmentation and membrane transport in plants are poorly explored. In particular, mechanisms of PAs uptake or active efflux across the PM are unknown (Igarashi and Kashiwagi, 2010). This knowledge is very important, however, to understand the exodus of PAs to the apoplast, where they are normally absent. In many cases, it can be proved that rather than PAs themselves, their catabolites (and especially ROS), exert the effect on plant membrane transporters. It is important to mention that the apparent specificity of diamine (Put) vs. PAs (Spd, Spm) effects in this case may be caused by a higher activity of the apoplastic DAO in dicots like Fabaceae or PAO in monocots like Poaceae (Moschou et al., 2008). Overall, the relation between PAs biosynthesis and catabolism (or, in other words, respective levels of PAs and their catabolites) may determine whether survival or PCD responses would be initiated (Moschou and Roubelakis-Angelakis, 2014). This is also applicable to the balance between PAs actions as ROS scavengers and antioxidant system activators, and PAs as a ROS source. In this regard, ROS speciation also becomes crucial. Whereas H_2O_2 is a relatively long-living and easy membrane-permeable molecule, •OH is short-lived and acts in the closest vicinity of the transient valency metal, which catalyzed its generation. A substantial evidence was obtained for differential effects of •OH and H_2O_2 on plant membrane transporters. In particular, a newly described dual cation and anion conductance, ROSIC, is activated only by •OH and PAs further modulate it in species- and tissue-dependent mode.

Apart of recently revealed cross-talks between PAs and ROS, an important link between PAs catabolism and stress response may be the PAs-induced generation of the NO (Wimalasekera et al., 2011), which in turn affects a variety of PM transporters, either directly or via the intracellular Ca^{2+} signal (**Figure 2**). In addition to ROS-activated Ca^{2+} influx channels, both ROS and PAs are capable to activate PM Ca^{2+} pumps. Thus, the fine tuning of Ca^{2+} signal may be achieved, which is worth of further experimental exploration.

It is conceivable that stress-induced changes of PAs and ROS metabolism were adapted for the stress resistance in a rather opportunistic way. Whereas few direct sensors for PAs and ROS evolved, other targets may be indirect and the net effect, e.g., on the K^+ transport across the PM, may be rather variable (Pandolfi et al., 2010). While searching for a solution of the equation with many parameters, one needs to take into the account PAs synthesis, transport, and catabolization. In addition, tissue-, species-, and physiological status-dependent expression of different ion channels and transporters as well as the modes of action of PAs and their catabolites should be also always kept in mind. Despite its complexity, this task is the only possible alternative, as the "spray and pray" strategy seems to be not applicable in the case of polyamines.

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Peroxisomal polyamine oxidase and NADPH-oxidase cross-talk for ROS homeostasis which affects respiration rate in *Arabidopsis thaliana*

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Homeostasis of reactive oxygen species (ROS) in the intracellular compartments is of critical importance as ROS have been linked with nearly all cellular processes and more importantly with diseases and aging. PAs are nitrogenous molecules with an evolutionary conserved role in the regulation of metabolic and energetic status of cells. Recent evidence also suggests that polyamines (PA) are major regulators of ROS homeostasis. In *Arabidopsis* the backconversion of the PAs spermidine (Spd) and spermine to putrescine and Spd, respectively, is catalyzed by two peroxisomal PA oxidases (AtPAO). However, the physiological role of this pathway remains largely elusive. Here we explore the role of peroxisomal PA backconversion and in particular that catalyzed by the highly expressed AtPAO3 in the regulation of ROS homeostasis and mitochondrial respiratory burst. Exogenous PAs exert an NADPH-oxidase dependent stimulation of oxygen consumption, with Spd exerting the strongest effect. This increase is attenuated by treatment with the NADPH-oxidase blocker diphenyleneiodonium iodide (DPI). Loss-of-function of *AtPAO3* gene results to increased NADPH-oxidase-dependent production of superoxide anions ($O_2^{\bullet-}$), but not H_2O_2, which activate the mitochondrial alternative oxidase pathway (AOX). On the contrary, overexpression of *AtPAO3* results to an increased but balanced production of both H_2O_2 and $O_2^{\bullet-}$. These results suggest that the ratio of $O_2^{\bullet-}/H_2O_2$ regulates respiratory chain in mitochondria, with PA-dependent production of $O_2^{\bullet-}$ by NADPH-oxidase tilting the balance of electron transfer chain in favor of the AOX pathway. In addition, AtPAO3 seems to be an important component in the regulating module of ROS homeostasis, while a conserved role for PA backconversion and ROS across kingdoms is discussed.

Keywords: polyamines, NADPH-oxidase, polyamine oxidases, respiration, ROS homeostasis, *Arabidopsis*

INTRODUCTION

Polyamines (PA) are low-molecular mass nitrogenous compounds, and the most abundant ones, across kingdoms, are putrescine (Put), spermidine (Spd), spermine (Spm), and thermospermine (t-Spm); they have been correlated with plethora of biological processes, including protein regulation (Baron and Stasolla, 2008; Takahashi and Kakehi, 2010), development (Wimalasekera et al., 2011; *inter alia*), ion channels (Wu et al., 2010; Zepeda-Jazo et al., 2011), control of nitrogen: carbon balance (Mattoo et al., 2006; for review see Moschou et al., 2012), stress responses (Alcazar et al., 2011b; Marco et al., 2011; Moschou and Roubelakis-Angelakis, 2013) and in particular homeostasis of reactive oxygen species (ROS; Chattopadhyay et al., 2006; *inter alia*).

Polyamines catabolism is mediated mainly by two classes of amine oxidases (AO), the diamine oxidases (DAO) and the PA oxidases (PAO; reviewed in Moschou et al., 2012). In *Arabidopsis*, the AO pathway consists of several, perhaps functionally redundant genes. For example, *Arabidopsis* has at least 10 *DAO* genes (four have been characterized; Møller and McPherson, 1998; Planas-Portell et al., 2013) and five *PAO* genes (*AtPAO1–AtPAO5*, all have been characterized; Ahou et al., 2014). DAOs oxidize Put and cadaverine (Cad), and with much lower affinity, Spd and Spm. The action of DAOs on Put yields pyrroline, H_2O_2, and ammonia (NH^{4+}; Cohen, 1998).

In contrast to DAOs, PAOs oxidize Spd, and Spm but not Put (Angelini et al., 2010). The apoplastic PAO catalyzes the terminal oxidation of PAs, yielding pyrroline and 1-(3-aminopropyl) pyrrollinium from Spd and Spm, respectively, along with 1,3-diaminopropane and H_2O_2. The plant intracellular (cytoplasmic or peroxisomal) PAOs interconvert PAs, producing H_2O_2. Interestingly, they interconvert Spm to Spd and Spd to Put, reversing the PA biosynthetic pathway (Tavladoraki et al., 2006; Kamada-Nobusada et al., 2008; Moschou et al., 2008c; Toumi et al., 2010; Fincato et al., 2012).

Polyamines catabolism has been correlated with numerous processes including cell growth, development, stress responses, and programmed cell death (PCD; Møller and McPherson, 1998; Yoda et al., 2003, 2006; Paschalidis and Roubelakis-Angelakis, 2005a,b; Tisi et al., 2011; Moschou et al., 2012; Moschou and Roubelakis-Angelakis, 2013). We have documented the contribution of tobacco apoplastic PAO (Moschou et al., 2008b) and peroxisomal (Wu et al., 2010) AtPAO3 in H_2O_2 production. The

apoplastic pathway has been mostly correlated with the execution of PCD (Yoda et al., 2003, 2006; Moschou et al., 2008b; Fincato et al., 2012; Moschou and Roubelakis-Angelakis, 2013). The peroxisomal AtPAO3 is critical for the elongation of pollen tube by modulating a plasma membrane H_2O_2-dependent Ca^{2+}-influx channel (Wu et al., 2010). In *Arabidopsis*, PA oxidation is mediated by PAO with diverse specificities and expression patterns (Fincato et al., 2011), thereby regulating ROS levels in a complex manner.

Superoxides ($O_2^{\bullet-}$) and H_2O_2 are the most well studied ROS; they are important players in physiological and pathological processes (Pitzschke et al., 2006; Dikalov et al., 2011; Suzuki et al., 2013). NADPH-oxidase catalyzes the conversion of molecular oxygen to $O_2^{\bullet-}$, and its activation accounts mostly for the large consumption of oxygen that characterizes the respiratory burst in mammalian phagocytic cells (Vignais, 2002). In mammals NADPH-oxidase is composed of membrane-bound and cytosolic proteins. In the center of the NADPH oxidase complex lies the heterodimeric NADPH-binding flavocytochrome b558, consisting of the glycosylated transmembrane protein gp91phox and the non-glycosylated p22phox subunit. Upon activation, the cytosolic proteins p47phox and p67phox become phosphorylated and translocate, together with p40phox and p21rac, to the membrane components, to form the active NADPH-oxidase complex (Segal and Abo, 1993). Plants deficient in gp91phox homologs have compromised responses to stress and have a reduced ability to accumulate ROS. Antisense tomato lines (*Lerboh1*) show reduced ROS accumulation and compromise wound response (Sagi et al., 2004). *Arabidopsis* plants disrupted in the *gp91phox* homologs, the respiratory burst oxidase homolog D (*AtrbohD*) and *AtrbohF*, exhibit reduced ROS production and treatment with the avirulent bacterium *Pseudomonas syringae* pv tomato DC3000 results to cell death (Torres et al., 2005), whereas they have diminished stomatal closure in response to abscissic acid (ABA; Kwak et al., 2003). These data suggest that NADPH-oxidase homologs in plants are important for ROS accumulation.

Another important source of ROS is the mitochondrial electron transport chain (ETC; Muller et al., 2004; Vacca et al., 2004). It consists of four complexes, tightly bound to the intermembrane space of mitochondria. Electrons derived from the tricarboxylic acid (TCA) cycle in the matrix move toward the ETC and in turn pass through the four complexes. Transfer of electrons between complex 3 and 4 of the ETC is facilitated *via* the electron carrier cytochrome-c (cyt-c pathway). The electron motion generates a proton gradient which in turn drives an ATPase. Dysfunction of the mitochondrial ETC leads to the leakage of electrons toward oxygen resulting in the generation of $O_2^{\bullet-}$ (Muller et al., 2004). In order to dissipate the excess electrons, the mitochondria possess another pathway, the alternative pathway, which depends on an alternative terminal oxidase (AOX; Atkin et al., 2002). AOX alleviates mitochondrial ETC from the excess electron load (Yip and Vanlerberghe, 2001).

Previous work from our lab suggested that a regulatory crosstalk between PAs and NADPH-oxidase takes place during tobacco protoplast regeneration (Papadakis and Roubelakis-Angelakis, 2005). PAs seem to be necessary for protoplasts to retain their totipotent state, and prevention of PCD. The interaction between main cellular sources of ROS, such as mitochondria and NADPH-oxidases, however, remains obscure. More importantly, a feed-forward regulation of different ROS sources has been reported (Dikalov et al., 2011). Therefore, the regulatory crosstalk between ROS sources merits further examination.

Here, we report that exogenous PAs stimulate oxygen consumption in *Arabidopsis* in an NADPH-oxidase dependent manner. Plants overexpressing the peroxisomal AtPAO3 show decreased oxygen consumption rate, in strict contrast to loss-of-function *Atpao3* plants which show increased consumption through the AOX pathway. Surprisingly, this increase is attenuated by diphenyleneiodonium iodide (DPI) but not by ascorbate (ASA), suggesting that NADPH-oxidase is upstream of a respiratory increase mediated by AOX. By delving the regulatory function of $O_2^{\bullet-}$ in oxygen consumption rate, we found that *AtPAO3* overexpressing plants show a balanced production of both $O_2^{\bullet-}$ and H_2O_2, while *Atpao3* loss-of-function plants show a high ratio of $O_2^{\bullet-}$ *versus* H_2O_2 production. These data suggest that NADPH-oxidase and AtPAO3 cross-talk for balancing intracellular $O_2^{\bullet-}/H_2O_2$ which in turn affect the cyt-c/AOX pathways.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Arabidopsis thaliana wild type (WT) plants of the ecotype Columbia (Col-0) were used along with transgenic plants overexpressing the peroxisomal *AtPAO3* (S-*AtPAO3*) and *Atpao3* T-DNA loss-of-function insertional mutants, previously described (Moschou et al., 2008c; Wu et al., 2010; Fincato et al., 2011). Plants were grown in a cabinet using an 8/16 h (light/dark) photoperiod and a constant temperature of 23°C. Developing seedlings were transferred to 96-well plates filled with 1/4 strength Murashige and Skoog (MS; Murashige and Skoog, 1962) culture medium. All treatments were carried out by supplementing the culture medium with the corresponding agent. More specifically, the PAs Put, Spd, and Spm were added as aqueous solutions at a final concentration of 1mM. Control plants were mock treated with dH_2O.

POLAROGRAPHIC MEASUREMENT OF RESPIRATORY OXYGEN CONSUMPTION

The rate of oxygen consumption was essentially determined as previously described (Andronis and Roubelakis-Angelakis, 2010). In brief, polarography was performed at 30°C with a Clark type electrode system (Hansatech Instruments, Kings's Lynn, Norfolk, UK), in the presence and absence of the alternative respiratory inhibitor salicylhydroxamic acid (SHAM). Oxygen consumption was measured for a period of 5 min. For inhibitor treatments, leaves were incubated in 15 mM SHAM in 3% (v/v) methanol for a period of 10 min prior to measurement. Control leaves were incubated in dH_2O or 3% (v/v) methanol. In all cases, the rate of oxygen consumption was expressed as per g fresh weight.

NADPH OXIDASE NATIVE PAGE AND ACTIVITY STAINING

Separation of NADPH oxidase isoenzymes and activity staining were carried out according to Carter et al. (2007). *Arabidopsis* leaf

tissue was collected and ground using liquid N_2. The powder was homogenized in a buffer containing 50 mM sodium phosphate, pH 6.8, supplemented with 0.5% (v/v) Triton X-100. 100 μg of protein were separated using native PAGE at 40 mA. Gels were then incubated in 0.5 mg mL^{-1} nitroblue tetrazolium (NBT) in 10 mM Tris, pH 7.4, and 134 mM NADPH until bands were detected.

IN SITU DETECTION OF H_2O_2 AND $O_2^{\bullet-}$

In situ accumulation of H_2O_2 was detected using the method of Thordal-Christensen et al. (1997) and of $O_2^{\bullet-}$ according to Jabs et al. (1996). *Arabidopsis* seedlings were destained using boiling pure ethanol and photographed using a Nikon Coolpix 4500 digital camera.

PROTEIN GEL BLOT ANALYSES AND IN-GEL ACTIVITIES OF APX AND SOD

Protein extractions and gel blots were performed as previously described (Moschou et al., 2013). One hundred mg of leaf material was mixed with 100 μL of urea extraction buffer [4 M urea, 100 mM DTT, and 1% (v/v) Triton X-100] and incubated in ice for 10 min. The samples were boiled with Laemmli sample buffer for 10 min and centrifuged at 13,000 rpm for 15 min. Equal amounts of the supernatants were loaded on 10% (v/v) polyacrylamide gels and blotted on a polyvinylidene difluoride (PVDF) membrane.

For the activity staining of ascorbate peroxidase (APX), 10 mM ASA were added to isoelectric focusing electrophoresis buffer (Rao et al., 1995), and 10% gels were pre-run for 30 min at 20 mA. Subsequently, the gels were incubated in the dark in a solution containing 50 mM potassium phosphate buffer, pH 7.0, and 2 mM ASA; the gels were incubated in the dark for another 30 min in 50 mM potassium phosphate buffer, pH 7.0, 4 mM ASA, and 2 mM H_2O_2. Bands were visualized after the incubation of gels in coloring solution (50 mM potassium phosphate buffer, pH 7.8, 14 mM tetramethylethylenediamine, TEMED; and 1.2 mM NBT). The activity staining of superoxide dismutase (SOD) has been previously described (Beauchamp and Fridovich, 1971).

IMAGE AND STATISTICAL ANALYSIS

The image analysis was performed using ImageJ v 1.41 software[1]. Statistical analysis was performed with SPSS v14[2] or JMP v 9 software[3]. We used Dunett's test with alpha values set at $\alpha = 0.1$.

RESULTS

EXOGENOUS PAs STIMULATE OXYGEN CONSUMPTION RATE

Our previous work established the effect of abiotic stress on the respiratory activity of WT tobacco plants (Andronis and Roubelakis-Angelakis, 2010). Under abiotic stress conditions, cyt-c, an electron carrier located between complexes III and IV of the ETC, dissociates leading to malfunction of the mitochondrial ETC and accumulation of ROS. As a result, the AOX pathway is activated in order to dissipate the excess electrons "leaking" from the ETC. Taking into consideration the link between PAs and plant responses to stresses, we attempted to reveal a potential correlation between PAs and respiratory activity in *Arabidopsis thaliana*.

Two-week old Col-0 *Arabidopsis* seedlings were treated with exogenous Put, Spd, and Spm and oxygen consumption rate was determined 10 min post-treatment. Respiration rate increased in the presence of all PAs used, in terms of oxygen consumption. Spd exerted the strongest effect on the respiration rate, resulting in a 2.6-fold increase compared to the mock treated plants (**Figure 1**). Put and Spm increased respiration, by 1.8- and 2.1-fold, respectively, compared to mock treated plants. These findings revealed an apparent link between PAs and ETC regulation in plants.

STIMULATION OF OXYGEN CONSUMPTION RATE DEPENDS ON PA-INDUCED GENERATION OF $O_2^{\bullet-}$

Previously, we have shown that exogenous application of Spd to tobacco plants leads to a significant increase in H_2O_2 content generated by the action of PAO (Moschou et al., 2008b). A plausible hypothesis could be that PAO-dependent ROS production is a component of the pathway which is responsible for the observed increase of the oxygen consumption rate. To test this hypothesis, we firstly examined whether exogenous Spd could induce H_2O_2 and $O_2^{\bullet-}$ accumulation in *Arabidopsis*. To this end, we employed *in situ* detection protocols of H_2O_2 and $O_2^{\bullet-}$. Indeed, a 10 min treatment with exogenous Spd (1 mM) led to a significant accumulation of H_2O_2, as a result of PA oxidation. The use of 1 mM Spd was based on our previous findings that this concentration is enough to enter peroxisomes and be backconverted to Put in *Arabidopsis* (Fincato et al., 2011). Surprisingly, we found a significant increase in $O_2^{\bullet-}$ content, as well (**Figures 2A,B**). From the aforementioned it is evident that the increase in plant respiration coincided with elevated H_2O_2 and/or $O_2^{\bullet-}$ in treated plants.

To prove the link between the produced ROS and increased respiration, we tested whether quenching of ROS would alleviate the

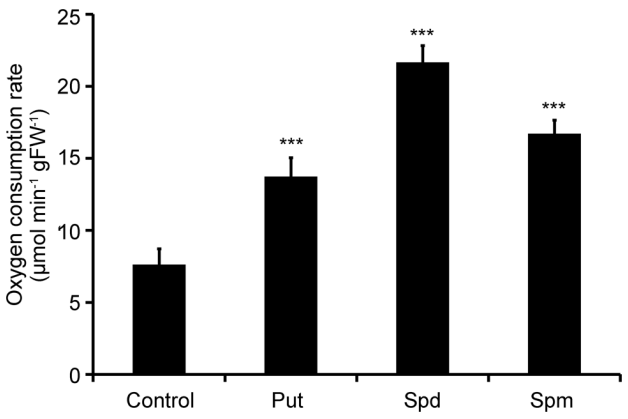

FIGURE 1 | Effect of exogenous polyamines on oxygen consumption rate in *Arabidopsis*. *Arabidopsis* Col-0 seedlings were treated with 1 mM Put, Spd, or Spm for a period of 10 min, and oxygen consumption rate was estimated by a Clark-type electrode. Data are the means of three independent experiments ±SD. Asterisks indicate statistical significant differences (***$P < 0.001$).

[1]http://rsb.info.nih.gov/ij/index.html

[2]www.spss.com

[3]www.jmp.com

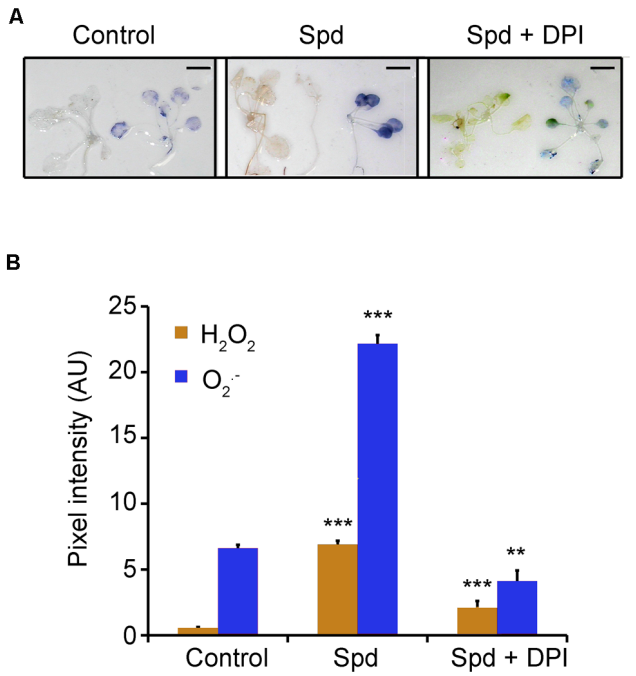

FIGURE 2 | Effect of exogenous Spd and DPI on ROS homeostasis in *Arabidopsis* plants. (A) Plants were treated with 1 mM Spd for a period of 10 min and H_2O_2 or $O_2^{\bullet-}$ were estimated by *in situ* detection methods. Scale bars, 2.8 cm. **(B)** Relative pixel intensity of the brownish (detection of H_2O_2) or bluish (detection of $O_2^{\bullet-}$) adduct formed after application of 1 mM Spd. AU, arbitrary units. Data are the means of three independent experiments ±SD. Asterisks indicate statistical significant differences (***$P < 0.001$; **$P < 0.01$).

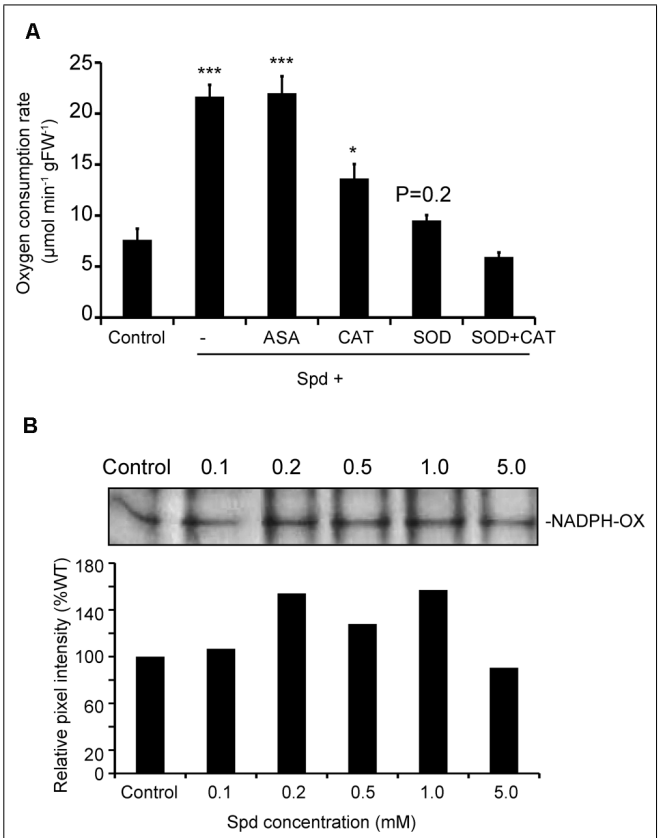

FIGURE 3 | Combined effect of exogenous Spd and ASA, CAT or SOD on oxygen consumption rate of *Arabidopsis* plants and dose-dependent response of NADPH-oxidase activity by Spd. **(A)** Oxygen consumption rate in plants incubated in the respective medium for 10 min. Data are the means of three independent experiments ±SD. Asterisks indicate statistical significant differences (***$P < 0.001$; *$P < 0.05$). For Spd+SOD treatment P value is indicated. **(B)** Effect of exogenous Spd on NADPH-oxidase activity in Col-0 *Arabidopsis* plants. Plants were treated with 0.1, 0.2, 0.5, 1, and 5 mM Spd for a period of 10 min and relative pixel intensity of lane profile after application of 1 mM Spd was assessed.

effect of Spd on respiration. Spd was added in combination with ASA, a scavenger of $O_2^{\bullet-}$ and H_2O_2, catalase (CAT), a scavenger of H_2O_2 and SOD, a scavenger of $O_2^{\bullet-}$. Addition of ASA to the medium failed to produce a significant effect on Spd-induced respiration, whereas CAT led to a 37% decrease over the rate found in Spd treatments (**Figure 3A**). The addition of SOD to the Spd-containing medium alleviated the effect of Spd to a greater extent leading to an overall reduction of 57% over the Spd treated plants, to a rate similar to that determined for the untreated plants (control). Finally, treatment with both, SOD and CAT in addition to Spd further reduced the respiration rate, rendering it lower than that determined in the untreated plants. These results suggest that PA-dependent ROS and particularly $O_2^{\bullet-}$ are required for induction of the increased oxygen consumption rate.

Previous work from our laboratory established the role of PAs and NADPH-oxidase in the developmental fate of isolated protoplasts (Papadakis and Roubelakis-Angelakis, 2005). Considering that Spd led to a more significant increase of $O_2^{\bullet-}$, than of H_2O_2 we hypothesized that $O_2^{\bullet-}$ is produced *via* the activation of the NADPH- oxidase. Indeed simultaneous treatment of Col-0 *Arabidopsis* plants with Spd and the NADPH-oxidase blocker DPI led to a significant reduction in both H_2O_2 and $O_2^{\bullet-}$ in the treated plants (**Figures 2A,B**), providing strong evidence for the participation of the NADPH-oxidase in the Spd-induced ROS accumulation.

So far there are strong indications that there is interplay between Spd and NADPH- oxidase in the generation of ROS which induces enhancement of respiration in *Arabidopsis*. To further verify this result we studied the effect of Spd as a dose-response on NADPH-oxidase activity in Col-0 *Arabidopsis* plants. Treatment with a range of Spd concentrations resulted to a dose-dependent effect in the increase of NADPH-oxidase activity up to a saturation point, and decreased thereafter as indicated by an in gel enzymatic assay (**Figure 3B**). Lower concentrations of Spd (0.1, 0.2, and 1 mM) increased NADPH-oxidase activity compared with the control plants, whereas higher concentration (5 mM and above) decreased NADPH-oxidase activity. These results suggest that low concentrations of Spd can induce NADPH-oxidase.

AtPAO3 REGULATES THE BALANCE BETWEEN H_2O_2 AND $O_2^{\bullet-}$

Recent data have shed light on the biochemical role of plant intracellular PAOs, which interconvert Spm to Spd and Spd

to Put, reversing the PA biosynthetic pathway (Fincato et al., 2011; Ahou et al., 2014). We have previously shown that plants overexpressing *AtPAO3* efficiently oxidize Spd to Put producing H_2O_2 (Moschou et al., 2008c; Wu et al., 2010; Fincato et al., 2011). S-*AtPAO3* plants overexpressing the peroxisomal *AtPAO3* and loss-of-function *Atpao3* seem to be valuable tools in the study of PA-induced respiration in *Arabidopsis* due to the localization of AtPAO3 in the peroxisomes which are in proximity to mitochondria. Indeed, *in situ* detection of ROS in the S-*AtPAO3 Arabidopsis* plants showed that they exhibited a higher but balanced production of H_2O_2 and $O_2^{\bullet-}$ compared with Col-0 plants (**Figure 4A**). In contrast, *Atpao3* plants accumulated significantly lower levels of H_2O_2 when compared with Col-0 plants, but increased levels of $O_2^{\bullet-}$. Therefore, S-*AtPAO3* and *Atpao3* plants are good models for studying the differential effects of H_2O_2 and $O_2^{\bullet-}$ in oxygen consumption.

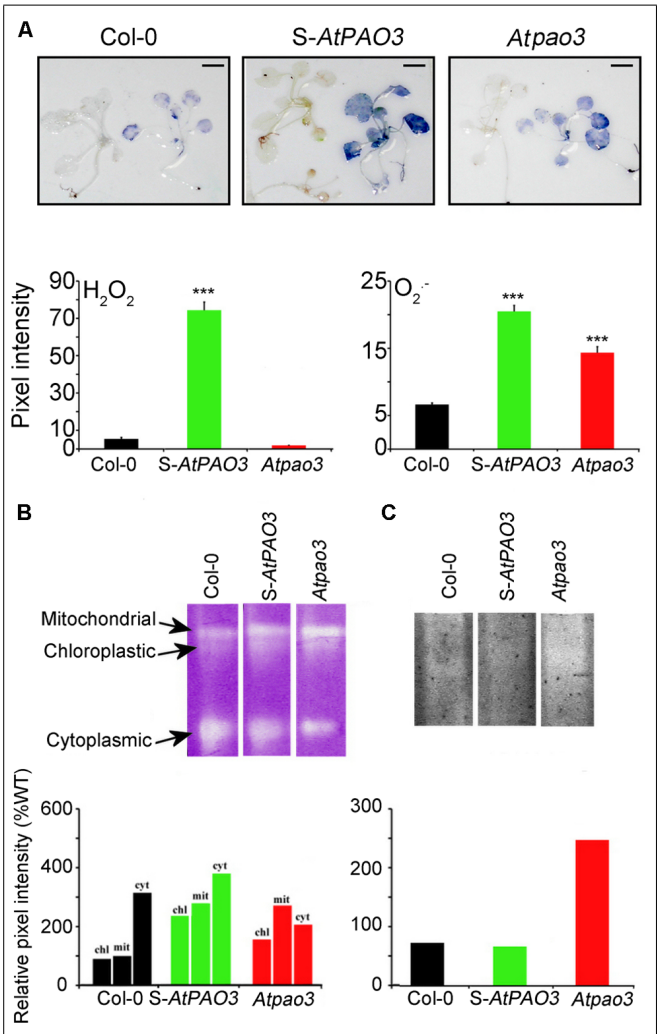

FIGURE 4 | *In situ* ROS in WT, S-*AtPAO3*, and *Atpao3 Arabidopsis* plants. (A) *In situ* ROS detection in WT, S-*AtPAO3* and *Atpao3* plants. Data are from a single representative experiment, repeated three times, and densitometric analysis. Data are the means of three different positions on a leave. Asterisks indicate statistical significant differences from the Col-0 (***$P < 0.001$). Scale bars, 2.8 cm. **(B)** Native electrophoresis and activity staining of SOD and densitometric analysis of isoenzymes. **(C)** Native electrophoresis and activity staining of APX and densitometric analysis.

Next, we examined a possible contribution of the antioxidant machinery in the observed differences in ROS levels in S-*AtPAO3* and *Atpao3* plants. We determined the contribution of SOD and APX, a well-established H_2O_2 scavenger, in the observed differences, between Col-0, S-*AtPAO3* and *Atpao3* in H_2O_2 and $O_2^{\bullet-}$ levels. Accumulation of $O_2^{\bullet-}$ in S-*AtPAO3* and *Atpao3* plants coincided with increased SOD activity in mitochondria and chloroplasts (**Figure 4B**). In contrast, APX was elevated in the *Atpao3*, but reduced in S-*AtPAO3* plants (**Figure 4C**). APX has a high affinity for H_2O_2 and therefore is downregulated at higher levels of H_2O_2 (Asada, 1992), which perhaps can explain the decrease of APX observed in S-*AtPAO3* plants, with increased H_2O_2 levels. These results suggest that at least APX and SOD mirror the changes of ROS levels observed in S-*AtPAO3* and *Atpao3*.

DEREGULATION OF AtPAO3 RESULTS TO CHANGES IN OXYGEN CONSUMPTION RATE

Overall the data presented so far support the role of Spd as an inducer of mitochondrial respiration *via* the NADPH-oxidase generated $O_2^{\bullet-}$ in Col-0 plants. This response is alleviated mostly by the action of the NADPH-oxidase blocker, DPI. Furthermore, loss-of-function mutant plants for the peroxisomal *AtPAO3* gene accumulate $O_2^{\bullet-}$, but not H_2O_2 in contrast to S-*AtPAO3* overexpressing plants, which accumulate both $O_2^{\bullet-}$ and H_2O_2. Considering the above, we determined the oxygen consumption rate in the S-*AtPAO3* transgenics and the *Atpao3* mutants to test whether the differential accumulation of ROS in the two genotypes leads to altered oxygen consumption. Indeed, the three tested genotypes exhibited notable differences in their capacity to consume oxygen (**Figure 5A**). The *Atpao3* plants exhibited the highest rate of oxygen consumption among the tested plants, showing a 2.7-fold increase over the Col-0 plants and a 4.3-fold increase over the S-*AtPAO3* plants. Interestingly, the increase in oxygen consumption of *Atpao3* plants resembled the effect of exogenous Spd in Col-0 plants.

Next, we examined whether the increase in oxygen consumption of *Atpao3* plants is $O_2^{\bullet-}$-dependent. To test this, we treated *Atpao3* with DPI, and as a control, we treated Col-0 plants simultaneously with Spd and DPI (**Figure 5A**). Treatment of *Atpao3* plants with DPI reduced the respiratory activity to the control levels, suggesting that increase of oxygen consumption in *Atpao3* plants depends on the production of $O_2^{\bullet-}$ by NADPH-oxidase. Similarly, Spd plus DPI treated Col-0 plants showed similar oxygen consumption rate to the untreated Col-0 plants, producing an effect equivalent to the DPI-induced decrease in the $O_2^{\bullet-}$ accumulation reported earlier.

We hypothesized that the increase observed in the *Atpao3* plants could be due to an increased contribution of the AOX pathway. To test this, we used the AOX pathway blocker SHAM. Indeed, application of SHAM to the *Atpao3* plants exerted a dramatic decrease in the oxygen consumption rate, suggesting the participation of the AOX pathway in the $O_2^{\bullet-}$-induced oxygen consumption (**Figure 5A**). In addition, the levels of the immunoreactive AOX protein in *Atpao3* were significantly higher compared to the rest

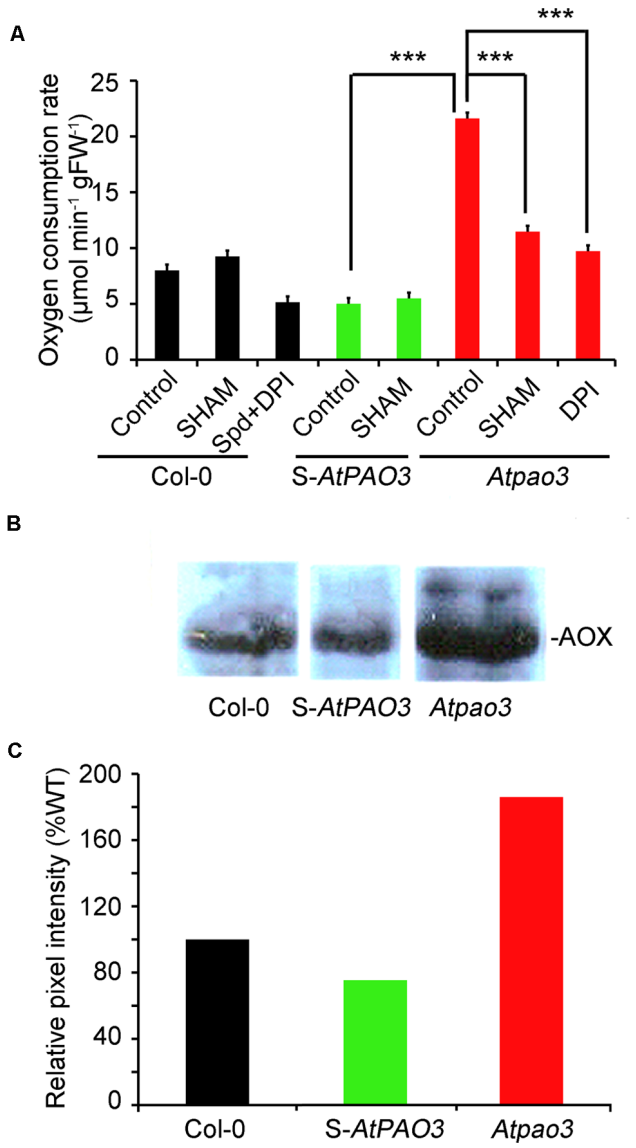

FIGURE 5 | Oxygen consumption rate in Col-0, S-*AtPAO3* and *Atpao3* *Arabidopsis* plants. (A) Oxygen consumption rate in Col-0, S-*AtPAO3* and *Atpao3* plants. Rate of oxygen consumption was determined in untreated plants and plants treated with the AOX blocker SHAM, exogenous Spd and DPI. Data are the means of three different positions on a leave. Asterisks indicate statistical significant differences from the S-*AtPAO3* control (***$P < 0.001$). **(B)** Western blot of AOX immunoreactive protein levels in Col-0, S-*AtPAO3* and *Atpao3* plants. Data are from a single representative experiment, repeated three times. **(C)** Densitometric analysis of AOX immunoreactive protein in **(B)**. Data are from a single representative experiment, repeated three times.

of the tested plants (**Figures 5B,C**). These results suggest that the $O_2^{\bullet-}$-dependent increase of oxygen consumption in *Atpao3* plants is exerted through the AOX pathway.

DISCUSSION

Exogenous Spm application to tobacco plants leads to mitochondrial dysfunction and to transcriptional activation of the AOX pathway, while small molecular weight antioxidants efficiently attenuate this induction, suggesting a possible involvement of ROS in this Spm-signaling pathway (Takahashi et al., 2003). A long standing notion proposed that PA oxidation results in induction of signaling cascades through H_2O_2, since H_2O_2 is a direct product of PA oxidation (Moschou et al., 2008b). However, recent evidence suggests that other ROS types may as well contribute to PA-dependent signaling cascades and that the PAs-ROS crosstalk stretches beyond H_2O_2 (Zepeda-Jazo et al., 2011; Velarde-Buendia et al., 2012).

In this work, application of exogenous PAs to Col-0 *Arabidopsis* plants stimulated the oxygen consumption rate; the stronger effect was exerted by Spd. Exogenous application of Spd is expected to increase H_2O_2 content through the PA oxidation/backconvertion pathway (Yoda et al., 2003; Moschou et al., 2008b). Therefore, we hypothesized that this product could be responsible for the increase in oxygen consumption. Surprisingly, H_2O_2 scavenging did not significantly attenuate the PA-dependent oxygen increase. On the other hand, exogenous SOD or DPI ameliorated the oxygen increase, caused by the exogenous Spd application. This denotes that the increase in oxygen consumption relies on $O_2^{\bullet-}$ production, and suggests that exogenous PA induces $O_2^{\bullet-}$ production by NADPH-oxidase intriguingly, DPI exerts a stronger effect than SOD. This may be due to the fact that SOD is expected to scavenge intercellular $O_2^{\bullet-}$ produced by NADPH-oxidase, giving rise to H_2O_2, while DPI is a plasma membrane permeable suicidal NADPH-oxidase inhibitor, that could efficiently prevent production of higher amount of $O_2^{\bullet-}$. Surprisingly, CAT mimicked the DPI effect. Nevertheless, it should be noted that recent evidence suggests that CAT participates in the induction of cell death (Hackenberg et al., 2013), therefore perplexing the interpretation of the data obtained with the use of CAT. ASA, on the other hand, did not alleviate oxygen consumption increase. It should be noted that ASA is a reducing agent, thereby directly affecting the ETC, and scavenges both $O_2^{\bullet-}$ and H_2O_2 (Foyer and Noctor, 2011). These results suggest that the PA-derived oxygen consumption increase depends mostly on $O_2^{\bullet-}$ production by the PA-induced NADPH-oxidase.

Exogenous PA at relatively high concentrations stimulated production of $O_2^{\bullet-}$ in human neutrophils (Guarnieri et al., 1987). In our study, exogenous application of Spd in *Arabidopsis* increased the content of H_2O_2 produced through PAO, as previously suggested for other plant species (Yoda et al., 2006; Wu et al., 2010; Moschou and Roubelakis-Angelakis, 2011; Tisi et al., 2011). Nevertheless, exogenous Spd induced a significant increase in the levels of $O_2^{\bullet-}$ along with an increase in NADPH-oxidase activity. Interestingly, previous studies suggested that Spd induces autophagy (self-consumption) in non-plant models and is an important surveillance mechanism that rather restricts ROS production (Eisenberg et al., 2009). In accordance, during protoplast isolation from tobacco (Papadakis and Roubelakis-Angelakis, 2005), induction of NADPH-oxidase and the concomitant production of $O_2^{\bullet-}$ was highly suppressed by PAs. This discrepancy between our current and previous work could be due to the fact that in Papadakis and Roubelakis-Angelakis paper, PAs were added to the protoplasts and then NADPH-oxidase was purified

and assayed. On the contrary, in this study PAs were added exogenously and NADPH-oxidase assessment was performed by an in-gel assay omitting further purification steps. This allows us to hypothesize that exogenous PAs may antagonize for Ca^{2+} in NADPH-oxidase preparations. Calcium is required for NADPH-oxidase activation. Therefore, we assume that NADPH-oxidase cannot accommodate Ca^{2+} in the presence of PAs on its binding. In addition, in the previous study $O_2^{\bullet-}$ content was determined by a chemiluminescence assay, while in this work we used an *in situ* detection protocol, which seems to be a more accurate and reliable method (Song et al., 2006). Therefore, PAs and especially Spd seem to positively affect NADPH-oxidase *in planta*, unlike in *in vitro* systems. In addition, a species-related differential effect on NADPH-oxidase of PA cannot be ruled out (tobacco versus *Arabidopsis*).

From the aforementioned, it is conceivable that production of $O_2^{\bullet-}$ may depend on a PAO system. There are two main PA oxidation regimes: an apoplastic and an intracellular one. Several plant species, especially monocots, possess the ability to oxidize higher PAs in their apoplastic compartments. The oxidative reaction is executed by DAOs and PAOs residing in this compartment. However, the former enzymes show low affinity for higher PAs such as Spd. Notably, apoplastic PAOs in *Arabidopsis* are missing. There are five genes encoding for PAOs in *Arabidopsis.* The inducible *AtPAO1* (Tavladoraki et al., 2006) and constitutively expressed *AtPAO5* (Ahou et al., 2014) encode for cytoplasmic enzymes, oxidizing Spm, while *AtPAO2, 3, 4* encode peroxisomal proteins (Kamada-Nobusada et al., 2008; Moschou et al., 2008c), oxidizing Spd (AtPAO2, 3) and Spm (AtPAO2,3,4). On the other hand, in *Arabidopsis* there are ten genes encoding DAOs, four of which have been characterized. One of them is an apoplastic enzyme; however, six remain to be characterized (Moschou et al., 2013; Planas-Portell et al., 2013). These suggest that most likely the contribution of the apoplast to the Spd-dependent production of $O_2^{\bullet-}$ is rather minimal in *Arabidopsis.*

To further examine whether intracellular PAOs are responsible for inducing production of $O_2^{\bullet-}$, we employed genetic means. The best characterized so far enzymatic activity that oxidizes mostly Spd and to a smaller extent Spm in *Arabidopsis* is that of the peroxisomal AtPAO3 (Moschou et al., 2008c; Fincato et al., 2012). In mammals and plants, PA oxidation has been implicated in the execution of PCD (Yoda et al., 2003; Tavladoraki et al., 2006; Moschou and Roubelakis-Angelakis, 2013). It has been exemplified that there is a direct relationship between PCD and the levels of cytotoxic PA catabolic products, i.e., H_2O_2 and aminoaldehydes. For example, during *Helicobacter pylori* infection that contributes to gastric cancer, PA-derived H_2O_2 coincides with PCD induction (Chaturvedi et al., 2004). However, PA-derived H_2O_2 seems to be a double-edged sword since oxidation by SMO could perhaps contribute to the eradication of tumor cells (Babbar et al., 2007). In tobacco, overexpression of apoplastic PAO is accompanied by premature cell death of xylem tissue (Tisi et al., 2011). Interestingly, exogenous supply of Spd to maize root tips highly expressing *PAO* alters cell cycle distribution, toward quiescence and induces PCD (Tisi et al., 2011). In addition, premature cell death of xylem hinders the proper differentiation of the secondary cell wall, which is normally deposited before PCD induction in xylem. Importantly, a H_2O_2 scavenger partially ameliorates Spd-induced effects. In addition, 4-aminobutanal which is an additional oxidation product of Spd, failed to mimic Spd effects, indicating that PAO-derived H_2O_2 is sufficient to induce PCD independently of aminoaldehydes.

In contrast to the previous, the PA backconversion pathway seems to have completely distinct functions, which remain largely elusive. It was shown that AtPAO3 is an important component of pollen tube elongation (Wu et al., 2010). More specifically, AtPAO3 generates H_2O_2 which positively affects the permeability of a plasma membrane-residing Ca^{2+} influx channel. As a result, the intracellular concentration of Ca^{2+} increases, thereby promoting pollen tube elongation. In loss-of-function *Atpao3* reduction of pollen tube elongation, and in a physiological context reduced fertility was evident. In addition, a role for the PA backconversion pathway was hypothesized with respect to dehydration response of *Arabidopsis* (Alcazar et al., 2011a). The putative paralog of *AtPAO3* gene, *AtPAO2* is upregulated by drought stress in a similar fashion as *RD29A* and *RD22.*

A number of PAOs have been implicated in the PA backconversion pathway in *Arabidopsis* and, unlike in mammals, plant PAOs did not require acetylated derivatives (Moschou et al., 2008c). We observed that plants overexpressing *AtPAO3* showed increased content of H_2O_2 consistent with its role in PAs oxidation. Surprisingly, this H_2O_2 production led to a significant $O_2^{\bullet-}$ increase, while *Atpao3* mutants showed reduced levels of H_2O_2 but increased $O_2^{\bullet-}$. This implies that loss of AtPAO3 caused an increment of $O_2^{\bullet-}$ *versus* H_2O_2. In animal cells, ROS have also been shown to play an important role in maintaining the balance between cell proliferation and differentiation. A redox-dependent signaling pathway controls the induction of cell division through the regulation of *cyclinD1* expression (Burch and Heintz, 2005). Distribution of specific ROS appears to act as an important signal at the transcriptional and posttranscriptional levels during cell-cycle progression (Menon and Goswami, 2007). In *Drosophila*, changing ROS balance can switch the status of hematopoietic cells from proliferation to differentiation (Owusu-Ansah and Banerjee, 2009). In *Arabidopsis*, it was shown that $O_2^{\bullet-}$ accumulates primarily in the root meristematic zone, whereas H_2O_2 accumulates mainly in the elongation zone (Tsukagoshi et al., 2010). Moreover, it has been shown that Mn-SOD activity regulates cell-cycle progression through modulation of ROS levels, which control expression of both the *cyclinB1* and *cyclinD1* genes in mouse cells (Sarsour et al., 2008). The authors proposed that $O_2^{\bullet-}$ regulates the proliferative cycle, whereas H_2O_2 induces quiescence and differentiation. Therefore, in the root elongation zone, the ratio between $O_2^{\bullet-}$ and H_2O_2 is decreased (Tsukagoshi et al., 2010).

In our study, AtPAO3 was shown to be an important factor for balancing $O_2^{\bullet-}$ and H_2O_2. Increased levels of $O_2^{\bullet-}$ *versus* H_2O_2 were detected in the absence of AtPAO3, perhaps due to the increased activity of APX, which scavenges H_2O_2. S-*AtPAO3* plants show reduced expression of APX but increased expression of mitochondrial and chloroplastic SOD isoenzymes, while *Atpao3* show significantly increased expression of APX, mitochondrial and chloroplastic SOD. Noteworthy, the increased isoenzymes are in proximity to peroxisomes. These changes are in accordance

with the ROS levels detected in these plants. However, further studies are required to elucidate whether the increased/decreased expression of these antioxidants controls ROS levels or alternatively, whether ROS levels control the induction/reduction of these genes/enzymes. Although this may sound like a "chicken or the egg" question it merits careful examination to further understand the regulation of ROS homeostasis. We can speculate that similar regulation of the $O_2^{\bullet-}/H_2O_2$ ratio takes place during other developmental transitions, apart the ones reported in the root (Tsukagoshi et al., 2010) like for example during pollen tube growth, which could contribute to the failure of *Atpao3* pollen tube elongation (Wu et al., 2010).

Overexpression of *SSAT* in mice, an acetylase required to direct PAs in non-plant models toward the PAO pathway, leads to increased H_2O_2 and carbonyl content, and reduced SOD, CAT, and cyt CYP450 2E1 expression, responsible for xenobiotic metabolism. This suggests that transgenic mice are hypersensitive to stress, leading to cell death, and they also are sluggish and less hostile (Kaasinen et al., 2004). Interestingly, although S-*AtPAO3* plants accumulate significantly higher amounts of ROS, they do not show symptoms of chronic stress. Tobacco plants overexpressing apoplastic PAO exhibit increased SOD and CAT expression, which do not exert a protective effect, but rather this increased expression represents an attempt to scavenge surplus H_2O_2 produced by continuous PA oxidation. The previous suggests that as in animals, constitutive apoplastic PA oxidation in plants can lead to chronic oxidative stress (Moschou et al., 2008a).

On the contrary, the AtPAO3 backconversion pathway seems to have a completely different function. We show that Spd oxidation by AtPAO3 is required for a balanced respiration through the cyt-c and AOX pathways. Notably, overproduction of PA-derived H_2O_2 in the S-*AtPAO3* plants results in a small decrease of oxygen rate consumption, but not in induction of the AOX pathway. To the contrary, *Atpao3* plants show increased oxygen consumption through the AOX pathway. Interestingly, this increase is attenuated by application of DPI, which specifically blocks $O_2^{\bullet-}$ generation by NADPH-oxidase. It was reported that a microtubule associated kinesin and a mitochondrial channel are able to regulate the balance between cyt-c and AOX pathways (Yang et al., 2011). In addition, it has been hypothesized that the ratio of (singlet + $O_2^{\bullet-}$)/H_2O_2 determines PCD initiation during stress (Sabater and Martin, 2013). The previous allow us to propose that an increased ratio of $O_2^{\bullet-}/H_2O_2$ leads to increased oxygen consumption through the AOX pathway. Likewise, it has been reported that $O_2^{\bullet-}$ is sufficient to induce *AOX1a/b* genes in rice (Li et al., 2013). These results demonstrate that depletion of AtPAO3 leads to higher production of $O_2^{\bullet-}$, which in turn activates the AOX pathway.

In conclusion, our results allow us to propose that AtPAO3 is required for balancing $O_2^{\bullet-}/H_2O_2$ production. An imbalance of the $O_2^{\bullet-}$ *versus* H_2O_2 production leads to activation of AOX pathway and increases oxygen consumption. The next critical step to advance our understanding on the role of PA backconversion, and its interplay and crosstalk with ROS will be the genetic dissection of PA backconverting pathways, and their molecular effectors.

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Changes in free polyamine levels, expression of polyamine biosynthesis genes, and performance of rice cultivars under salt stress: a comparison with responses to drought

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Soil salinity affects a large proportion of rural area and limits agricultural productivity. To investigate differential adaptation to soil salinity, we studied salt tolerance of 18 varieties of *Oryza sativa* using a hydroponic culture system. Based on visual inspection and photosynthetic parameters, cultivars were classified according to their tolerance level. Additionally, biomass parameters were correlated with salt tolerance. Polyamines have frequently been demonstrated to be involved in plant stress responses and therefore soluble leaf polyamines were measured. Under salinity, putrescine (Put) content was unchanged or increased in tolerant, while dropped in sensitive cultivars. Spermidine (Spd) content was unchanged at lower NaCl concentrations in all, while reduced at 100 mM NaCl in sensitive cultivars. Spermine (Spm) content was increased in all cultivars. A comparison with data from 21 cultivars under long-term, moderate drought stress revealed an increase of Spm under both stress conditions. While Spm became the most prominent polyamine under drought, levels of all three polyamines were relatively similar under salt stress. Put levels were reduced under both, drought and salt stress, while changes in Spd were different under drought (decrease) or salt (unchanged) conditions. Regulation of polyamine metabolism at the transcript level during exposure to salinity was studied for genes encoding enzymes involved in the biosynthesis of polyamines and compared to expression under drought stress. Based on expression profiles, investigated genes were divided into generally stress-induced genes (*ADC2, SPD/SPM2, SPD/SPM3*), one generally stress-repressed gene (*ADC1*), constitutively expressed genes (*CPA1, CPA2, CPA4, SAMDC1, SPD/SPM1*), specifically drought-induced genes (*SAMDC2, AIH*), one specifically drought-repressed gene (*CPA3*) and one specifically salt-stress repressed gene (*SAMDC4*), revealing both overlapping and specific stress responses under these conditions.

Keywords: polyamines, salt stress, drought stress, gene expression, rice, natural variety

INTRODUCTION

Cultivation of rice (*Oryza sativa* L.) is limited by environmental stresses, of which salinity and drought represent some of the most devastating ones. Erosion, soil degradation and salinization affect approximately 3.6 billion of the world's 5.2 billion ha of dryland used for agriculture (Riadh et al., 2010). 10% of the land surface (950 Mha) and 50% of all irrigated land (230 Mha) are salt-affected (Ruan et al., 2010). Global annual losses from soil salinity are estimated at US$12 billion (Qadir et al., 2008). Rice (*Oryza sativa* L.) is considered to be moderately sensitive to salinity (Akita and Cabuslay, 1990) with a clear distinction between initial effects of salinity and long-term effects that result from the accumulation of salt in expanded leaves (Yeo et al., 1991). Salt stress causes reduction in leaf expansion, relative growth rate (Akita and Cabuslay, 1990) and photosynthesis (Nakamura et al., 2002; Cha-Um et al., 2006), as well as enhanced senescence (Lutts et al., 1996). Three major processes have been considered to participate in protection against high cytosolic Na^+: (1) the minimization of Na^+ entry into cells; (2) the compartmentation of Na^+ into the vacuole; and (3) the increased efflux of Na^+ out of the cell driven by specific ion transporters (Chinnusamy et al., 2005).

Polyamines are involved in a wide range of biological processes, including growth, development and programmed cell death (Galston and Sawhney, 1990; Bouchereau et al., 1999; Kaur-Sawhney et al., 2003; Moschou and Roubelakis-Angelakis, 2013), as well as abiotic stress responses (for recent reviews see Alcázar et al., 2010; Gill and Tuteja, 2010; Hussain et al., 2011; Gupta et al., 2013). Nevertheless, reported responses of polyamines to salt stress are often contradictory, even within one species. In rice, either a decrease of putrescine (Put) and/or spermidine (Spd)

Abbreviations: ABA, abscisic acid; ADC, arginine decarboxylase; AIH, agmatine iminohydrolase; CPA, N-carbamoylputrescine amidohydrolase; ODC, ornithine decarboxylase; Put, putrescine; qRT-PCR, quantitative RT-PCR; ROS, reactive oxygen species; SAMDC, S-adenosyl-methionine decarboxylase; SPD, spermidine synthase; Spd, spermidine; SPM, spermine synthase; Spm, spermine.

(Lin and Kao, 1995; Maiale et al., 2004), or of all three major polyamines (Prakash et al., 1988), but also a salt-induced increase of Put (Basu et al., 1988; Basu and Ghosh, 1991), Spd, and spermine (Spm) (Katiyar and Dubey, 1990; Maiale et al., 2004) has been reported. Also a differential modification of polyamines upon salt stress depending on the tolerance level was described (Krishnamurthy and Bhagwat, 1989). Considering water stress in rice in general, including osmotic stress, dehydration, and withholding water, accumulation of polyamines was reported for most scenarios (Capell et al., 2004) but also a decrease of Put with a parallel increase of Spm (Do et al., 2013). Modifications of polyamines in response to osmotic stress were considered to be affected by dose and time of treatment (Lefèvre et al., 2001). A transcriptome analysis of polyamine over-accumulators revealed that endogenous polyamines participate in stress signaling through a crosstalk with abscisic acid (ABA), Ca^{2+} signaling and other hormonal pathways in plant defense and development (Marco et al., 2011).

Positively charged polyamines are able to interact with negatively charged molecules, such as nucleic acids, acidic phospholipids, proteins, and cell wall components (Martin-Tanguy, 2001; Kakkar and Sawhney, 2002). The physiological function of the various polyamines in stress response is not resolved yet, but their involvement in protein phosphorylation, conformational transitions of DNA (Martin-Tanguy, 2001), maintenance of ion balance, radical scavenging and prevention of senescence, stabilization of membranes (Bouchereau et al., 1999), and gene regulation by enhancing DNA-binding activity of transcription factors (Panagiotidis et al., 1995) was shown and a role as compatible solutes is discussed. Additionally, polyamines affect ion channel conductivity due to their positive charge and are able to block vacuolar channels, e.g., calcium channels (Hussain et al., 2011).

Polyamine catabolism in the apoplast is a common mechanism within reactive oxygen species (ROS) signaling (Pottosin et al., 2014). Furthermore Spd and Spm were described as nitric oxide (NO) inducers in plants which are part of a complex network also containing ABA and H_2O_2 (Hussain et al., 2011; Pottosin et al., 2014). In addition, polyamines are major players in the turnover of nitrogenous compounds in plants under optimal as well as stress conditions (Moschou et al., 2012).

Put biosynthesis either from ornithine or indirectly from arginine via agmatine is catalyzed by ornithine (ODC; EC 4.1.1.17) and arginine decarboxylase (ADC; EC 4.1.1.19), respectively. Agmatine is then sequentially converted to Put by agmatine iminohydrolase (AIH; EC3.5.3.12) and N-carbamoylputrescine amidohydrolase (CPA; EC 3.5.1.53). Spd and Spm are synthesized from Put by the addition of aminopropyl groups, transferred from decarboxylated S-adenosylmethionine (SAM), which is produced from SAM by S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50). Spd synthase (SPD; EC 2.5.1.16) and Spm synthase (SPM; EC 2.5.1.22) catalyze the final steps of the Spd and Spm synthesis. Polyamine metabolism and transport have been recently reviewed in Gupta et al. (2013), and the integration with other metabolic networks was shown in *Arabidopsis* (Bitrián et al., 2012).

An induction of the transcript level and/or activity of ADC could be shown for rice (Chattopadhyay et al., 1997) as well as for other species (Mo and Pua, 2002; Urano et al., 2004; Hao et al., 2005a; Legocka and Kluk, 2005; Liu et al., 2006) under salinity. Transcript levels of other polyamine biosynthesis-related genes are also increased under salt stress, e.g., *SAMDC* in rice (Li and Chen, 2000b), soybean (Tian et al., 2004), wheat (Li and Chen, 2000a), *Arabidopsis* (Urano et al., 2003) and apple (Hao et al., 2005b), *SPD* and *SPM* in *Arabidopsis* (Urano et al., 2003), and maize (Rodríguez-Kessler et al., 2006). Tolerance to drought was improved by constitutive over-expression of oat *ADC* in rice (Capell et al., 1998), with a simultaneous effect on plant development. When polyamine accumulation was induced by over-expression of oat *ADC* or *Tritodermum SAMDC* under the control of an ABA-inducible promoter, rice plants were more resistant to high salinity (Roy and Wu, 2001, 2002). Furthermore, over-expression of the *Datura stramonium ADC* gene under the control of the stress activated maize ubiqitin-1 promoter conferred tolerance to osmotic stress in rice (Capell et al., 2004). For a broader overview of transgenic approaches; see Gill and Tuteja (2010) and Marco et al. (2012).

Here we investigated changes in polyamine content and expression levels of all genes encoding enzymes involved in polyamine biosynthesis in a wide range of rice cultivars under long-term moderate salt stress using two different NaCl concentrations and we explored the possible correlations between physiological parameters, polyamine content, and gene expression levels and salt sensitivity of those 18 rice cultivars. In addition, by comparing polyamine levels and changes in gene expression with results obtained under mild drought stress conditions, we were able to classify the different genes into either salt- or drought- or generally stress-responsive.

MATERIALS AND METHODS

PLANT MATERIAL, CULTIVATION, AND SALT STRESS TREATMENT

Eighteen rice (*Oryza sativa* L.) cultivars originating either from the IBT (Institute of Biotechnology, Hanoi, Vietnam) or from the IRRI (International Rice Research Institute, Manila, Philippines) {Nipponbare (IRGC accession 12731) (NB), Taipei 309 (IRGC accession 42576) (TP), IR57311-95-2-3 [IRGC accession 17509 (INGER)] (IR) and Zhonghua} were grown under control and salt stress conditions in three independent experiments in a climate chamber. For a complete list of cultivars see **Table 1**. The design was a randomized complete block design with five blocks, each containing one hydroponic culture box with 0, 50, and 100 mM NaCl, respectively. Boxes were randomized within the blocks.

Seeds were germinated at 28°C for 10 days. Plantlets were transferred to a climate chamber with 12 h light phase at a photon flux density of 600 μE m^{-2} s^{-1} (Lamps: Iwasaki Eye MT 400 DL/BH E40, DHL Licht, Wülfrath, Germany); temperature was 26°C (day) and 22°C (night), with a relative humidity of 70%. Plants were grown hydroponically in 10 l polypropylene boxes filled with medium according to Yang et al. (1994) and covered with a lid. Plantlets were fixed to holes in the lid with a piece of foam material and covered with a transparent lid for 2 days. After 14 days the growth medium was exchanged for medium with the

Table 1 | List of cultivars of *Oryza sativa* L. used for salt stress experiments.

Cultivar	Number	Subspecies	Origin
CR203	1	*Indica**	IBT
DR2	2	*Indica**	IBT
C70	4	*Indica**	IBT
C71	5	*Indica**	IBT
Doc Do	7	*Indica*	IBT
Doc Phung	8	*Indica*	IBT
Cuom	14	*Indica/japonica**	IBT
Nuoc Man	21	*Indica*	IBT
Lua Man	22	*Indica**	IBT
Nep Man	23	*Indica**	IBT
Nuoc Man 1	25	*Indica*	IBT
Cham	26	*Indica*	IBT
Cham Bien	27	*Indica*	IBT
Cha Va	28	*Indica*	IBT
Nipponbare	50	*Japonica*	IRRI
Taipei 309	51	*Japonica*	IRRI
IR57311-95-2-3	52	*Indica*	IRRI
Zhonghua	53	*Japonica*	IRRI

For genotyping of the Vietnamese cultivars marked with an asterisk, see Degenkolbe et al. (2013). IBT, Institute of Biotechnology, Hanoi, Vietnam; IRRI, International Rice Research Institute, Manila, Philippines.

appropriate salt concentration (0, 50, 100 mM NaCl), which was renewed after an additional week.

After 14 days of salt stress treatment, plants were sampled 4–6 h after the beginning of the light period. The middle sections of leaves were selected for physiological measurements to avoid taking material from the elongation zone at the base of the leaf blade or senescent tissue at the top of the leaves, especially in stressed plants. Fully expanded green leaf blades were harvested and immediately frozen in liquid nitrogen for transcript and polyamine analysis. The remaining plant was harvested to determine shoot fresh (FW) and dry weight (DW, 48 h, 80°C).

For a detailed description of the drought stress experiments please refer to Do et al. (2013).

21 rice cultivars were grown under control and drought conditions in controlled climate chambers in 12 h days (600 μE m^{-2} s^{-1}) at 26°C and 75% relative humidity and 12 h nights at 22°C and 70% relative humidity. Plants were grown in 540 g sand mixed with 8 g of Lewatit HD 50 (Lanxess, Langenfeld, Germany) and 0.4 g Fetrilon Combi (Compo, Münster, Germany). Pots were positioned. Rice plants were grown in boxes filled with water to the level of the substrate surface. 26 days after sowing drought stress was applied by water removal from half of the boxes. When the permanent wilting point for 50% of the plants was reached soil water content was kept constant for 14 days by weighing each pot at the end of the light period and adding the amount of water lost during the last 24 h.

PHYSIOLOGICAL CHARACTERIZATION OF THE PLANTS AND SAMPLING

The leaf phenotype of stressed and control plants was visually assessed for individual plants before and during salt stress treatment 24 DAS (before stress treatment), 30 DAS (6 days after stress treatment), and 37 DAS (13 days after stress treatment) based on the stress damage score of the IRRI (Mitchell et al., 1998). An average stress damage score considering chlorosis and necrosis and using a scale from 1 to 9 was given to every plant with "1" representing plants with undamaged leaves, "9" almost or completely dead plants. The number of tillers was counted, and plant height (Wopereis et al., 1996) measured at the same time.

Chlorophyll-*a* fluorescence and leaf temperature were measured with a pulse-amplitude-modulated Dual- PAM-100 fluorometer (WALZ, Effeltrich, Germany) on the middle section of the second fully expanded leaf during mid-day without dark adaptation and under climate chamber conditions. The effective quantum yield of PS II ($\Delta F/Fm'$) was determined from the maximum light-adapted fluorescence yield (Fm') and the current fluorescence yield (Ft) as [$\Delta F/Fm' = (Fm' - Ft)/Fm'$].

Salt score values were ranked and the average rank calculated within each experiment. Results of three experiments were combined and mean and standard error of the average ranks was calculated for every cultivar. The relative DW of the shoot was calculated by dividing the average shoot DW under salt condition by the respective control value for each cultivar and experiment.

A description of the physiological characterization and the ranking of cultivars under drought stress conditions are given in Do et al. (2013).

POLYAMINE ANALYSIS

Free polyamines (Put, Spd, and Spm) were quantified by High Performance Liquid Chromatography (HPLC) as described in Do et al. (2013). Data were analyzed using the Chromeleon software (Dionex, Germering, Germany) and calibration curves obtained from the pure substances. Detailed data on polyamines levels under drought conditions are given in Do et al. (2013).

QUANTITATIVE RT-PCR (qRT-PCR)

qRT-PCR was performed with the ABI Prism 7900HT (Applied Biosystems, Foster City, CA) as described in Do et al. (2013). Primers were designed using PrimerExpress (Version 2.0, Applied Biosystems) and all primer sequences are given in Do et al. (2013). Data were analyzed using the SDS 2.0 software (Applied Biosystems) and normalized based on the expression of the housekeeping genes actin 1 and cyclophilin. Normalized gene expression was calculated by dividing the average relative expression (primer efficiency P to the power of cycle number Ct) of the two housekeeping genes (H1 and H2) by the relative expression of the gene of interest (GOI) (Degenkolbe et al., 2009). Primer efficiency was calculated using LinRegPCR (Ramakers et al., 2003). Fold change was calculated as $\log_2$ of the ratio of relative expression of genes under stress conditions to relative expression under control conditions. Detailed gene expression data of drought-tolerant and drought-sensitive cultivars are given in Do et al. (2013).

STATISTICS

Spearman correlations between the mean rank of the salt stress damage score and the respective means of all performance parameters across three experiments were determined using the rcorr

function provided by the R-package "Hmisc" after a correction for outliers. The significance of differences between treatments was analyzed using unpaired, two-sided *t*-tests in Microsoft Excel. Significance levels in the figures are indicated as: $0.05 > p > 0.01$ (*), $0.01 > p > 0.001$ (**), $p < 0.001$ (***).

RESULTS

PHENOTYPING OF RICE CULTIVARS UNDER SALT CONDITIONS

18 rice cultivars representing either *japonica* or *indica* subspecies, including cultivars from a Vietnamese breeding program and four well characterized IRRI cultivars (**Table 1**), were subjected to two different salt stress conditions, 50 and 100 mM NaCl, in the vegetative stage using hydroponic culture in controlled growth chambers. Before salt treatment and after 6 and 13 days of salt stress plants were characterized and compared to control plants by scoring their leaf phenotype and by measuring plant height, tiller number, FW, and DW. Differences between cultivars were highest after 14 days at 100 mM NaCl. At this time point cultivars could be clearly classified based on their salt sensitivity using the average rank of visual scoring (**Figure 1**). Mean ranks from 13.8 to 73.7 indicated a large natural variation for salt tolerance between the selected cultivars. The *indica* cultivars Cham bien (27), Cham (26), and Nuoc man (21) were the most tolerant with scoring values from 13.8 to 14.9, whereas the *japonica* cultivars Nipponbare (50), Taipei 309 (51), and Zhonghua (53) were the most sensitive, with scoring values ranging from 58.3 to 73.7. All cultivars of the *japonica* subspecies could be classified as sensitive. The most sensitive *indica* cultivars were C70 (4), DR2 (2), and Lua Man (22).

Integrity of the photosynthetic machinery was investigated by measuring the effective quantum yield of photosynthesis after 13 days of stress for both NaCl concentrations (**Figure 2**). While in most cultivars quantum yield was not impaired by salt treatment, a significant reduction occurred at 100 mM NaCl in the six most sensitive cultivars, including both *indica* and *japonica* subspecies. At mild salt stress conditions of 50 mM NaCl no significant reduction of effective quantum yield was observed.

Plant height was cultivar dependent under control conditions with largest plants in cultivar 25 and smallest in cultivar 4. A reduction due to 100 mM NaCl treatment occurred in four cultivars (14, 8, 22, 50) and showed no tolerance dependent pattern (not shown). Tiller number under control conditions was also highly cultivar dependent with the highest number in cultivar 22 and the lowest in 51, and it was reduced by 14 to 41% across cultivars under salt stress (data not shown). FW was significantly affected by salt treatments of 50 and 100 mM NaCl in most cultivars (**Figure 3A**). In the most sensitive cultivar Nipponbare (50) it was reduced to 22% of the control value. Strikingly, relative DW as %FW was increased at 100 mM NaCl with descending tolerance between no change in the most tolerant and an increase of 39% in the most sensitive cultivar (**Figure 3B**). The most tolerant cultivars showed no significant change of relative DW under salt stress. Spearman correlation analysis for the sensitivity rank at 100 mM NaCl and the ratio of all available growth parameters in comparison to control conditions revealed significant negative correlations between sensitivity rank and photosynthetic quantum yield at 50 and 100 mM NaCl and FW at 100 mM NaCl, and a significant positive correlation with the relative DW at 100 mM NaCl (**Table 2**).

POLYAMINE CONTENT OF RICE CHANGES DURING SALT STRESS

Pool sizes of predominant free polyamines (Put, Spd, Spm) were measured in leaves of all cultivars after 14 days of salt stress in comparison to the control. **Figure 4** shows the respective values in all investigated cultivars sorted by their tolerance. Put showed the highest genotypic variation, especially under control conditions, with values ranging from 120 to 4230 nmol g^{-1} DW. Under control conditions, Put content was significantly higher in the more sensitive cultivars including *indica* and *japonica* ssp. compared to more tolerant ones. For the eight most sensitive cultivars, Put values under control conditions ranged from 1450 to 4230 nmol g^{-1} DW, whereas values in the eight most tolerant cultivars reached only values from 120 to 1166 nmol g^{-1} DW. After 14 days of salt treatment a strong decrease of Put at both NaCl levels was restricted to the eight most sensitive cultivars

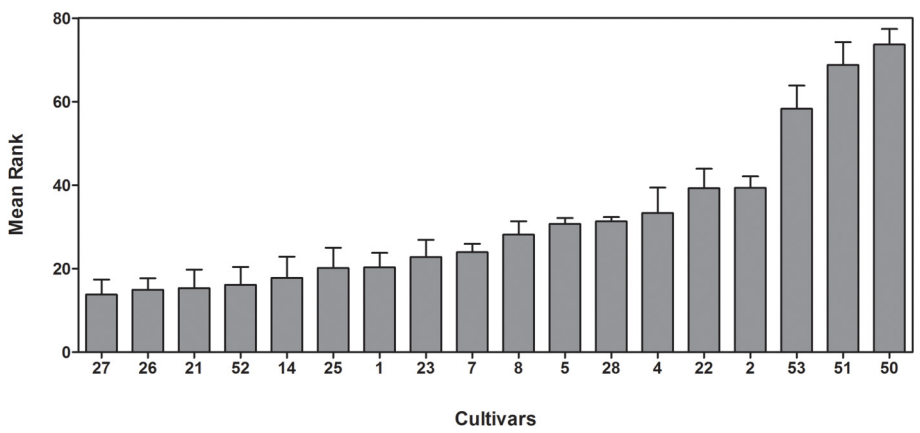

FIGURE 1 | Classification of 18 rice cultivars after 13 days at 100 mM NaCl in hydroponic culture. Scores were ranked and the average rank of three independent experiments is shown with standard errors. Cultivars are sorted from the most tolerant to the most sensitive from left to right.

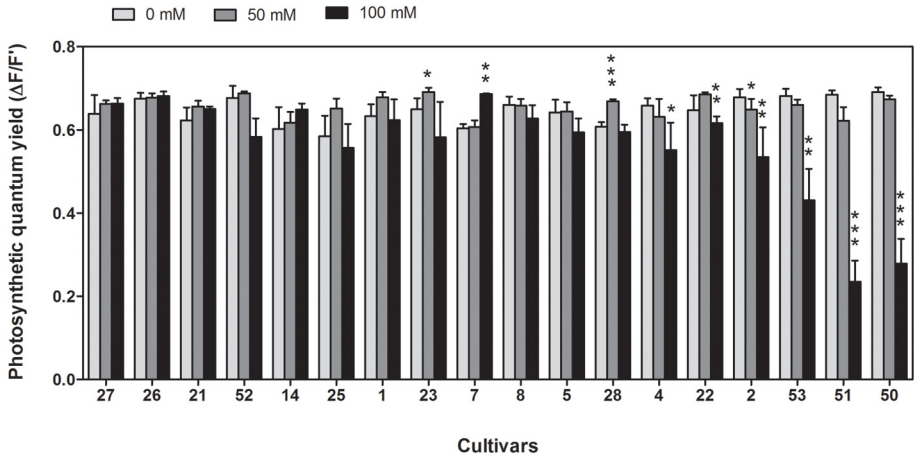

FIGURE 2 | Photosynthetic quantum yield of 18 rice cultivars under control conditions and after 13 days at 50 mM or 100 mM NaCl in hydroponic culture. Means of three independent experiments with five replicate plants each are shown with standard errors. Significance levels are indicated as: $p < 0.001$ (***), $0.001 < p < 0.01$ (**), $0.01 < p < 0.05$ (*) in comparison to control. Cultivars are sorted from the most tolerant to the most sensitive from left to right.

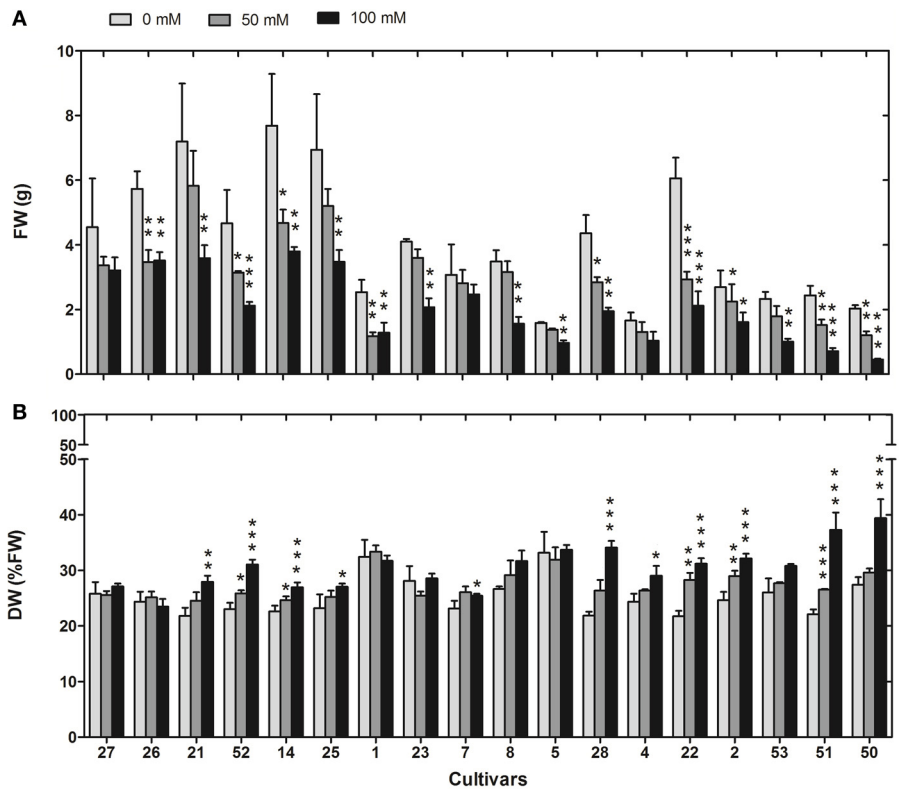

FIGURE 3 | FW (A) and DW as %FW (B) of 18 rice cultivars under control conditions and after 13 days at 50 mM or 100 mM NaCl in hydroponic culture. Means of three independent experiments with five replicate plants each are shown with standard errors. Significance levels are indicated as: $p < 0.001$ (***), $0.001 < p < 0.01$ (**), $0.01 < p < 0.05$ (*) in comparison to control. Cultivars are sorted from the most tolerant to the most sensitive from left to right.

with 11 and 1.5% of the control values for cultivars 2 and 50 at 100 mM NaCl, respectively. All other cultivars showed either an increase (26, 27) or no significant change. The ratio of Put at 50 and 100 mM NaCl in comparison to control conditions was negatively correlated with the salt sensitivity score, confirming a stronger relative decrease of Put content in sensitive as compared to tolerant cultivars (**Table 2**). Strikingly, the most sensitive cultivars contained the highest absolute amounts of Put under control

Table 2 | Spearman correlation analysis between the mean sensitivity rank at 100 mM NaCl and the ratio of all available growth parameters under stress compared to control conditions, absolute polyamine content in nmol g^{-1} DW and ratio of polyamine content under stress in comparison to control conditions.

Parameter (Absolute content or ratio to control)	*r*	*p*-value
Ratio photosynthetic yield 50 mM	−0.589	*0.0101*
Ratio photosynthetic yield 100 mM	−0.736	**0.0005**
Ratio tiller number 50 mM	0.059	0.8167
Ratio tiller number 50 mM	−0.143	0.5701
Ratio plant height 50 mM	−0.032	0.8997
Ratio plant height 100 mM	−0.257	0.3033
Ratio FW 50 mM	−0.346	0.1600
Ratio FW 100 mM	−0.585	*0.0107*
Ratio DW 50 mM	0.422	0.0810
Ratio DW 100 mM	0.587	*0.0104*
Put 0 mM	0.820	<**0.0001**
Spd 0 mM	0.150	0.5534
Spm 0 mM	−0.276	0.2684
Put 50 mM	0.298	0.2293
Spd 50 mM	0.069	0.7851
Spm 50 mM	−0.013	0.9579
Put 100 mM	−0.575	*0.0126*
Spd 100 mM	−0.560	*0.0156*
Spm 100 mM	−0.422	0.0810
Ratio Put 50 mM	−0.800	**0.0001**
Ratio Put 100 mM	−0.843	<**0.0001**
Ratio Spd 50 mM	−0.061	0.8103
Ratio Spd 100 mM	−0.676	***0.0021***
Ratio Spm 50 mM	0.179	0.4784
Ratio Spm 100 mM	−0.094	0.7109

P-values below 0.05 are highlighted in italic, below 0.01 highlighted in boldface and italic, and below 0.001 highlighted in boldface numbers.

conditions, indicated by a positive correlation of Put content with the salt sensitivity score ($r = 0.82$, $p = 0.00003$).

In contrast to Put, Spd content was not changed under salinity, except for a significant reduction at 100 mM NaCl in the most sensitive cultivars 51 and 50 (to 40 and 28%, respectively) and an increase at 100 mM NaCl in the most tolerant cultivar 27 (to 181%) that was, however, not significant (**Figure 4**). Surprisingly, absolute Spd levels at 100 mM NaCl showed a significant negative correlation ($r = -0.56$, $p = 0.0155$) with salt sensitivity and also the ratio of Spd at 100 mM NaCl in comparison to control was negatively correlated with salt sensitivity (**Table 2**).

General increases of Spm levels were observed in all cultivars under both salt stress conditions with up to 3-fold increases at 100 mM NaCl. However, the predominant compound in most cultivars under salinity conditions (100 mM) was still Put followed by Spd and Spm with the exception of the three most sensitive cultivars 53, 51, and 50 with a higher Spm content, followed by Spd and Put.

EXPRESSION ANALYSIS OF POLYAMINE BIOSYNTHESIS GENES

Expression of 16 genes encoding enzymes involved in polyamine biosynthesis was analyzed in leaves of eight cultivars after 14 days of salt treatment (50 and 100 mM NaCl) using qRT-PCR. The eight cultivars were selected based on their widely differing salt tolerance and included both subspecies with cultivar 52, 14, and 1 belonging to the more tolerant, 5, 4, 22, 2, and 50 to the more and most sensitive ones. The $\log_2$ fold change between gene expression under salt compared to control conditions is shown in **Figure 5**. In general, increased gene expression is more obvious at 50 mM than at 100 mM NaCl with the exception of *ODC1* in some cultivars and *SPD/SPM2* and *SPD/SPM3* in all cultivars. At 50 mM NaCl *ADC2*, a gene involved in the synthesis of Put, was induced in almost all cultivars except cultivar 5 and the most sensitive cultivar 50. Also *SPD/SPM2* and *SPD/SPM3* were induced in almost all cultivars under this condition. There are two alternative pathways to synthesize Put, either indirectly from arginine, catalyzed by the enzymes ADC, AIH, and CPA, or directly from ornithine, catalyzed by ODC. In two of the tolerant cultivars, genes encoding enzymes for both pathways (*ADC2, ODC1*) were induced by salt stress, whereas in sensitive cultivars the expression of only one of these genes, either *ADC2* or *ODC1* was increased.

The fold change in the expression of genes encoding enzymes involved in the following steps of the polyamine biosynthesis pathway, leading to the synthesis of Spd and Spm, varied for cultivars and genes with *SAMDC2* being induced in most of the cultivars at both salt levels. Additionally, *SPD/SPM2* and *SPD/SPM3* were induced in all cultivars under both conditions. No correlations could be found between the $\log_2$ fold change of gene expression and the salt sensitivity of the cultivars.

COMPARISON OF THE SALT AND DROUGHT RESPONSES OF POLYAMINE METABOLISM AND GENE EXPRESSION

For comparison of the responses of rice to salt and drought stress at the level of polyamine metabolism, data from three salt-tolerant (52, 14, 1) and three salt-sensitive cultivars (22, 2, 50) were summarized and averaged. These cultivars were chosen to allow comparison of their responses to drought stress investigated in a previous study (Do et al., 2013). Do and co-workers analyzed drought tolerance and the response of polyamine metabolism in 21 rice cultivars with an overlap of nine cultivars between the two studies. For the comparison, three drought-sensitive cultivars (22, 2, 50) and three drought-tolerant cultivars (1, 4, 52) were selected. Cultivar 14 in our selection of salt-tolerant cultivars, had to be replaced by 4 in the selection of drought-tolerant cultivars, because cultivar 14 was not drought-tolerant.

Average polyamine levels (Put, Spd, Spm) of the three tolerant and sensitive cultivars were compared under drought and salt (50, 100 mM NaCl) conditions (**Figure 6**). Under control conditions Put levels were similar for the different growth conditions (growth in sand for drought and hydroponic culture for salt stress) with values between 100 and 800 nmol g^{-1} DW except for sensitive cultivars in hydroponic culture with over 4150 nmol Put g^{-1} DW. Put levels were clearly reduced under both drought and salt conditions with the exception of tolerant cultivars at 50 and 100 mM NaCl. Spd values were slightly lower in hydroponic culture compared to plants grown in sand under control conditions. While Spd was significantly reduced after 18 days of drought stress in all cultivars, no significant changes were observed under salt stress. Spm levels were also similar for both growth conditions in the absence of stress and were significantly elevated in sensitive

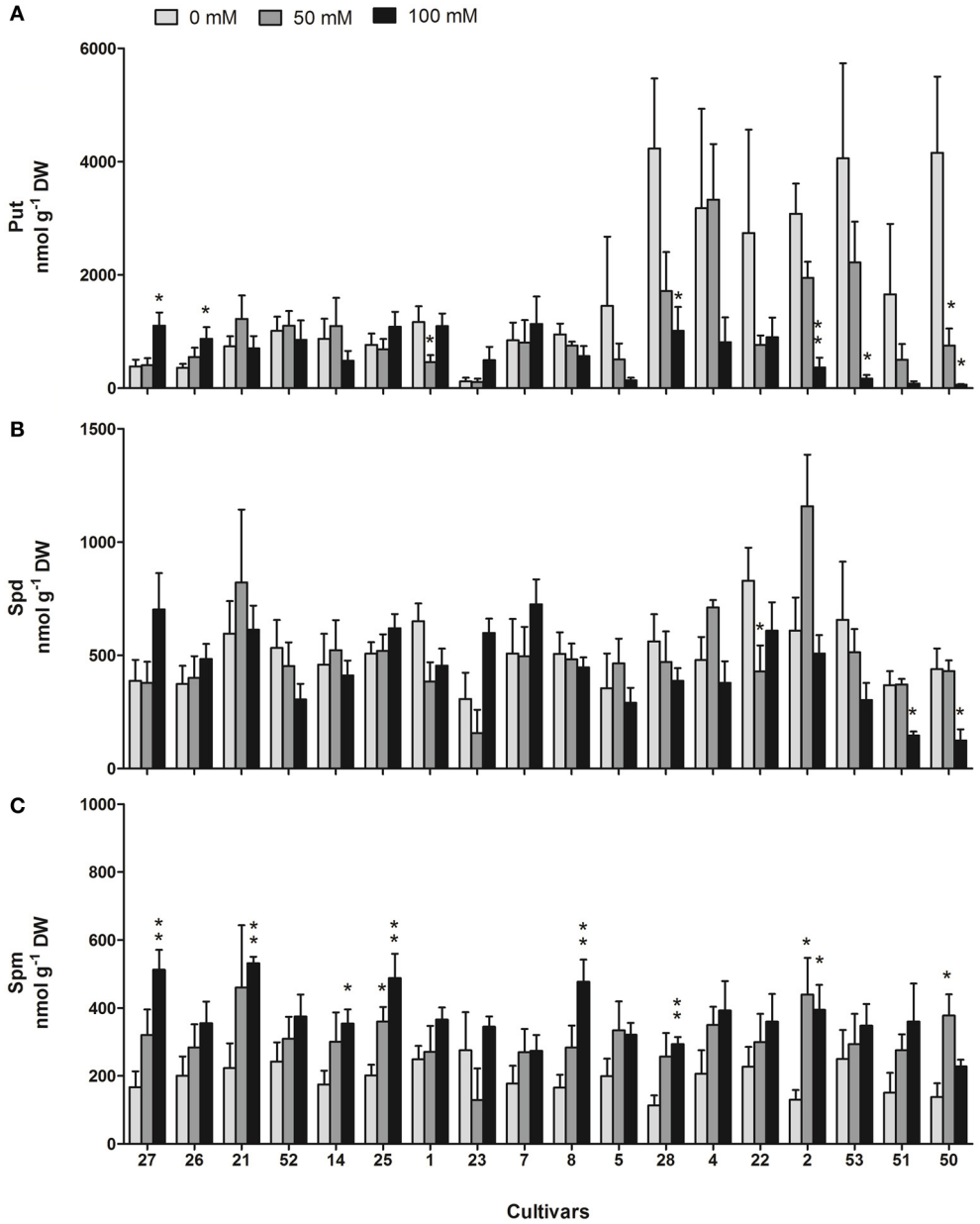

FIGURE 4 | Polyamine content under control and salt stress (50 and 100 mM NaCl) conditions. The panels show Put **(A)**, Spd **(B)**, and Spm **(C)** content in leaves of 18 rice cultivars. Each value represents the mean (±s.e.m.) of one experiments with five replicate plants each. Significance levels are indicated as: $0.001 < p < 0.01$ (**), $0.01 < p < 0.05$ (*) in comparison to control. Cultivars are sorted from the most tolerant to the most sensitive from left to right.

cultivars under drought and both salt treatments, while they rose only at 100 mM NaCl in the tolerant cultivars.

In summary, tolerant cultivars are able to keep their Put levels constant under salt stress and increase their Spm levels only slightly at the high salt concentration, whereas in sensitive cultivars Put levels are strongly decreased and Spm levels increased. Under drought conditions, polyamine levels show the same pattern in sensitive cultivars with reduced Put and increased Spm levels. In contrast, the pattern in drought tolerant cultivars under drought is different from the salt response with strongly decreased Put and Spd levels and no changes of Spm.

To compare gene expression levels under different culture conditions, we calculated the average gene expression of the three tolerant and sensitive cultivars, respectively. A positive value of relative expression ($\log_2$) represents a higher expression of a GOI in comparison to the housekeeping genes and a negative value a lower expression. Differences in gene expression between growth conditions could be observed for *ADC1*, with a lower expression in sand-grown plants compared to plants grown hydroponically,

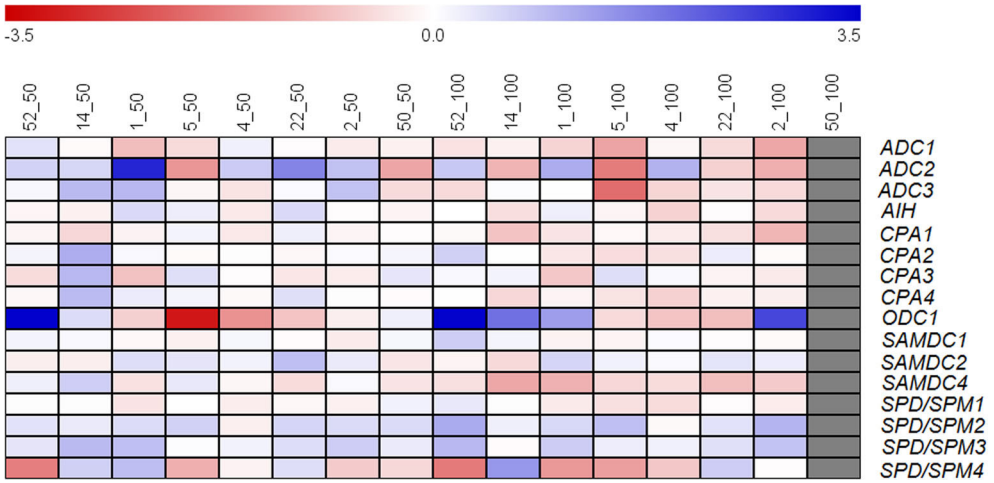

FIGURE 5 | Log_2 fold change of expression of genes, encoding enzymes involved in polyamine biosynthesis, under salt stress (50, 100 mM) as compared to control conditions in eight rice cultivars differing in salt tolerance. Leaves from five different plants were pooled for RNA extraction. Each value represents the mean of three technical replicates. Cultivars are sorted from the most tolerant to the most sensitive from left to right.

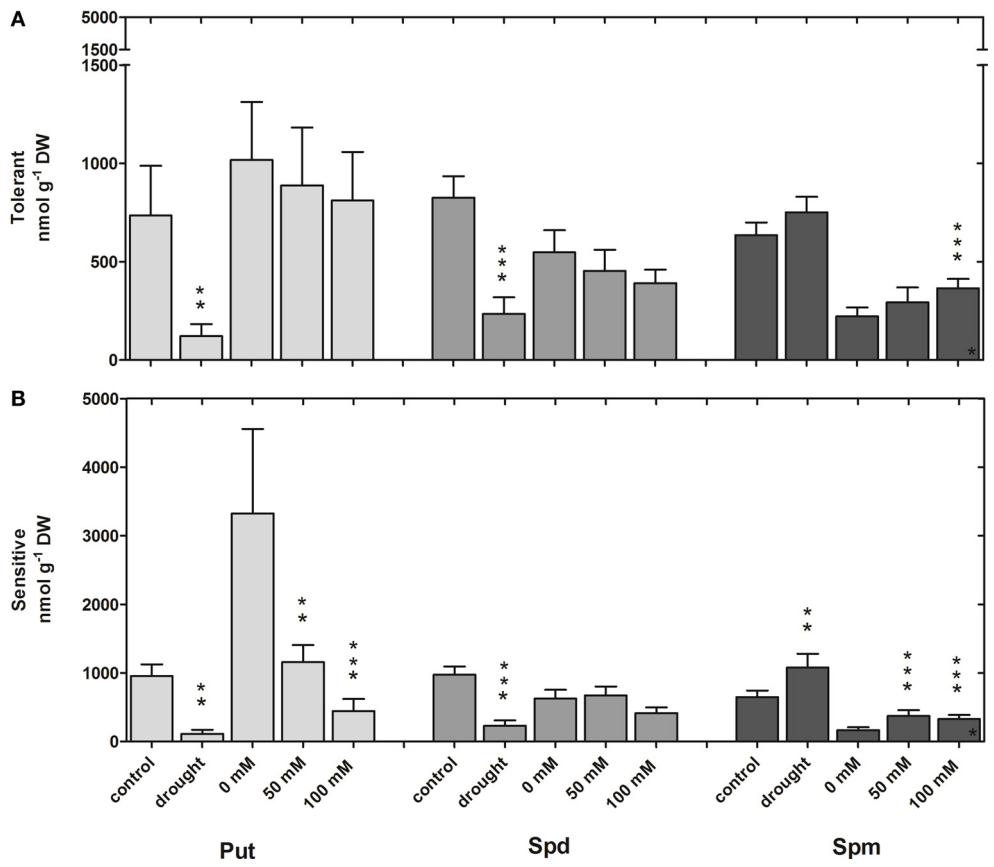

FIGURE 6 | Comparison of polyamine contents under drought and salinity stress. Put, Spd, and Spm contents are shown for drought- and salt-tolerant (52, 4, 1) **(A)** and drought- (22, 2, 50) and salt-sensitive cultivars (22, 2, 50) **(B)** under control and stress conditions. Each value represents the mean (±s.e.m.) of three cultivars with five replicate plants each. Significance levels are indicated as: $p < 0.001$ (***), $0.001 < p < 0.01$ (**), $0.01 < p < 0.05$ (*) in comparison to the respective control.

and *ODC1*, showing a higher expression in salt-sensitive cultivars grown in hydroponic culture (**Figure 7**).

Additionally, $\log_2$ fold change values of average gene expression of the selected tolerant and sensitive cultivars under both stresses were calculated (**Figure 8**). At drought and salt conditions compared to control *ADC1* expression was reduced in both groups with the highest reduction in sensitive cultivars at 100 mM NaCl, while *ADC2* was highly induced under stress with the exception of sensitive cultivars at 100 mM NaCl. Log_2fold change of gene expression of *ADC3* showed a fluctuating pattern with the strongest reduction again in sensitive cultivars at 100 mM NaCl. This seemed to be partly compensated by a high induction of *ODC1*, which catalyzes an alternative pathway of Put synthesis. On the other hand, *ODC1* is also induced under all other conditions except for drought in tolerant and at 50 mM NaCl in sensitive cultivars. Whereas *SAMDC2* was always induced in sensitive cultivars under all conditions, this induction was only observed under drought in tolerant cultivars. Log_2 fold change of gene expression of *SAMDC4* showed a clear stress specific pattern, whereas *SPD/SPM2*, *SPD/SPM3,* and *SPD/SPM4* (with one exception) were generally stress induced in both tolerance groups.

Based on expression profiles of cultivars differing in tolerance, the response in gene expression after drought and salt stress could be differentiated for 13 genes. The investigated genes were divided into generally stress-induced genes *(ADC2*, *ODC1*, *SPD/SPM2*, *SPD/SPM3*) with two genes showing tolerance related differences under salt (*ADC2*) or drought conditions (*ODC1)*, one generally stress-repressed gene *(ADC1*), constitutively expressed genes *(ADC3, CPA1, CPA2, CPA4, SAMDC1, SPD/SPM1*), one specifically drought-induced gene *(AIH*), one specifically drought-repressed gene *(CPA3*), and one specifically salt-stress repressed gene *(SAMDC4*) revealing both overlapping and specific stress responses.

DISCUSSION

Despite several published studies on the response of polyamine metabolism to salt stress in rice, comparative analyses of a large number of cultivars are rare. For the present investigation of the polyamine response in 18 cultivars, a detailed physiological characterization at two different salt concentrations in an early vegetative stage was performed and revealed a large variation of salt tolerance among the cultivars based on a rank of scoring data that indicated a higher salt sensitivity of *japonica* as compared to *indica* cultivars. It was previously reported that salt tolerance of *indica* cultivars was higher than that of *japonica* cultivars, denoted by a lower reduction of growth and a better Na^+ exclusion (Lee et al., 2003). Among the cultivars investigated in the present study, Nipponbare was known to be salt-sensitive (Karan et al., 2012), while for all other cultivars no salt tolerance classification was available. Our phenotypic ranking was supported by a decrease of photosynthetic quantum yield and a reduction of FW under salt stress, especially in sensitive cultivars. Decreased photosynthetic yield is well known from previous studies (Lutts et al., 1996; Yamamoto et al., 2004) and the lack of an effect in a salt tolerant cultivar (Pokkali) was also shown (Dionisio-Sese and Tobita, 2000). Reduced FW under salinity was also described by Su and Wu (2004), who used this parameter for the classification of salt tolerance. The three most sensitive cultivars, 50, 51, and 53, all *japonica* ssp., as well as the two most tolerant cultivars, 26 and 27 (*indica*), were also clustered according to their metabolomic profiles considering changes of four metabolite pools under salinity suggesting a similar metabolic state (Zuther et al., 2007).

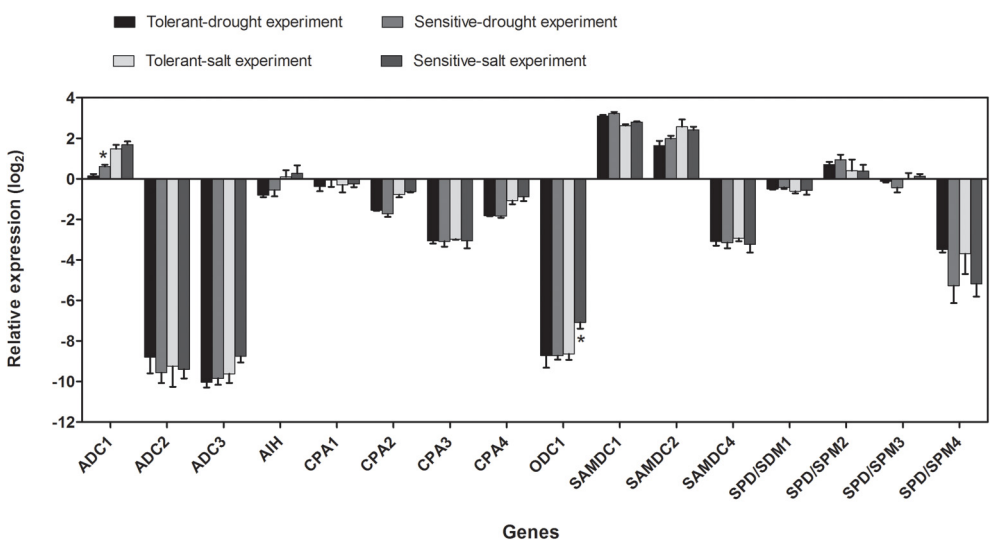

FIGURE 7 | Relative average expression ($\log_2$) of genes encoding enzymes involved in polyamine biosynthesis under control conditions in three tolerant vs. sensitive cultivars used in drought and salt stress experiments. Relative gene expression ($\log_2$) in comparison to the housekeeping genes is shown for drought- and salt-tolerant (52, 4, 1) and drought- (22, 2, 50) and salt-sensitive cultivars (22, 2, 50) under control conditions. Data represent the means of three cultivars from either one or two biological replicates with three technical replicates each. Significance levels are indicated as: $0.01 < p < 0.05$ (*) in comparison to the respective tolerant cultivars.

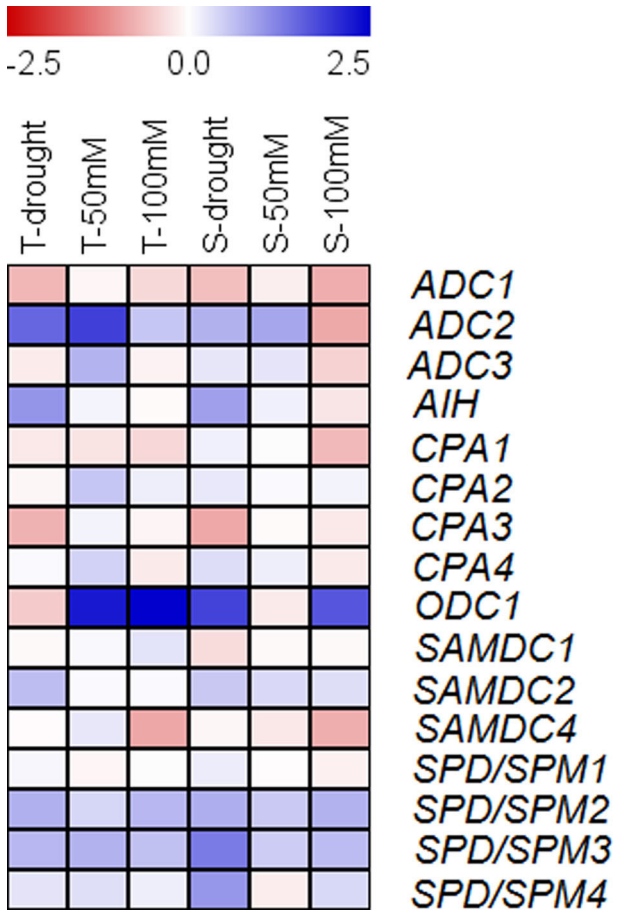

FIGURE 8 | Log_2 fold change of expression of genes, encoding enzymes involved in polyamine biosynthesis, under drought and salt conditions as compared to control in three tolerant vs. sensitive cultivars of the respective stress condition. Log_2 fold change of gene expression is shown for drought- and salt-tolerant (T) (52, 4, 1) and drought- (22, 2, 50) and salt-sensitive cultivars (S) (22, 2, 50). Data represent the means of three cultivars from either one or two biological replicates with two technical replicates each.

EFFECTS OF SALINITY STRESS ON FREE POLYAMINE CONTENTS IN LEAVES

A general increase of Spm was observed under salinity conditions for all cultivars in the present study, which is in agreement with previous results obtained in rice (Krishnamurthy and Bhagwat, 1989; Maiale et al., 2004) and other species (Sanchez et al., 2005). Spm may be involved in the stabilization of membranes (Tassoni et al., 1998; Rajasekaran and Blake, 1999) and nucleic acids (Hultgren and Rau, 2004), scavenging of free radicals (Lester, 2000), osmotic adjustment (Aziz et al., 1999), regulation of ion nutrition (Chattopadhyay et al., 2002), and regulation of senescence (Lahiri et al., 2004). It can improve the viability of protoplasts (Tiburcio et al., 1986) and participates in the prevention of electrolyte leakage and chlorophyll loss (Chattopadhyay et al., 2002) as well as enhanced stem elongation growth (Rajasekaran and Blake, 1999). Polyamines are also able to block ion channels thereby reducing NaCl-induced K^+ efflux proportional to their charge (Zhao et al., 2007). Furthermore Spm can provoke a net Ca^{2+}-efflux which might influence ROS and PA signaling (Pottosin et al., 2012).

These multiple functions suggest a beneficial role for Spm under stress conditons, independent of the tolerance level. Spm was also identified as the polyamine responsible for salt acclimation in *Arabidopsis* using transgenic lines overexpressing oat ADC (Alet et al., 2011b). Furthermore it was shown that *Arabidopsis* mutants, defective in the synthesis of Spm (*spms-1*), accumulated more Na^+ and were impaired in survival experiments compared to control (Alet et al., 2012).

Despite only slight changes of Spd content in most of the cultivars under salinity, a significant negative correlation of Spd levels and of the changes under salt stress with salt sensitivity was found. This is in agreement with findings of Krishnamurthy and Bhagwat (1989) who reported that salt-tolerant rice accumulates high levels of Spd and Spm. Over-expression of *SPD* from *Cucurbita ficifolia* in *Arabidopsis* results in significantly increased Spd content in leaves and in enhanced tolerance to various abiotic stresses (Kasukabe et al., 2004). Spd treatment also induced recovery from salinity-induced damage of the plasma membrane and PM-bound H^+-ATPase in salt-tolerant as well as salt-sensitive cultivars (Roy et al., 2005).

Strikingly, polyamine levels changed differently in tolerant and sensitive cultivars, with higher levels of Put under control conditions and a stronger decrease under salt stress in sensitive cultivars. This resulted in a shift of the predominant polyamine at 100 mM NaCl from Put in tolerant to Spm in the three most sensitive cultivars. These results may help to explain contradictory findings for changes of Put levels in the literature. It was earlier suggested that endogenous levels of Put might be limiting for salt resistance (Gupta et al., 2013). Our results are in agreement with Maiale et al. (2004), who also found a larger decrease of Put in sensitive as compared to tolerant cultivars. However, it has also been reported that Put strongly accumulates in response to osmotic (Flores and Galston, 1982; Aziz and Larher, 1995; Liu et al., 2004) and salinity stress (Basu et al., 1988; Krishnamurthy and Bhagwat, 1989; Katiyar and Dubey, 1990; Lefèvre et al., 2001). This accumulation was considered to be protective, conferring a selective advantage to the stressed plants. Put was reported to stabilize membranes (Prakash and Prathapsenan, 1988) and to counteract the Na^+ and Cl^- accumulation and induction of the K^+ efflux (Prakash and Prathapsenan, 1988; Ndayiragije and Lutts, 2006). Polyamine specificity is more and more discussed with dominant polyamines generating a specific signature for the response to a specific stress (Pottosin et al., 2014). Tolerance dependent differences of Put levels under control conditions were independent of subspecies and might be used as potential markers for future breeding efforts.

EXPRESSION LEVELS OF GENES ENCODING ENZYMES INVOLVED IN POLYAMINE BIOSYNTHESIS UNDER SALINITY CONDITIONS

To elucidate the molecular basis for changes in polyamine biosynthesis in response to salinity conditions, expression levels of genes encoding enzymes involved in polyamine biosynthesis were analyzed. The expression of many of these genes under different stress conditions and at different growth stages has been analyzed before

(Li and Chen, 2000a,b; Kwak and Lee, 2001; Piotrowski et al., 2003; Tian et al., 2004; Hao et al., 2005a; Rodríguez-Kessler et al., 2006). However, the expression profiles of all genes involved in polyamine biosynthesis were previously only studied in rice under drought (Do et al., 2013), but not under salt stress. In *Arabidopsis*, the expression of all genes was investigated under dehydration (Alcázar et al., 2006b), while studies with *Arabidopsis* under salt, dehydration, cold and ABA treatments (Urano et al., 2003) and with maize under salt stress (Rodríguez-Kessler et al., 2006) did not include expressions of *AIH* and *CPA*.

We found expression levels of *ADC2*, *SAMDC2*, *SPD/SPM2,* and *SPD/SPM3* induced at 50 mM NaCl in most of the cultivars. Among the three *ADC* genes, mainly *ADC2* was up-regulated. *ADC2* was also found to be stress-induced in *A. thaliana* (Soyka and Heyer, 1999), and mustard (Mo and Pua, 2002). Whereas we found no correlation between the $\log_2$ fold change of gene expression and the salt sensitivity of the cultivars, another study reported a correlation of the accumulation of an *ADC* transcript with salt stress tolerance in rice (Chattopadhyay et al., 1997). In addition, tolerant cultivars activated two pathways to synthesize Put via arginine and ornithine by higher expression of *ADC2* and *ODC1*, whereas sensitive cultivars only induced one of these genes. Increased synthesis of Put catalyzed by two alternative pathways might be advantageous for the further accumulation of Spm catalyzed by, e.g., *SAMDC2*, *SPD/SPM2,* and *SPD/SPM3*. Enzyme activity measurements will be necessary to confidently link gene expression data to polyamine pool sizes.

COMPARATIVE ANALYSIS OF POLYAMINES UNDER DROUGHT AND SALT STRESS

Our results clearly show that Spm content was significantly increased under both, drought, and salinity conditions, except for tolerant cultivars under drought, which kept their already high initial levels. Under drought stress Spm became the most prominent polyamine, whereas this was only true for the most sensitive cultivars at 100 mM NaCl. The increase in Spm content is consistent with a report by Maiale et al. (2004) for rice under salinity conditions, but contradictory to reports by Krishnamurthy and Bhagwat (1989) for salt and by Liu et al. (2004) for osmotic stress. In these studies, tolerant rice cultivars accumulated higher levels of Spd and Spm, while sensitive rice cultivars showed low levels of these substances and an increase in Put levels. Nevertheless, Spm accumulation seems to be a general feature of plant responses to drought and salinity stress, although its physiological role under stress is still partly unknown. Elevated polyamine levels under salt stress seem to have self-protecting effects due to the modulation of ion channels thereby mediating ion flux homeostasis (Zhao et al., 2007). In roots the immediate effect of polyamines on NaCl-induced K^+ efflux was dependent on the plant and the polyamine and ranged from beneficial to detrimental (Pottosin et al., 2014). In barley alterations of K^+-homeostasis, caused by interaction between polyamines and ROS, contributed substantially to genetic variability in salt-sensitivity (Velarde-Buendía et al., 2012).

Together with Spm, Spd may also be involved in the response of plants to stress, e.g., through the induction of stomatal closure (Liu et al., 2000), prevention of chlorophyll loss (Chattopadhyay et al., 2002), stabilization of membranes (Rajasekaran and Blake, 1999) and scavenging of free radicals (Velikova et al., 1998). Several studies reported an accumulation of Spd under salt (Krishnamurthy and Bhagwat, 1989; Basu and Ghosh, 1991) and osmotic stress (Tiburcio et al., 1986; Li and Chen, 2000a). In contrast, decreased Spd levels in response to stress were also reported under salt (Maiale et al., 2004; Sanchez et al., 2005), osmotic (Aziz et al., 1997), and drought stress (Turner and Stewart, 1986). In this study, a reduction of Spd was only observed under drought conditions, whereas under salinity condition the Spd content was unchanged. The ability of Spd to prevent the uptake of Na^+ and the loss of K^+ (Chattopadhyay et al., 2002) may suggest that high Spd levels could be more important under salt than under drought stress.

Put levels decreased in our experiments in leaves under drought stress independent of tolerance of the cultivar, while under salinity conditions they were not changed in tolerant and sharply decreased in sensitive cultivars. For salt-sensitive cultivars threefold higher Put levels were observed under control conditions in hydroponic culture compared to cultivation in sand, with Put levels at 50 mM NaCl reaching values comparable to values under control conditions in sand grown plants. Nevertheless this will not affect the comparison between different stress conditions due to the restriction to relative changes in Put levels in comparison to control levels. The decrease of Put levels in all cultivars under drought and in sensitive cultivars under salinity conditions could be caused by the higher substrate need for the Spm synthesis. A strong metabolic canalization of Put into Spm synthesis induced by drought was also described for *Arabidopsis* and *Craterostigma plantagineum* but did not lead to Spm accumulation in *Arabidopsis* due to a Spm-to-Put back-conversion (Alcázar et al., 2011).

A Spm-to-Put back-conversion by polyamine oxidase (PAO) might have also occurred, indicated by the fact that Spm accumulation after salt stress was lower in salt sensitive than in salt tolerant cultivars. On the other hand, Put levels in poplar and tomato did not affect Spm levels, while Spd and Spm levels are inter-dependent (Mattoo et al., 2010). Reduced Put levels could be also reached by the action of DAO yielding pyrroline, H_2O_2 and ammonia (Moschou et al., 2012) but avoiding high Put levels, which might be toxic for plants (Slocum et al., 1984; Panicot et al., 2002). For an estimation of degradation processes an analysis of gene expression and enzyme activities of DAO and PAO would be necessary. Due to the involvement of polyamines in stress response as well as programmed cell death the balance between intracellular polyamine concentrations and polyamine catabolism resulting in ROS generation in the apoplast will be crucial for the survival of plants (Pottosin et al., 2014).

Another interesting finding of our study was a descending gradient of Put levels from sensitive to tolerant cultivars already under control conditions. This was also shown previously for the Put levels and drought tolerance of a set of 21 rice cultivars (Do et al., 2013). The higher Put content in sensitive cultivars under control conditions might be a useful tool for breeders to select against sensitivity in breeding programs. In conclusion, we have shown that polyamines are strongly involved in the response of rice to drought and salinity stress. From our results and previous

reports we hypothesize that Spm contributes to the drought and salinity tolerance of rice, while the involvement of Spd and Put particularly in salt tolerance still needs to be clarified.

COMPARISON OF EXPRESSION OF GENES ENCODING ENZYMES INVOLVED IN POLYAMINE BIOSYNTHESIS BETWEEN SALT AND DROUGHT STRESS

The comparison of tolerant and sensitive cultivars under the different stress conditions indicated a general up-regulation of *ADC2*, except for sensitive cultivars at 100 mM NaCl. This higher expression could provide an advantage for stress adaptation, e.g., through the *de novo* synthesis of Put as a substrate for longer chain polyamines. Arabidopsis plants over-expressing the *ADC* gene from oat under the control of a stress-inducible promoter were more resistant to dehydration stress associated with an increase of putrescine levels (Alet et al., 2011a). *ADC2* was additionally identified as salt- and drought-induced in *Arabidopsis* in several studies, as reviewed in Alcázar et al. (2006a). Under salt stress at 100 mM NaCl cultivars of the sensitive group seem to compensate the lower *ADC2* induction by the induction of *ODC1*, while tolerant cultivars activated both pathways under salinity which is in contradiction to reports showing that the ADC pathway for polyamine biosynthesis is predominant in higher plants (Birecka et al., 1985; Rajam, 1993; Rodríguez-Kessler et al., 2006). The existence of two alternative routes for the synthesis of Put could be explained by the differential compartmentation of the two enzymes resulting in the specific regulation of different plant processes. With the localization of ADC in chloroplasts, polyamines synthesized via the ADC pathway seem to play a role in maintaining photosynthetic activity (Borrell et al., 1995). Additionally, ADC is thought to be the enzyme primarily responsible for abiotic stress-induced Put accumulation (Galston and Sawhney, 1990; Tiburcio et al., 1997). ODC is found in the nucleus (Slocum, 1991) and mitochondria (Acosta et al., 2005). It has been suggested that ODC is involved in the regulation of cell division/proliferation in growing plant tissues, while ADC is involved in cell expansion (Cohen, 1998).

The transcript level of *AIH* was reported to be up-regulated under drought stress in tolerant as well as sensitive cultivars (Do et al., 2013), but was unchanged in response to salt stress. One report is available for *A. thaliana* under dehydration stress (Alcázar et al., 2006b), where the expression level of *AIH* was slightly increased, in agreement with the results from rice under drought stress.

The *CPA* genes of rice show different expression levels under control conditions both in plants grown in sand and in hydroponic culture. The expression levels of the four genes are related as follows: *CPA*1 > *CPA*2 ≥ *CPA*4 > *CPA*3. Except for a reduction in the expression level of *CPA3* under drought stress (Do et al., 2013), no change was detectable for any *CPA* gene under drought or salt stress, showing that these genes are constitutively expressed. This result is in accordance with the observation that the transcript level of the *CPA* gene in *A. thaliana*, which is similar to *CPA1* from rice, was not altered by osmotic stress (Piotrowski et al., 2003). A drought-dependent repression of *CPA3* in rice has not been reported before.

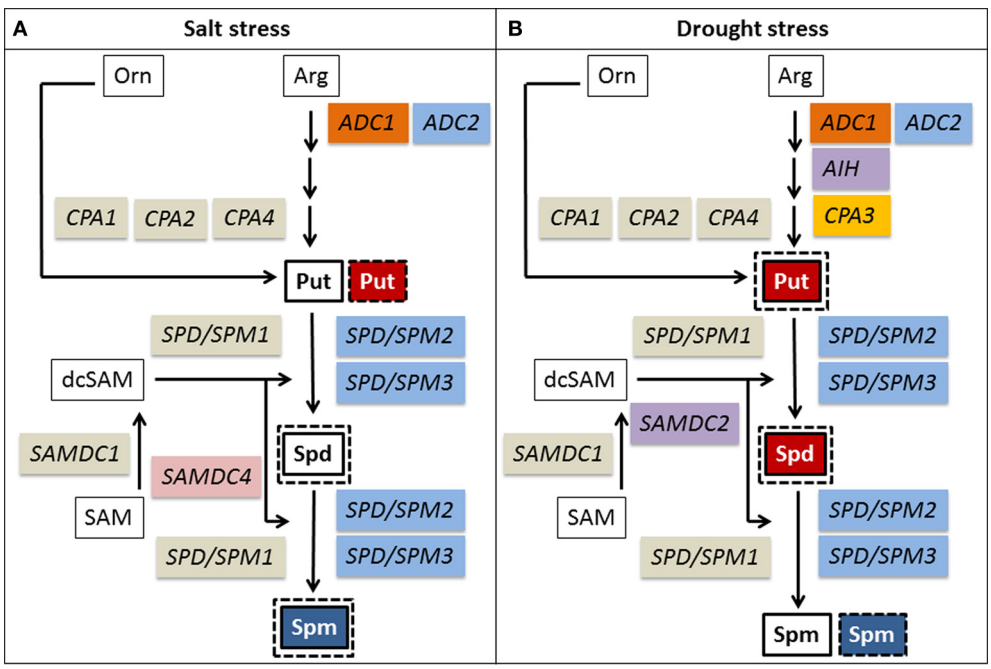

FIGURE 9 | Changes of polyamine metabolism in response to salt (A) and drought stress (B) in rice leaves of tolerant and sensitive cultivars. Changes of polyamines are shown for tolerant (solid line) or sensitive cultivars (dotted line). A decrease is shown in red, an increase in blue. Changes in gene expression under stress conditions are color coded as follows: constitutively expressed—gray, induced by drought and salt stress—blue, induced by drought—purple, repressed by salt and drought stress—orange, repressed by drought stress—yellow, repressed by salt stress—pink. Arg, arginine; Orn, ornithine; SAM, S-adenosylmethionine; dcSAM, decarboxylated S-adenosylmethionine.

Several enzymes are involved in the pathway from Put to Spd and Spm, including *SAMDC* and *SPD/SPM*. In response to drought and salt stress, the expression levels of *SAMDC2*, *SPD/SPM2*, and *SPD/SPM3* were up-regulated with the exception of *SAMDC2* in tolerant cultivars under salt stress. Increased expression of *SAMDC* was also reported in rice (Li and Chen, 2000b; Kawasaki et al., 2001; Rabbani et al., 2003; Shiozaki et al., 2005), wheat (Li and Chen, 2000a), soybean (Tian et al., 2004), and *Arabidopsis* (Alcázar et al., 2006b) under drought and salt, and in maize (Rodríguez-Kessler et al., 2006) for *SPD/SPM* under salt conditions. In addition, a tolerant rice cultivar responded more quickly to salt and reached the highest expression level of the *SAMDC* gene under short-term stress, while the sensitive cultivar reached higher levels after a prolonged time of stress (Li and Chen, 2000b). In our study, a differential regulation of *SAMDC* genes among the different tolerance groups was not observed. However, *SAMDC4* was specifically down-regulated at 100 mM NaCl. Under all conditions and over all tolerance groups an induction of *SPD*/SPM*2*, *SPD/SPM3*, and *SPD/SPM4* genes was observed. A spermine synthase was also shown to be salt- and drought-induced in *Arabidopsis*, as reviewed in Alcázar et al. (2006a), and a spermine synthase mutant of *Arabidopsis* was shown to be more sensitive to drought and salt stress (Yamaguchi et al., 2007).

Changes in gene expression and in polyamine levels in differentially tolerant cultivars under salt or drought stress conditions are summarized in **Figure 9**. By comparing the gene expression analyses under salt and drought stress in tolerant and sensitive cultivars, three genes that had previously been classified as drought-induced (Do et al., 2013) are now identified as generally stress-induced (*ADC2*, *SPD/SPM2*, *SPD/SPM3*), whereas *AIH* was confirmed as specifically drought-induced. *SAMDC2*, on the other hand, was induced by drought and was only induced by salt stress in sensitive cultivars. *ADC1*, which was previously classified as drought-repressed could now be identified as generally stress-repressed, whereas *CPA3* was confirmed as specifically drought-repressed. Only *SAMDC4* could be identified as salt-stress repressed gene. Five genes that were previously classified in drought stress experiments (Do et al., 2013) as constitutively expressed (*CPA1, CPA2, CPA4, SAMDC1, SPD/SPM1*) were confirmed under salt stress.

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Physiological and molecular implications of plant polyamine metabolism during biotic interactions

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During ontogeny, plants interact with a wide variety of microorganisms. The association with mutualistic microbes results in benefits for the plant. By contrast, pathogens may cause a remarkable impairment of plant growth and development. Both types of plant–microbe interactions provoke notable changes in the polyamine (PA) metabolism of the host and/or the microbe, being each interaction a complex and dynamic process. It has been well documented that the levels of free and conjugated PAs undergo profound changes in plant tissues during the interaction with microorganisms. In general, this is correlated with a precise and coordinated regulation of PA biosynthetic and catabolic enzymes. Interestingly, some evidence suggests that the relative importance of these metabolic pathways may depend on the nature of the microorganism, a concept that stems from the fact that these amines mediate the activation of plant defense mechanisms. This effect is mediated mostly through PA oxidation, even though part of the response is activated by non-oxidized PAs. In the last years, a great deal of effort has been devoted to profile plant gene expression following microorganism recognition. In addition, the phenotypes of transgenic and mutant plants in PA metabolism genes have been assessed. In this review, we integrate the current knowledge on this field and analyze the possible roles of these amines during the interaction of plants with microbes.

Keywords: polyamines, spermidine, spermine, putrescine, thermospermine, plant pathogen, mutualism

INTRODUCTION

The aliphatic polycationic compounds collectively known as polyamines (PAs) have proven to be essential for growth and development in all living organisms (Cohen, 1998). PAs participate in many fundamental processes such as chromatin condensation, DNA replication and transcription, RNA processing, protein synthesis and post-translational modification (Igarashi and Kashiwagi, 2000; Childs et al., 2003; Wallace et al., 2003). The most common PAs in nature are the diamine putrescine (Put), and those traditionally called higher PAs as the triamine spermidine (Spd) and the tetraamines spermine (Spm) and thermospermine (TSpm). In addition, other compounds are also regarded as PAs, such as the diamines 1,3-diaminopropane (DAP) and cadaverine (Cad). Uncommon PAs show a limited distribution in nature and consists of Spd and Spm-related molecules as nor-Spd, nor-Spm, homo-Spd, homo-Spm, and longer penta- and hexaamines (Bagni and Tassoni, 2001; Fuell et al., 2010; Sagor et al., 2013).

In plant tissues, the relative abundance of PAs depends on the species and the developmental stage. They are detected in actively growing plant tissues and in plants exposed to biotic or abiotic stress (Jimenez-Bremont et al., 2007; Rodriguez-Kessler et al., 2008; Alcázar et al., 2010; Gill and Tuteja, 2010; Hussain et al., 2011). It has been demonstrated that PAs are important for the normal course of diverse plant ontogenic processes such as cell division and elongation, organogenesis and somatic and zygotic embryogenesis (Hummel et al., 2004; Paschalidis and Roubelakis-Angelakis, 2005; Mattoo et al., 2010). In general terms, the levels of Put in plants are higher than those of the other PAs (Cohen, 1998). Cad can also be found in considerable high concentrations even though it seems to be limited to legumes and a few species belonging to other plant families (Tomar et al., 2013). Additionally, PAs may exist in soluble as well as insoluble fractions. The soluble fraction consists of free PAs and PAs conjugated to organic acids, mainly hydroxycinnamic acid. In turn, the insoluble fraction is formed by PAs bound to macromolecules such as proteins, cell wall polysaccharides and plasma membrane (Martin-Tanguy, 2001).

Although the exact mechanism of action of PAs remains still elusive, many authors have proposed that the spatial separation of their positive charges under physiological pH plays a crucial role. This feature gives them the capacity to bind negatively charged molecules such as nucleic acids, phospholipids, and proteins; and it is thought that by these means PAs affect the structure and function of these macromolecules. Moreover, the formation of these compounds protects macromolecules

from degradation and modification (Ruiz-Herrera et al., 1995; D'Agostino et al., 2005).

There is a considerable amount of information about the role played by PAs during the interactions of plants with either pathogenic or beneficial microbes. These data indicate that changes in PA metabolism constitute a key adaptive response of the plant, and that their occurrence determines the development of the interaction. In turn, evidence suggests that some microorganisms are able to perturb plant PA metabolism in order to adjust it to their own requirements.

In this review, we integrate the current knowledge on the modulation of plant PA metabolism upon pathogen recognition and its importance for plant defense, giving particular emphasis in the advances achieved by the scientific community in the last few years. In addition, we discuss the importance of PAs in beneficial plant interactions, as those established by nitrogen-fixing bacteria and mycorrhizal fungi. The impact of biotechnological approaches and genetic engineered plants with altered PA levels in plant–microbe interactions is also reviewed.

PA METABOLISM IN PLANTS

The concentration of PAs is finely controlled in eukaryotic cells in order to fulfill the requirements at any time. Precise regulation of PA levels is important since not only its depletion, but also extensive PA accumulation may be deleterious (Ditomaso et al., 1989; He et al., 1993; Poulin et al., 1993). Therefore, several processes such as PA synthesis, catabolism, transport, compartmentalization, and conjugation act in concert to maintain the concentration of these compounds in suitable levels (reviewed by Kusano et al., 2007; Carbonell and Blázquez, 2009). We briefly summarize below the principal aspects of plant PA metabolism.

BIOSYNTHESIS OF PAs

In plants, the synthesis of Put may be conducted by two pathways (Martin-Tanguy, 2001). One of these metabolic routes starts with the decarboxylation of the amino acid arginine by the enzyme arginine decarboxylase (ADC, EC 4.1.1.19), located mainly in the chloroplast in photosynthetic cells but directed to the nucleus in non-photosynthetic cells (Borrell et al., 1995; Bortolotti et al., 2004). This reaction renders agmatine, which is then metabolized to N-carbamoylputrescine by agmatine iminohydrolase (AIH, EC 3.5.3.12). Consequently, N-carbamoylputrescine is converted in Put by the enzyme N-carbamoylputrescine amidohydrolase (NCPAH, EC 3.5.1.53). The other metabolic pathway begins with the amino acid ornithine converted into Put in a single-step reaction catalyzed by the enzyme ornithine decarboxylase (ODC, EC 4.1.1.17), which is mainly found in the plant cytosol. The synthesis of higher PAs requires decarboxylated S-adenosylmethionine (dcSAM) as aminopropyl donor, generated by the action of the enzyme S-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50). dcSAM is then used by the enzyme spermidine synthase (SPDS, EC 2.5.1.16) to add an aminopropyl moiety to Put, hence forming Spd. In a consecutive aminopropylation reaction, the enzyme spermine synthase (SPMS, EC 2.5.1.22) adds an aminopropyl group to Spd converting it to Spm. Alternatively, thermospermine synthase (tSPMS, EC 2.5.1.79) forms TSpm by adding the aminopropyl group to the opposite end of the molecule of Spd.

The relative importance of ADC and ODC activities for Put synthesis varies according to the plant species and the tissue involved (Cohen, 1998; Hanfrey et al., 2001; Flemetakis et al., 2004). In some plant species the *ODC* gene is absent, as occur in many members of the *Brassicaseae* family including *Arabidopsis thaliana*, and in non-vascular plants such as *Physcomitrella patens* (Jiménez-Bremont et al., 2004; Fuell et al., 2010). In addition, whereas in most plants there is only one gene coding for ADC, two paralogous genes with different patterns of expression occurs in *Brassicaseae* members (Galloway et al., 1998; Hummel et al., 2001). The disruption of each *ADC* gene in *Arabidopsis* does not cause a major phenotypic change, but knocking down both has a lethal effect (Urano et al., 2005).

The *Arabidopsis* genome contains four genes coding for SAMDCs, two SPDSs, one SPMS and one tSPMS (known as *ACAULIS5*; Fuell et al., 2010). SPDS and SPMS seem to be localized in the cytoplasm and the nucleus (Belda-Palazón et al., 2012), while SAMDC appears to be mostly cytoplasmic (Torrigiani et al., 1986; Belda-Palazón et al., 2012). Plant SAMDCs are usually regulated at the translational level by PAs (Hanfrey et al., 2002, 2003). It has been shown that a small upstream open reading frame (uORF) in the SAMDC mRNA 5′ leader sequence is responsible for RNA translation repression mediated by PAs. This mechanism regulates the availability of dcSAM for Spd and Spm synthesis, which seems to constitute the main point of regulation for the synthesis of higher PAs in these organisms (Fuell et al., 2010). The double mutants *atsamdc2*(*bud2*)-*atsamdc1* (Ge et al., 2006) and *atspds1-atspds2* (Imai et al., 2004b) are embryo lethal, indicating that higher PAs are essential for plant embryogenesis. This effect could be due to a deficiency in Spd synthesis and not Spm, since no phenotypic effect is evident in *spms* plants (Imai et al., 2004a). In turn, disruption of the *ACAULIS5* gene in *Arabidopsis* plants lead to a notable dwarf phenotype, demonstrating the importance of TSpm synthesis for plant growth (Imai et al., 2004a).

CATABOLISM OF PAs

Polyamine catabolism is mediated by diamine oxidases (DAOs, EC 1.4.3.6) and PA oxidases (PAOs, EC 1.5.3.3). DAOs are homodimers with copper as the prosthetic group. These enzymes show a preference for diamines such as Put and Cad, and have low affinity for Spd and Spm. The oxidation of Put renders H_2O_2, NH_4^+ and γ-aminobutanal, the latter being spontaneously converted to Δ^1-pyrroline. DAOs from legumes and *Arabidopsis* have been described to be directed to the extracellular space, remaining in this compartment loosely associated to cell walls (Federico and Angelini, 1991; Moschou et al., 2008b). Recently, it was demonstrated that *Arabidopsis* has at least ten putative *DAO* genes, four of which have been characterized: *AtCuAO1* and *AtAO1* encode for apoplastic proteins, whereas the products of *AtCuAO2* and *AtCuAO3* are found at the peroxisome (Planas-Portell et al., 2013). Interestingly, *DAO* genes show different expression profiles in response to external stimuli (Planas-Portell et al., 2013).

Polyamine oxidases use FAD as cofactor and oxidize mainly Spd and Spm. These enzymes may be involved in two different

catabolic pathways: (1) terminal catabolism and (2) back-conversion of PAs. PAOs acting in the terminal catabolism pathway oxidize Spd or Spm using molecular O_2 as electron donor. In this case, DAP and H_2O_2 are released as final products, besides either 4-aminobutanal or (3-aminopropyl)-4-aminobutanal depending on whether Spd or Spm act as substrate, respectively. Alternatively, enzymes back-converting PAs transform Spm into Spd, and Spd into Put with the concomitant production of 3-aminopropanal and H_2O_2. Both types of reactions produce H_2O_2 as final product, which plays an essential role in plant–microbe interactions. Five genes coding for PAOs have been identified in *Arabidopsis*, showing cytosolic (AtPAO1 and AtPAO5) and peroxisomal (AtPAO2, AtPAO3, and AtPAO4) localization (Moschou et al., 2008c; Takahashi et al., 2010; Fincato et al., 2011). It has been demonstrated that AtPAO1 to AtPAO4 catalyze the back conversion of PAs (Tavladoraki et al., 2006; Kamada-Nobusada et al., 2008; Moschou et al., 2008c; Fincato et al., 2011). The peroxisomal back-converting pathway has also been described in rice (Ono et al., 2012), even though all other enzymes characterized in monocotyledonous plants so far are involved in the terminal catabolic pathway (Tavladoraki et al., 1998; Šebela et al., 2001). It was recently found that *AtPAO2* in *Arabidopsis* is negatively modulated in a PA-dependent manner by a uORF located in the 5′-UTR sequence (Guerrero-González et al., 2014). A comparative analysis of several PAO transcripts from different plant species indicated that uORF sequences are very conserved, suggesting a common regulatory mechanism for these enzymes.

TRANSPORT AND CONJUGATION OF PAs

The transport of PAs into different cell compartments is a crucial step to regulate several cell processes (Igarashi and Kashiwagi, 2010; Mulangi et al., 2012b). Only PA importers have been characterized so far at the functional level in plant cells. The first evidence of PA import was found in carrot protoplasts and isolated vacuoles, and it was suggested that PA entrance is driven by the transmembrane electrical gradient and a possible antiport mechanism (Pistocchi et al., 1988). It was found that external pH and the concentration of calcium modulate PA uptake (Antognoni et al., 1994; Pistocchi et al., 1988). Recently, the identification and characterization of specific importers has been achieved in rice and *Arabidopsis* (Fujita et al., 2012; Mulangi et al., 2012a). The first of these proteins reported was OsPUT1 (polyamine uptake transporter) from rice. Radiological uptake and competitive inhibition experiments in a heterologous system revealed that OsPUT1 functions mainly as a Spd importer and that the gene is expressed in all tissues except for mature roots and seeds (Mulangi et al., 2012b). Five additional importers from *Arabidopsis* and rice were described to form part of the same clade with *OsPUT1* and show high affinity for Spd (Mulangi et al., 2012a). A LAT (L-type amino acid transporter) protein in *Arabidopsis* named RMV1 is involved in the proton-dependent incorporation of PAs with a high affinity for Spm (Fujita et al., 2012). Lastly, PA transport into phloem and xylem tissues has been reported (Friedman et al., 1986), but the cell components involved in this processes have not yet been characterized.

Conjugation of PAs is an additional mechanism used by plant cells to regulate the intracellular concentration of the free forms, and these conjugates may comprehend up to 90% of the total PA cell content in some plant species (Bagni and Tassoni, 2001). It has been shown in *Nicotiana attenuata* that different hydroxycinnamoyl transferases (HCTs, EC 2.3.1.138) catalyze the conjugation of caffeoyl, cinammoyl, and feruloyl-CoA to Put or Spd (Onkokesung et al., 2012). It is unknown whether the deconjugation of these compounds is catalyzed by the same transferases. Interestingly, the genes coding for HCTs in *N. attenuata* are induced by the transcription factor R2R3-NaMYB8, which is known to mediate the defense responses against herbivore attack (Kaur et al., 2010).

PAs AND PLANT–MICROBE INTERACTIONS

Plant PA metabolism undergoes remarkably changes during plant–microbe interactions (Walters, 2003; Hussain et al., 2011). The roles played by PAs during these processes are considerably intricate. This is due to the fact that PAs are not only essential to maintain cell viability, but function as signaling molecules regulating many of the responses that help the plant to cope with biotic stress. As will be discussed later, this effect may be exerted either directly by PAs or being mediated through their catabolism. The modulation of PA metabolism is so important for the outcome of the pathogenic interactions that not only the plant modifies PA concentration in response to pathogens, but some microorganisms have developed mechanisms to induce modifications of PA levels in host tissues. Therefore, it seems that when plants are attacked by pathogens, the organism that takes control of the PA machinery has a great opportunity to take the lead. The interaction of plants with beneficial microorganisms also induces changes in PA metabolism, and the establishment of mutualism with symbionts such as Rhizobia and Mycorrhizae appear to depend on PA levels. In the following section we aboard the evidences generated so far regarding the role played by PAs in plant associations with both pathogenic and beneficial microorganisms.

CHANGES IN PLANT PA METABOLISM DURING PATHOGENIC INTERACTIONS

Research indicates that PAs participate actively during the interactions between plants and pathogens. It has been shown that PAs levels and the activity of PA metabolic enzymes augment in infected tissues during microbial colonization, which seems to be independent of the nature of the pathogen, i.e., biotroph or necrotroph (Walters, 2003; Hussain et al., 2011).

Biotrophic microorganisms feed on nutrients produced by the host living cells (Glazebrook, 2005) and the establishment of this type of interaction leads to the accumulation of PAs in plants. For instance, Greenland and Lewis (1984) reported an increase of Spd in barley (*Hordeum vulgare* L.) leaves infected with the rust fungus *Puccinia hordei*, while Spd and Put were increased in this plant species during the infection caused by the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (Walters et al., 1985). Similar effects were demonstrated to occur in the interaction between wheat and *Puccinia graminis* f. sp. *tritici* (Machatschke et al., 1990; Foster and Walters, 1992) and in bean plants treated with fungal

elicitors (Broetto et al., 2005). Importantly, the main increments in PA concentration during the interaction with biotrophs are found in leaf areas close to the pathogen entry point. These areas have been termed "green islands" and are correlated with the induction of photosynthesis and delay in senescence. The accumulation of PAs was explained on the basis of higher activities in plant PA biosynthetic enzymes, in particular that of ADC (Walters and Wylie, 1986; Coghlan and Walters, 1990). Nevertheless, this is not necessarily true for all the interactions involving microorganisms of this kind. For instance, the ODC seems to be responsible for the increment of PAs in wheat leaves infected by *Puccinia graminis* f. sp. *tritici* (Foster and Walters, 1992). In this particular case, the authors reported a reduction of ADC activity but a significant induction of ODC activity in the pustules, the sites were fungal spores brake through the plant epidermis. On these grounds, it was hypothesized that the ODC from the pathogen would be responsible for PA production at the infection site.

Variations in PA levels between resistant and susceptible cultivars in response to pathogens has been described. In this trend, the analysis of two genotypes of barley during the interaction with *B. graminis* f. sp. *hordei* revealed that the resistant cultivar accumulates higher Put and Spd levels than the susceptible one following pathogen infection (Cowley and Walters, 2002a,b; Asthir et al., 2004). Similarly, PAs are accumulated in resistant (but not susceptible) tobacco plants in response to tobacco mosaic virus (TMV; Marini et al., 2001). Accordingly, highly susceptible sugarcane buds infected with the smut fungus *Ustilago scitaminea* reduce free PA contents while increase their conjugation to phenolic acids (Legaz et al., 1998). The authors attributed the susceptibility of the plant to a reduction in the availability of free phenolic compounds with importance for defense rather than a diminution in free PA concentration. In contrast, *Arabidopsis* ecotypes Col-0 and Bur-0, which are susceptible and partially resistant to *Plasmodiophora brassicae* infection, respectively, did not show significant differences in PA contents when challenged with the pathogen. However, susceptible plants displayed a transient accumulation of the PA precursor agmatine and strong arginase activity (the enzyme catalyzing the transformation of arginine in ornithine), whereas partially resistant plants showed continuous agmatine production and weak arginase activity. Based on these observations, it was proposed that symptom development is associated to differences in arginine metabolism and agmatine production (Jubault et al., 2008).

Many studies have shown that any situation leading to free PA accumulation in plants is usually accompanied of a rise in the concentration of conjugated PAs and the induction of PA oxidase activity. This is important because both phenomena could account for the improvement in plant tolerance. Thus, conjugated PAs are accumulated in plant tissues in response to viral and fungal infections (Walters, 2003; Rodriguez-Kessler et al., 2008; Muroi et al., 2009), and it was shown that these compounds affect microbial growth and prevent the development of disease in plants (Martin-Tanguy et al., 1976; Mackintosh et al., 1997; Walters et al., 2001). Muroi et al. (2009) reported the accumulation of conjugates of agmatine and Put in leaves of *A. thaliana* infected with *Alternaria brassicicola*. The importance of these compounds in the defense against this pathogen was clearly demonstrated by the interruption of the *AtACT* gene, encoding an enzyme that conjugates PAs to hydroxycinnamic acid, which enhanced the plant susceptibility. In addition, it has been suggested that PA conjugates contribute to the formation of a phenolic barrier that makes cell walls more resistant to microbial enzymatic hydrolysis (Walters et al., 2001). In turn, PA oxidation could also have a positive effect on plant defense. DAO and PAO activities were induced accompanying the accumulation of free PAs in tobacco and barley plants resistant to TMV and *B. graminis* f. sp. *hordei*, respectively (Marini et al., 2001; Cowley and Walters, 2002a,b). Interestingly, plant resistance to pathogens in these cases depends on the activation of the hypersensitive response (HR), which is characterized by host cell death at the infection site. As one of the key steps triggering the HR is the production of H_2O_2, it is conceivable that the activity of PA catabolic enzymes contributes to elicit this response. In addition, H_2O_2 may promote plant resistance by HR-independent mechanisms, involving cell wall reinforcement, MAPK activation, and modulation of gene expression (Cona et al., 2006a; Nanda et al., 2010). In this regard, PA oxidation was also induced in barley against an incompatible strain of *B. graminis*, where resistance is not associated to HR induction but depends on the ability to avoid penetration of leaf epidermal cells (Cowley and Walters, 2002a). In the same line of evidences, DAO activity is induced in chickpea in response to the necrotrophic pathogen *Ascochyta rabiei*, and its inhibition by 2-bromoethylamine reduces plant resistance (Rea et al., 2002). Necrotrophic pathogens kill the host cells to feed on the remaining, and it has been demonstrated that the activation of HR ultimately favors host colonization (Govrin and Levine, 2000). Therefore, it might be expected that the resistance to *Ascochyta rabiei* mediated by PA oxidation would be due to a mechanism other than HR.

DIRECT MODULATION OF HOST PA METABOLISM BY PHYTOPATHOGENIC MICROBES

Plant tumor-like structures provoked by pathogens show a remarkably induction of PA synthesis and increase in PA concentration. For instance, PAs accumulate in turnip roots infected by *Plasmodiophora brassicae Woron.*, a microorganism that produce hypertrophy and hyperplasia of infected tissues in members of the plant family *Brassicaceae* (Walters and Shuttleton, 1985). Similar increments in free and conjugated PAs were described in maize tumors produced by the fungus *Ustilago maydis* (Rodriguez-Kessler et al., 2008), where ADC and SAMDC activities played a major role. These observations raise an interesting question that has remained elusive so far: are pathogens able to modulate plant PA metabolism in their own favor? Recent studies demonstrate that it might be the case for some interactions. Cytokinins produced by *Rhodococcus fascians* induce Put accumulation in *Arabidopsis* by activating *ADC* expression, which contributes to symptom development (Stes et al., 2011). Moreover, PA synthesis is induced in cabbage protoplasts infected by TYMV and it was demonstrated that these newly synthesized PAs are incorporated by viral particles (Balint and Cohen, 1985). It has not been proven yet if this is triggered by signals generated by this pathogen to assure survival in the host cells. In turn, perhaps

one of the most direct evidences demonstrating the modulation of plant PA metabolism by a pathogen is the interaction between the protein 10A06 secreted by the nematode *Heterodera schachtii* and the product of the *SPDS2* gene of *Arabidopsis*. Interestingly, expression of 10A06 in *Arabidopsis* enhanced plant susceptibility, which was associated to the activation of *SPDS2* gene expression, the increment in Spd concentration and the activation of PAO activity (Hewezi et al., 2010). Surprisingly, over-expression of *SPDS2* was sufficient to affect plant defense. A further examination of these phenotypes demonstrated that *10A06* or *SPDS2* expression increases the abundance of several transcripts coding for host antioxidant enzymes. Based on this, the authors hypothesized that the induction of PAO activity imposes oxidative stress to plant cells and that this is counteracted by the activation of the antioxidant machinery, which might create a less hostile environment for the pathogen. In this same line, it was demonstrated that the protein C2 of the beet severe curly top virus (BSCTV) interacts with SAMDC1 in *Arabidopsis*, which contributes to increment plant susceptibility (Zhang et al., 2011b). In this case, it was found that C2 interacts with a region of SAMDC1 containing a PEST sequence (enriched in the amino acids Pro, Glu, Ser, and Thr), thereby attenuating its degradation at the proteasome. In this context, the stabilization of SAMDC activity leads to a low SAM/dcSAM ratio, thus reducing host and viral DNA methylation. It is though that this hypomethylated environment facilitates viral replication. Whether direct interaction with host PA metabolism enzymes is a general virulence mechanism for pathogens has not been further evaluated. Interestingly, a recent work by Kim et al. (2013a) demonstrated that the interaction between PA metabolic enzymes and pathogen effectors could also be used by plants to trigger defense. Thus, the effector protein AvrBsT of *Xanthomonas campestris* pv. *vesicatoria* is recognized by the ADC1 protein at the cytoplasm of pepper cells, and this interaction enhances AvrBsT-mediated cell death and plant resistance.

TRANSGENIC MODULATION OF PLANT PA LEVELS TO IMPROVE THE TOLERANCE TO PATHOGENS

Several genetically engineered plant lines with altered PA levels show increased tolerance to microorganism attack. This fact constitutes another line of evidence demonstrating the importance of PAs during plant–pathogen interactions. In relation to this, tobacco plants over-expressing the human *SAMDC* gene accumulate free and conjugated PAs and show higher tolerance to pathogens (Waie and Rajam, 2003). Similarly, transgenic eggplants over-expressing the oat *ADC* gene acquired resistance to wilt disease caused by the fungus *Fusarium oxysporum* (Prabhavathi and Rajam, 2007). These plants showed increased ADC activity and accumulation of PAs, particularly the conjugated forms of Put and Spm. As the DAO activity was also enhanced, it was suggested that the acquisition of resistance might be due to an integrated mechanism involving PA biosynthesis and degradation rather than a single event. In turn, over-expression of human *SAMDC* gene in tomato conferred resistance to the fungal pathogens *F. oxysporum* and *Alternaria solani* (Hazarika and Rajam, 2011), and the over-expression of an apple *SPDS* gene in sweet orange enhances plant tolerance to *Xanthomonas axonopodis* pv. *citri* (Fu et al., 2011; Fu and Liu, 2013). At last, the over-expression of the *SPMS* gene in *A. thaliana* resulted in higher Spm levels and enhanced resistance against *Pseudomonas viridiflava* (Gonzalez et al., 2011). Promoting PA oxidation might also favor plant defense. Regarding this, the over-expression of an extracellular PAO from maize in tobacco plants leads to an increment in the expression of genes related to systemic acquired resistance and cell wall-based defense (Moschou et al., 2009). These changes confer disease resistance against *Pseudomonas syringae pv. tabaci* and *Phytophthora parasitica*, but not against infections caused by the *cucumber mosaic virus*.

PAs AND DEFENSE HORMONE METABOLISM

Polyamine metabolism is modulated by exogenous application of phytohormones known to be associated to plant defense. However, only a few works addressed the relationship between PAs and defense hormones in a plant-pathogen system. For instance, treatment of barley primary leaves with methyl-jasmonate (MeJA) induces the increment in free and conjugated Put and Spd, as well as in ODC, ADC, SAMDC, and DAO activities (Walters et al., 2002). Conjugated PA accumulation was extended to non-treated leaves and correlated with a reduction in powdery mildew infection in these tissues. It was also shown that application of MeJA entails PA accumulation in loquat fruits and reduction of the symptoms associated to anthracnose rot caused by *Colletotrichum acutatum* (Cao et al., 2014). Similarly, treatment of wheat with MeJA increases the concentrations of both free and conjugated PAs and induces ODC and PAO activities. The change in PA metabolism was correlated with the induction of pathogenesis related (PR) proteins such as peroxidases and chitinases and a reduction in the incidence of infections by *Puccinia recondita* (Haggag and Abd-El-Kareem, 2009). The over-accumulation of conjugated PAs and induction of PA oxidation mediated by jasmonates (Jas) was also reported to occur in *Hycoscyamus muticus* L. root cultures, tobacco leaf disks and chickpea plants (Biondi et al., 2000, 2003; Biondi and Scaramagli, 2001; Rea et al., 2002). The effect of MeJA on conjugated PA levels is exacerbated in tobacco transgenic lines expressing a *SAMDC* gene in antisense, which showed low SAMDC activity and a relative accumulation of Put (Torrigiani et al., 2005). Interestingly, treatment of Arabidopsis plants with MeJA increases the expression of *ADC2* while *ADC1* remains unaltered, suggesting the existence of different regulatory pathway for both genes (Perez-Amador et al., 2002). Importantly, *ADC2* is regulated by a stress-induced kinase cascade involving MPK3 and MPK6 and is important for defense against *P. syringae* pv. tomato DC3000 (Kim et al., 2013b). Two *ODC* genes are also induced in tobacco in response to MeJA, but its importance for plant defense has not been explored (Xu et al., 2004). The effect of MeJA on PA metabolism may be quite different in other plant species. Thus, it has been shown that MeJA caused a transient inhibition at the transcriptional levels of the expression of *ADC*, *SAMDC*, and *SPDS* in rice (Peremarti et al., 2010). These data could be explained by the existence of contrasting regulatory mechanisms operating over the metabolic enzymes in different plant species. In addition, it should also be considered that the expression of PA metabolism genes not only

depends on the action of one hormone, but it is influenced by several signaling pathways. Thus, it has been demonstrated that the effect of MeJA on ODC and SAMDC activities function synergistically with that of auxins in tobacco leaf disks, but it is counteracted by cytokinins (Biondi et al., 2003). In *Arabidopsis*, MeJA induces the expression of *CuAO1*, *CuAO2,* and *CuAO3*, and it was shown that *CuAO1* and *CuAO3* also respond to abscisic acid (ABA; Wimalasekera et al., 2011b; Planas-Portell et al., 2013).

Salicylic acid (SA) plays a fundamental role in plant defense (Glazebrook, 2005). Unfortunately, the current information concerning the relationship between SA and PAs is scarce. It has been shown that SA is able to induce the accumulation of PAs by activating the expression of *ADC* and *ODC* in maize, tobacco, and tomato (Németh et al., 2002; Jang et al., 2009; Zhang et al., 2011a). Nevertheless, it has not been evaluated yet whether JA and SA act synergistic on the activities of these enzymes. In chickpea plants, however, the application of SA repressed the induction of PA oxidation mediated by JA (Rea et al., 2002). In turn, ABA has been recently demonstrated to induce DAO expression in *Arabidopsis* (Wimalasekera et al., 2011b). ABA is also able to induce Put oxidation at the apoplast of *Vicia faba* (An et al., 2008). This was demonstrated to be important for stomatal closure, a known mechanism contributing to prevent plant colonization by bacterial pathogens. Future research is necessary to understand the connections between SA and ABA metabolism with PAs during plant biotic stress.

The infection of tomato and purple passion (*Gynura aurantiaca*) plants with the citrus exocortis viroid (CEVd) induces ethylene production and represses PR expression. This is accompanied by a reduction in ODC activity, which leads to a decline in Put contents (Belles et al., 1991, 1993). Importantly, the reduction in Put concentration is absent with the use of ethylene biosynthesis inhibitors or compounds that prevent the action of the hormone, suggesting that ethylene exerts an inhibitory effect on ODC activity. Conversely, PAs may also interfere with ethylene metabolism. This was demonstrated in a tomato transgenic line expressing the yeast *SPDS* gene and showing relatively high levels of Spd (Nambeesan et al., 2012). This line exhibits high susceptibility to the attack of the fungus *Botrytis cinerea*, which seems to be due to an attenuated expression of ethylene biosynthetic and signaling genes.

PAs AS MEDIATORS OF PLANT DEFENSE ACTIVATION

Increasing evidences demonstrate that PAs and their catabolic products (i.e., H_2O_2) can act as defense signaling molecules. This function has been mostly assigned to Spm, even though it has been shown that in some systems either Put, Spd, or TSpm participate in defense activation.

During TMV infection, Spm is accumulated in the apoplast of tobacco. This change induces the expression of acidic and basic PRs in a SA-independent pathway and is associated to resistance to virus infection (Yamakawa et al., 1998). Further studies demonstrated that Spm oxidation at the apoplast is crucial to elicit defense responses, and that this pathway involves the activation of key MAPK proteins as SIPK and WIPK (Takahashi et al., 2003; Moschou et al., 2008a). This signaling cascade also requires the opening of the mitochondrial pore and Ca^{2+} influx in this organelle, suggesting that a disruption of the electrochemical potential at this membrane participates in the process. Gene expression analysis in different plant species showed that Spm mediates the induction of several defense genes, such as members of the hairpin-induced 1 (HIN1) family (Takahashi et al., 2004a), proteinase inhibitors (Raju et al., 2009), the transcription repressor ZFT1 (Mitsuya et al., 2007), and HR-associated factors (Takahashi et al., 2004b). In tobacco, the activation of the Spm pathway ultimately leads to the induction of programmed cell death, thus promoting resistance to TMV. In relation to this, it has been shown that Spm is able to activate *NHL10* expression in *Arabidopsis*, another gene induced during HR (Zheng et al., 2005). The involvement of Spm in defense gene activation may be even broader in this species, where 90% of the genes regulated by this PA also respond during the HR elicited by an avirulent virus (Sagor et al., 2009). Most of these genes are important for cell redox homeostasis, protein metabolism and plant defense. Recent evidences demonstrated that the response triggered by Spm is not only restricted to HR-related genes. In this regard, Gonzalez et al. (2011) evaluated the transcriptome of an *Arabidopsis* line overexpressing the *SPMS* gene, which showed high tolerance to the virulent bacteria *P. viridiflava* by mechanisms that are independent of HR induction. This analysis revealed that Spm accumulation induces the expression of genes involved in pathogen perception and defense, including several regulatory proteins such as transcription factors and kinases. By the use of a PAO inhibitor the authors showed that the induction in gene expression may be mostly explained through Spm oxidation, even though part of the response if carried out by the native non-oxidized PA.

Put and Spd were also described to participate in plant defense in *Arabidopsis* and tobacco, respectively. In this trend, Put treatment reduces pathogen susceptibility of an *ADC2* mutant of *Arabidopsis*, suggesting that this diamine is involved in defense responses (Kim et al., 2013b). On the other hand, Spd accelerated the induction of HR and improves the tolerance against TMV of tobacco leaf disks (Lazzarato et al., 2009). In addition, tobacco plants treated with Spd showed higher levels of SA and expression of PRs, besides the induction of systemic acquired resistance. Constitutive expression of a *SPDS* gene in sweet orange also modifies the expression of a large group of genes, many of them with a potential function in plant defense (Fu and Liu, 2013). However, as Put and Spd function as precursors of Spm, it is not clear whether these PAs by their self are able to induce plant defense or rather it is carried out after its conversion to Spm.

In turn, TSpm has received particular attention in the last years. It has been shown that it is equally efficient at inducing defense gene expression as its isomer Spm (Sagor et al., 2012; Marina et al., 2013). In fact, exogenous TSpm is able to restrict CMV multiplication in *Arabidopsis* (Sagor et al., 2012) and improve the resistance of this species to *P. viridiflava* (Marina et al., 2013). In agreement with this, the over-expression of *ACAULIS5* in *Arabidopsis* enhances tolerance to bacterial attack while null mutant lines are more susceptible. Importantly, the use of PA oxidase inhibitors impairs resistance in the *ACAULIS5* transgenic

lines, suggesting that TSpm oxidation is crucial to elicit plant resistance (Marina et al., 2013). Other longer uncommon PA isomers (caldopentamine, caldohexamine, and homocaldopentamine) have recently been described to induce defense gene expression in an even stronger way than that of Spm. These molecules are also oxidized in plant tissues but at lower rates than other PAs (Sagor et al., 2013). In addition, they are able to induce the expression of NADPH oxidase genes, the enzyme that produce superoxide anion (O_2^-) and thus contribute to ROS accumulation.

Finally, it has been suggested that PA action is mediated through the generation of nitric oxide (NO), a key mediator of plant defense. This is based on studies in *Arabidopsis* seedlings where it was shown that exogenous Spd and Spm increased NO production (Wimalasekera et al., 2011a). However, the interplay between PA and NO metabolism during pathogenic interactions in plants has not been evaluated to the present.

Polyamine oxidation plays an essential role during PA signal transduction. Importantly, the activities of these enzymes are increased upon pathogen contact, particularly in resistant cultivars, and transgenic plants constitutively expressing PA oxidase genes present higher tolerance to pathogen attack (Yoda et al., 2006; Moschou et al., 2008a, 2009). Conversely, the use of oxidase inhibitors or oxidase gene deletion strongly impairs plant resistance (Yoda et al., 2009; Choi and Hwang, 2012). Because PAO and DAO activities result in the production of H_2O_2, a process that contributes to elicit host cell death, it has been proven that the importance of PA oxidation depends on the life-style of the pathogen. For instance, oxidation of PAs was demonstrated to be beneficial for plant defense in pathosystems involving biotrophic or hemibiotrophic pathogens (Yoda et al., 2006, 2009; Moschou et al., 2009). By contrast, the induction of host cell death by PA oxidation facilitated pathogen spreading in plants interacting with necrotrophic microorganisms (Marina et al., 2008). The activation of defense responses mediated by these enzymes may not be attributed only to H_2O_2 production, since other reaction products may give rise to intermediaries with a substantial role during biotic stress. For instance, Δ^1-pirroline derived from Put and Spd oxidation is further metabolized to γ-aminobutyric acid (GABA), a compound largely associated to plant defense (Bouche and Fromm, 2004). Additionally, DAP could be also converted in β-alanine and uncommon PAs, all of them proposed to be involved in plant resistance (Cona et al., 2006b).

In particular, PA oxidation might have an important role when occurring at the apoplast. In this trend, Marina et al. (2008) demonstrated that oxidase activities are induced in apoplast of tobacco leaf disks challenged with very dissimilar pathogens such as *P. viridiflava* and *Sclerotinia sclerotiorum*. By the use of specific inhibitors of PA oxidation, they showed that this is important to avoid colonization by the bacteria, which presents a biotrophic lifestyle, but favors the disease caused by the necrotrophic fungus *S. sclerotiorum*. Likewise, the extracellular diamine oxidase AtCuAO1 is induced in *Arabidopsis* by stress-related stimuli as treatment with SA, MeJA, flagellin, and ABA (Planas-Portell et al., 2013). In tobacco, the oxidation of apoplastic PAs induces the HR during TMV infection and this is essential for defense against the bacterium *P. syringae* pv. *tabaci* and the oomycete *Phytophthora parasitica* var. *nicotianae* (Yoda et al., 2003; Moschou et al., 2009). In this trend, the expression of an extracellular PAO from maize in tobacco cells is sufficient to induce programmed cell death when PAs are exogenously added (Rea et al., 2004). Besides the induction of HR, it has been demonstrated that the oxidation of PAs at the apoplast is important to strengthen cell wall during pathogen attack (Cona et al., 2006a; Angelini et al., 2010).

PLANT PA METABOLISM IN THE INTERACTION WITH BENEFICIAL MICROORGANISMS

Many soil-borne microorganisms have been well studied for their beneficial effects on plant growth and are classified into different categories, as nitrogen-fixing bacteria (rhizobia), mycorrhizal fungi, plant growth promoting rhizobacteria, mycoparasitic fungi, etc (Mendes et al., 2013). The current evidence demonstrating the influence of PA metabolism in the establishment and development of beneficial associations is relatively scarce in comparison with that obtained from studies on pathogenic associations. The research conducted in this area has been mainly focused in two groups: the nitrogen-fixing bacteria and mychorrizal fungi.

PAs AND NODULATION IN LEGUMES

Symbiotic nitrogen fixation results from the interaction between legume plants and soil-borne bacteria called rhizobia. This interaction leads to the formation of a novel plant organ, the root nodule, in which rhizobia differentiate into specific nitrogen-fixing bacteroids able to reduce atmospheric nitrogen to ammonia (Oldroyd and Downie, 2008). Many studies demonstrate that nodule initiation and development is regulated by the availability of nitrogen. In addition, it has been shown that changes in PA concentration might affect the control of root nodule number and biomass (Vassileva and Ignatov, 1999; Terakado et al., 2006).

It is well known that root nodules accumulate PAs to levels that are 5 to 10 times higher than in non-symbiotic organs, even though the composition of PAs depends on the legume species studied (Fujihara et al., 1994; Efrose et al., 2008). It has been proposed that PA accumulation in mature nitrogen-fixing nodules could be a mechanism to enhance nodule tolerance to the osmotic stress caused by the establishment of the symbiotic interaction. Studies of symbiosis in *Lotus japonicus* indicate the occurrence of a coordinated spatial and temporal induction of PA biosynthesis genes throughout the nodule life, where the expression of *LjSPDS* and *LjSPMS* is particularly high in the earlier stages of nodule development (i.e., 10 days post-infection) and decline thereafter. Interestingly, the expression pattern of these genes did not correlate with Spd and Spm levels, which are incremented in later stages of nodulation (Efrose et al., 2008). This observation may reflect higher Spd and Spm catabolism rates in early stages, in which H_2O_2 may promote cross-linking of a plant matrix glycoprotein associated with the lumen of the infection threads, the initial structure that allows rhizobia colonization of root hairs (Wisniewski et al., 2000). DAO deficient plant lines are less efficient in cross-linking the matrix glycoprotein but they does not show a difference in nodule formation when compared to control plants, suggesting that Put oxidation plays a

minor role in nodule initiation (Wisniewski and Brewin, 2000). These lines though, are less sensitive to the inhibition of nodule formation that Put exerts on wild type plants. These data indicates that products derived from Put oxidation may delay nodule development.

In the nitrogen-fixing interaction between *Galega orientalis* and *Rhizobium galegae,* the exogenous application of PAs (mainly Put) at concentrations ranging from 10 to 50 μM exerts stimulatory effects in the number and biomass of nodules. In addition, it stimulates nitrogen fixation and enhances the ability of the *Rhizobium* strain HAMBI 540 to attach to *Galega* roots (Vassileva and Ignatov, 1999). However, the exposition of this plant species to higher concentrations of PAs (100 μM) impairs nodule function and originates specific structural abnormalities, indicating that a fine balance in PA concentration must exist in the plant-rhizobia interphase to avoid deleterious effects on nodules and roots.

The relative concentration of each PA appears to be relevant to control nodulation in legumes. In this regard, it has been demonstrated that the soybean supernodulating mutant En6500 maintains high Put levels but low concentrations of Spd and Spm in leaves, roots and nodules in comparison to its parent plant Enrei (Terakado et al., 2006). Importantly, treatment of leaves with an inhibitor of Spd synthesis increases nodule formation in Enrei, whereas Spd and Spm applied to foliar tissues reduce nodulation in both mutant and parental lines. The supernodulating phenotype showed by the mutant line is probably caused by a deficiency in the metabolism of brassinosteroid, a hormone that probably induces Spd synthesis. In fact, exogenous application of the brassinosteriod brassinolide not only reduces root nodule number in this mutant, but also restores Spd levels (Terakado et al., 2006).

Uncommon PAs may also have important functions in nodule development. These PAs have been found in legume root nodules, and in many cases their synthesis and/or accumulation was restricted to these organs (Fujihara, 2009). For example, the tetraamines homo-Spd and the Spm analog canavalmine were identified exclusively in senescent nodules of the sword bean *Canavalia gladiata* (Fujihara et al., 1986). Unsaturated analogs of Spd, such as *N*-(3-aminopropyl)-1,4-diamino-2-butene and N-(4-aminobutyl)-1,4-diamino-2-butene, have been identified in root nodules induced by *Bradyrhizobium japonicum* (Fujihara and Yoneyama, 1999). It is still not clear if these PAs are produced by rhizobia and exported to the plant or whether they are actually produced by the plant cells. In *Medicago sativa* nodules, it was found that the high content of homo-Spd is provided by the bacteroid (López-Gómez et al., 2014).

Likewise, metabolites derived from PA catabolism appear to be important for nodulation. For instance, it has been proposed that GABA (which may be produced from 4-aminobutanal) has a dual role, acting as signaling molecule during plant-bacteria communication and also functioning as an amino acid precursor in bacterial metabolism (Sulieman, 2011). In this trend, feeding with GABA into the phloem sap of *Medicago truncatula* plants causes a short-term increase in nodule activity and a remarkably increment in the amino acid and organic acid content in nodules (Sulieman and Schulze, 2010).

PAs AS INDUCTORS OF THE PLANT-MYCORRHIZAL ASSOCIATION

Plant root symbioses with fungi, referred as mycorrhiza, occur in different forms (Parniske, 2008). In this mutualistic association, the fungi are adapted to live using nutrients provided by the plant, usually nitrogen and carbohydrates. In return, the fungus augments water and minerals availability for the plant. The establishment of this interaction is accompanied by a complex exchange of signaling molecules between the symbiont and the plant, and it has been suggested that PAs may play an important role in this process (Niemi et al., 2002, 2006).

Exogenous application of PAs has a positive effect during the formation of mycorrhizal symbiosis. El Ghachtouli et al. (1995) observed that PAs significantly increased the frequency of mycorrhizal colonization in pea roots. Similarly, application of Put and Spm to trifoliate orange (*Poncirus trifoliata*) seedlings increased mycorrhizal colonization by *Glomus versiforme*, whereas Spd did not influence this parameter (Wu and Zou, 2009). This study also showed that PA treatment enhances leaf Mn^{2+} and root Ca^{2+} uptake. Therefore, it was concluded that PAs stimulate the colonization of roots by the fungus, hence helping the host to absorb minerals. Moreover, it has been reported that the exogenous application of PAs in combination with mycorrhiza colonization alter the morphology of plant roots (Wu et al., 2012). Thus, *Citrus tangerine* plants treated with PAs were better colonized by the fungus *Paraglomus occultum* and showed an increment in root length, diameter, surface area, and volume, as well as higher root branching. Similarly, treatment of *Citrus limonia* seedlings with either Put or Spd increases root length and augments the mitotic index of root tip cells (Yao et al., 2010). These data indicates that PAs are involved in root elongation of citrus plants. Likewise, the exogenous addition of Put, Spm, and Spm at 1 μM improves rooting frequency and provokes root elongation in Virginia pine (*Pinus virginiana*; Tang and Newton, 2005).

Some evidence demonstrates that mycorrhizae formation can result in changes in both free and conjugated PA levels in plant tissues. Thus, Kytöviita and Sarjala (1997) observed that PA concentration (mainly Put and Spd) augmented in root tips of old pine (*Pinus sylvestris*) inoculated with *Suillus variegatus* in comparison with non-mycorrhizal tips. Total free PA pools were also increased in *Lotus glaber* following colonization by *Glomus intraradices*, augmenting the Spd/Spm to Put ratio. It has been suggested that this is one of the mechanisms by which mycorrhizal fungi enhance plant adaptation to saline soils (Sannazzaro et al., 2007). Up to now, little is known about the molecular events that precede the changes in the content of PAs during the establishment of mutualistic association with mycorrhiza-forming fungi.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The experimental work conducted so far clearly indicates that PAs exert important biological activities during the establishment of plant–microbe interactions (see **Figure 1**). In brief, these data indicate that:

(a) Changes in PA levels occur in plants following microbe recognition. PA biosynthesis tends to be induced during this

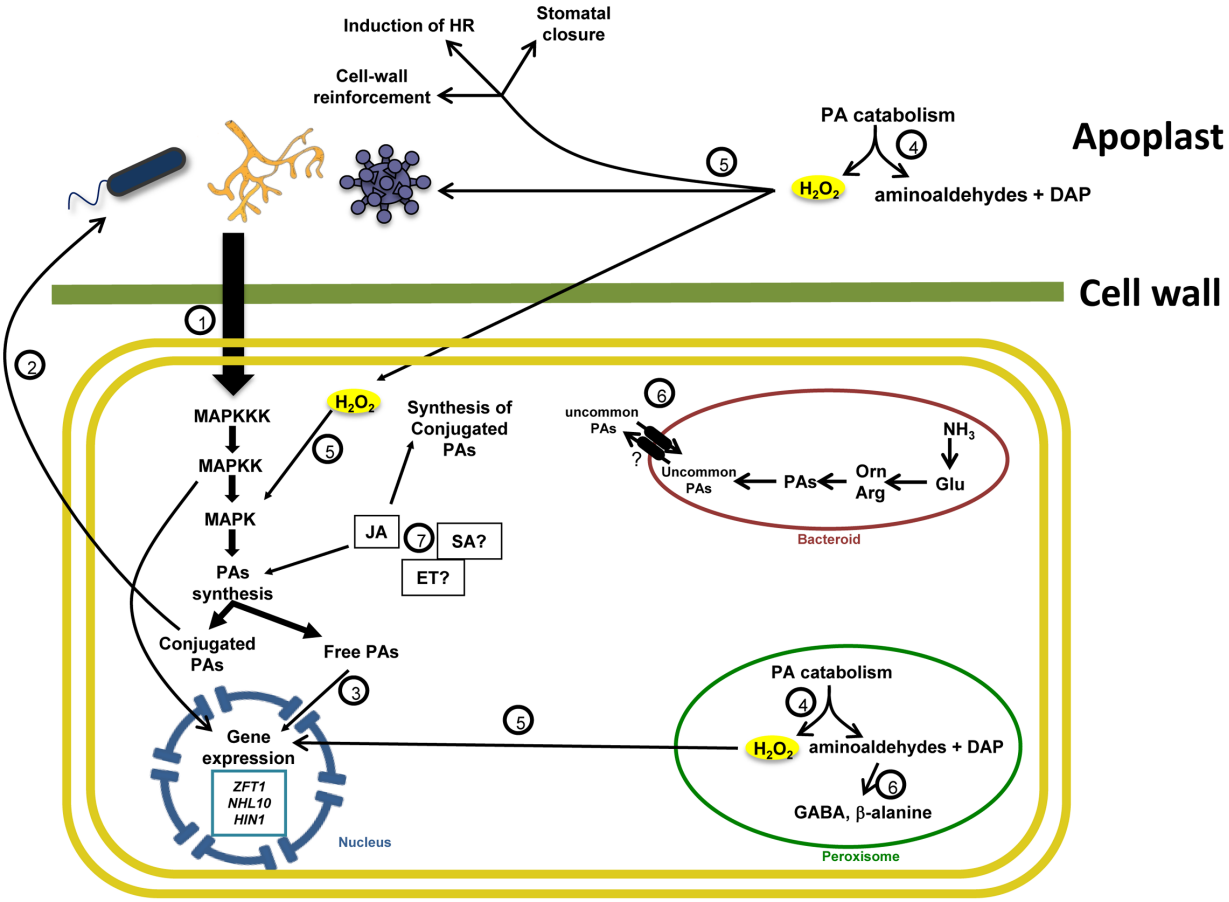

FIGURE 1 | Polyamine metabolism in plant-microorganism interactions. (1) The presence of microorganisms is perceived by specific plant receptors that activate signal transduction pathways. Many MAP kinases, such as WIPK and SIPK, participate in these pathways stimulating defense gene expression and PA biosynthesis. This process leads to the accumulation of free and conjugated PAs. (2) Some conjugated PAs have an antimicrobial effect. (3) In turn, free PAs might contribute to the activation of gene expression directly or through their catabolism. (4) PA oxidation by DAO and PAO occurs in the peroxisome and apoplast, with the generation of H_2O_2, DAP and aminoaldehydes. (5) H_2O_2 acts as signaling molecule and stimulates defense gene expression. In addition, this molecule affect pathogen growth, induces cell-wall reinforcement and stomatal closure, and activates the HR. Other products from the oxidation of PAs may play a defensive role. For instance, aminoaldehydes are precursors for the biosynthesis of GABA in the cytosol and peroxisome, which participate in plant defense and plant-bacteria communication. DAP is a substrate for the synthesis of uncommon PAs and β-alanine, compounds that appear to have an important role during plant biotic stress. (6) Phytohormones such as JA contribute to defense responses by stimulating PA biosynthesis and conjugation. The role of SA and Ethylene in PA metabolism is less clear.

process, which usually lead to the accumulation of the free and conjugated forms of these compounds. The timeline of accumulation as well as the nature of the PAs accumulated depend on the type of the interaction being established and the species involved in the interaction

(b) The role of PAs during plant–microbe interactions might be the result of multiple mechanisms, i.e., their interaction with macromolecules, regulation of gene expression and protein stability, and the induction of defense signaling pathways.

(c) The accumulation of PAs is often accompanied by the induction of PA oxidation. PA catabolism is essential to trigger plant defense responses particularly when occurring at the apoplast, even though part of this response is also induced by non-oxidized PAs.

(d) The establishment of beneficial and pathogenic interactions might be dependent on PAs. Recent evidences suggest that both plant and microorganisms have evolved mechanisms to modulate PA metabolism in their own advantage.

In spite of the above mentioned, there are still several issues to be addressed in order to have a full understanding of the participation of plant PAs in the outcome of these associations. For instance, little is known about the mechanisms used by cells to transport different PAs to organelles and apoplast, and whether these compounds are actively transported between the plant cells and microbes. Additional research is needed to identify the molecular mechanisms that regulate PA metabolism and the main targets of PAs during the induction of plant defense, including the cross-regulation between PAs and phytohormones. These studies should involve not only the most common PAs referred in this review, but also other non-canonical molecules. At last, it is also essential to know the significance of the mechanisms

deployed by microbes to perturb host PA metabolism, as well as the effects that PAs produced by these organisms may have on plant physiology.

Current genetic strategies such as the generation of transgenic and mutant plant lines in addition to genome-wide expression analyses of plants either under pathogenic or mutualistic interactions will help to unravel the regulatory networks that control PA metabolism in plants. The expression of genes of PA metabolism in transgenic lines should be driven by promoters specifically induced by microorganisms, since constitutive expression could offer undesirable characteristics. These studies will provide a better knowledge of the roles played by these biological amines in the development of plant–microbe interactions. It is conceivable that a deeper understanding in this field will make wonderful contributions to the design of better strategies to control plant diseases and favor beneficial plant–microbe associations.

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Stress and polyamine metabolism in fungi

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Fungi, as well as the rest of living organisms must deal with environmental challenges such as stressful stimuli. Fungi are excellent models to study the general mechanisms of the response to stress, because of their simple, but conserved, signal-transduction and metabolic pathways that are often equivalent to those present in other eukaryotic systems. A factor that has been demonstrated to be involved in these responses is polyamine metabolism, essentially of the three most common polyamines: putrescine, spermidine and spermine. The gathered evidences on this subject suggest that polyamines are able to control cellular signal transduction, as well as to modulate protein-protein interactions. In the present review, we will address the recent advances on the study of fungal metabolism of polyamines, ranging from mutant characterization to potential mechanism of action during different kinds of stress in selected fungal models.

Keywords: polyamines, stress response, polyamine mutants, fungi, metabolism

INTRODUCTION

Sudden changes in the external conditions can directly impact the internal environment of all living organisms, and can disrupt their homeostasis and normal physiology. Therefore, cells have developed complex systems to identify the status of their environment, and rapidly generate defense systems against environmental stress (Gasch, 2007; Lushchak, 2011; Montibus et al., 2013). An effective procedure to obtain information on the mechanisms regulating the stress response is the identification of pathways or specific genes suffering changes in their expression under stress conditions. Accordingly, it has been shown that a large number of genes are affected in their expression when an organism responds to an environmental stress. Many of these genes are conserved among fungi, including those involved in carbohydrate metabolism, protein metabolism, defense against reactive oxygen species (ROS), intracellular signaling, etc. (Giaever et al., 2002; Gasch, 2007). Among the known elements related with stress response are polyamines, as it has been widely demonstrated in plants (Galston and Sawhney, 1990; Alcazar et al., 2010; Gill and Tuteja, 2010a; Gupta et al., 2013). However, in recent years, investigations into the molecular genetics of fungal polyamine metabolism have led to the isolation of mutants altered in this function that show alterations in their response to stress. The importance of polyamine participation in the response to stress has been highlighted by the increasing number of reports describing the changes occurring in polyamine concentrations induced in response to diverse forms of stress. Accordingly, in the present review we describe the recent genetic and molecular evidences illustrating the role of polyamine metabolism in the responses of fungi to stress.

POLYAMINE METABOLISM IN FUNGI

BIOSYNTHESIS OF POLYAMINES

Putrescine, spermidine and spermine are low-molecular-mass aliphatic cations critical to cell survival (Tabor and Tabor, 1984), and the pathways for their biosynthesis have been analyzed in all the kingdoms of living organisms [e.g., see Valdes-Santiago et al., 2012a]. However, there are some basic differences in the distribution of polyamines among them. In general, it is accepted that in fungi, as well as in animals, there is only one mechanism to produce putrescine *de novo*: by decarboxylation of ornithine by the enzyme ornithine decarboxylase (Odc, E.C.4.1.1.17), which is the first and rate-limiting enzyme in the synthesis of polyamines. In most fungi decarboxylation of ornithine is the only route to putrescine synthesis, however, in plants there is an additional route to produce putrescine: the decarboxylation of L-arginine by arginine decarboxylase (for more details of the general pathway in plants refer Alcazar et al., 2010; Gupta et al., 2013). Nevertheless, some authors have reported arginine decarboxylase activity in fungi such as *Ceratocystis minor, Verticillium dahliae, Colletotrichum gleosporoides*, and *Gigaspora rosea*, suggesting that both, arginine and ornithine decarboxylase enzymes could be involved in putrescine biosynthesis (Khan and Minocha, 1989; Weerasooriya et al., 2003; Sannazzaro et al., 2004). Putrescine is converted to spermidine by the addition of an aminopropyl group. *S*-adenosylmethionine decarboxylase (Samdc; E.C.4.1.1.50) is responsible for the formation of the donor of the aminopropyl group, decarboxylated S-adenosylmethionine (dcSAM), and spermidine synthase (Spe, E.C.2.5.1.16) is the transferase of the aminopropyl group from dcSAM to putrescine. Finally, spermidine is converted to spermine by a similar reaction, resulting in the transfer of an

aminopropyl group to spermidine by spermine synthase (Sps; E.C.2.5.1.22) (see **Figure 1**). It should be noticed that this last step does not occur in most fungi, which accordingly contain only putrescine and spermidine (Nickerson et al., 1977). An ortholog of the gene encoding Sps is found only in the subphylum Saccharomycotina of the Ascomycota phylum which include few human pathogens and at least 10 phytopathogenic species (Suh et al., 2006; Pegg and Michael, 2010). Also interesting is to mention that *SPE* gene is present in members of Basidiomycota subphyla as a bifunctional gene encoding spermidine synthase and saccharopine dehydrogenase, the last enzyme involved in lysine biosynthesis (Leon-Ramirez et al., 2010). Regarding polyamine distribution, in general, eukaryotes have low putrescine content and a high content of spermidine and spermine, while prokaryotes have a higher concentration of putrescine than spermidine (Manni et al., 1987). A difference between fungi and plants is the presence of thermospermine, an isomer of spermine that has not been found in fungi (Fuell et al., 2010; Takano et al., 2012)

RETRO-CONVERSION OF POLYAMINES

In fungi, polyamines are oxidized to putrescine by the pathway shown in **Figure 1**. The first step is the acetylation of the aminopropyl group of polyamines, a reaction catalyzed by spermine or spermidine N^1—acetyltrasferase (Ssat; E.C. 2.3.1.57), to give either N^1—acetyl-spermidine or N^1—acetyl-spermine. These are in turn degraded by a polyamine oxidase (Pao; E.C. 1.5.3.11), with the formation of either putrescine or spermidine. Polyamine acetyltransferases and polyamine oxidases have been reported to be present in yeast as well as in other fungi (Yamada et al., 1980; Chattopadhyay et al., 2003b; Landry and Sternglanz, 2003; Liu et al., 2005; Valdes-Santiago et al., 2010). The ability to direct back-conversion of spermine to spermidine by spermine oxidase such as occurs in animals has been reported in plants and it is other dissimilarity between plants and fungi (Tavladoraki et al., 1998; Alcazar et al., 2006). In plants, redundancy of genes such as diamino oxidases or polyamine oxidases genes has been documented (Alcazar et al., 2006; Ono et al., 2012).

REGULATION OF POLYAMINE SYNTHESIS

Modulation of polyamine biosynthesis is mostly achieved by the degradation of Odc protein, as it has been reported in different species including *Schizosaccharomyces pombe, Neurospora crassa*, and *Saccharomyces cerevisiae* [(Barnett et al., 1988; Toth and Coffino, 1999) for review see (Ivanov et al., 2006)]. The regulator of Odc is the protein ornithine decarboxylase antizyme (Az) (Hayashi et al., 1996). Az interacts with Odc to be degraded by the proteasome in an ubiquitin independent manner (Zhang et al., 2003). Among fungi, the regulatory mechanism of Az is conserved [for review, see (Sorais et al., 2003)]. As indicated above, it was demonstrated that in *S. cerevisiae*, the degradation of Odc occurs without ubiquitination, as also happens in mammalian cells (Gandre and Kahana, 2002; Ivanov et al., 2006), and the identification of genes encoding Az from other fungi, suggests that the mechanism may be widely distributed in these organisms (Ivanov et al., 2006; Ivanov and Atkins, 2007). A peculiarity of fungi is that they present a single antizyme ortholog, while mammalian cells posses several antizyme encoding genes (Coffino, 2001a,b; Kurian et al., 2011). As expected, it is known that Az is positively regulated by polyamine levels. The mechanism for this regulation involves a translational frameshift in the open reading frame (ORF) in the encoding gene, through which a second ORF that encodes the active protein is established. In *S. cerevisiae* an unusual mechanism for the control of Az synthesis was unveiled (Kurian et al., 2011). Accordingly, the authors made the surprising discovery that at low polyamine levels Az acquires a

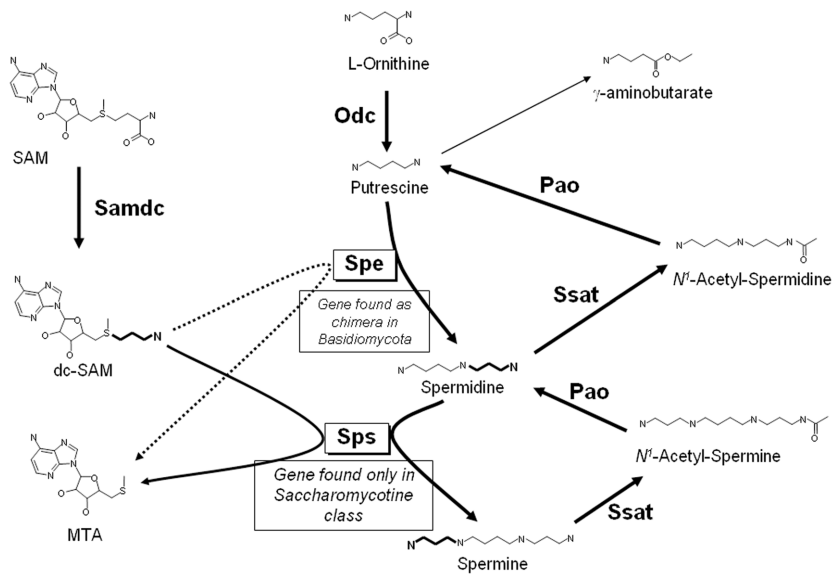

FIGURE 1 | General pathway for the biosynthesis and catabolism of polyamines in fungi.

conformation that arrest its own synthesis, but high concentrations of polyamines bind, not to the regulatory region of the gene, but directly to the Az polypeptide, thus avoiding that it acquires such conformation, and promoting the completion of its synthesis Other regulatory mechanisms of the metabolism of polyamines occur at the levels of Odc, Ssat, and Pao, due to their early and rapid responses to external stimuli (Vujcic et al., 2003; Wallace et al., 2003). Additionally, the existence of regulatory sequence elements in the 5′ and 3′ of *ODC* regions in *N. crassa* are related with changes in the rate of synthesis of Odc, and with changes in the abundance of *ODC* mRNA (Williams et al., 1992; Hoyt et al., 2000).

Contrary to fungi, plants posses several copies of the genes involved in polyamine metabolism which increase the complexity of polyamine regulation. As an example, in *Arabidopsis thaliana* there are two genes encoding *Adc1* and *Adc2,* the first one is presented in all tissues and it is constitutively expressed, while the second one responds to some abiotic stresses (Soyka and Heyer, 1999; Perez-Amador et al., 2002). Likewise, *Spd1* and *Spd2* are the genes encoding spermidine synthase in *A. thaliana* (Imai et al., 2004; Ge et al., 2006).

Regard SAMDC, it is synthesized as a proenzyme, it has been demonstrated that putrescine induces the cleavage of the proenzyme in a specific amino acid to produce the active and mature enzyme (Pegg et al., 1998). In plants there is an additional regulation control at transcriptional level. Plant *Samdc* contain a tiny 5′-uORF and introns in 5′leader sequence that regulate their expression (Hu et al., 2005). The absence of Az homolog in plant genomes corroborates the predominance of SAMDC as the regulator of polyamine homeostasis (Illingworth and Michael, 2012).

INTERACTIONS WITH OTHER METABOLIC PATHWAYS

The pleiotropic effects observed in polyamine mutants may be due to the relationships existing between polyamines and other metabolic pathways. As an example, ornithine, the precursor of putrescine not only is considered a key regulator of polyamine biosynthesis, but it may also regulate the pathways for glutamate transformation to arginine and to proline. Indirectly, it can also regulate putrescine catabolism, contributing to the aminobutiric acid content of the cells, since putrescine can be converted into Δ^1-pyrroline by an amino oxidase, and this compound is metabolized to γ-aminobuyrate by pyrroline dehydrogenase (Seiler et al., 1979; Fogel et al., 1981; Majumdar et al., 2013). Another compound related to polyamines is *S*-adenosylmethionine (SAM), which is essential for the synthesis of polyamines. *S*-adenosylmethionine synthetase (Sams; EC 2.5.1.6) catalyzes the biosynthesis of SAM from ATP and L-methionine (Tabor and Tabor, 1984). In *S. cerevisiae,* methionine regulates the expression of the two *SAMS* genes (*SAMS1* and *SAMS2*) (Thomas et al., 1988); and in turn *SAMS2* gene is subjected to the inositol-choline regulation given by the octameric sequence 5′-CATRTGAA-3′ contained in its upstream promoter region (Kodaki et al., 2003). Genes encoding Sams are evolutionarily well conserved (Mautino et al., 1996). In *S. pombe* mutants, the absence of Sams affected cell growth, mating and sporulation, and by over-expressing the gene, growth of the cells became methionine-sensitive (Hilti et al., 2000).

5′-Methylthioadenosine (MTA), is a product of SAM catabolism during polyamine biosynthesis (Heby, 1981). More than 98% of MTA in *S. cerevisiae* is a by product of SAM, originated from the polyamine biosynthetic pathway (Avila et al., 2004). MTA can affect gene expression, proliferation, differentiation and apoptosis, and these effects may be due to the inhibitory effect that intracellular accumulation of this nucleoside has over polyamine biosynthesis *in vivo* (Raina et al., 1982; Garcea et al., 1987); in addition in *S. cerevisiae*, MTA causes a specific inhibition of spermidine synthase (Chattopadhyay et al., 2006a).

Regard plants, SAM is the precursor of ethylene, hence polyamines, DNA methylation and ethylene share it as a common precursor (Pandey et al., 2000). Polyamines are precursors of many plant secondary metabolites such as nicotine and tropane alkaloids (Martin-Tanguy, 2001). Furthermore, plant polyamine metabolism is also connected with the production of nitric oxide and GABA [Reviewed by Alcazar et al. (2010)]. The regulation of polyamine biosynthesis, proline and cytokinins by abscisic acid as well as ethylene during UV-B stress and salt stress has been documented (Rakitin et al., 2009; Xue et al., 2009; Shevyakova et al., 2013).

POLYAMINES AND STRESS

TOLERANCE OF FUNGAL MUTANTS AFFECTED IN POLYAMINE METABOLISM TO DIFFERENT STRESS CONDITIONS

The study of the stress response in fungal mutants affected in polyamine metabolism was started with *S. cerevisiae*, where the authors had in mind evidences indicating some roles of polyamines in the protection of the cell and cell components, and cell differentiation (Balasundaram et al., 1993; Chattopadhyay et al., 2006b; Watanabe et al., 2012). In general the ability to deal with stress is diminished in mutants affected in any of the genes encoding enzymes related with polyamine metabolism. *U. maydis* is a well studied system in this regard, proving to be an important model organism for understanding polyamine metabolism, especially since this phytopathogenic fungus lacks spermine (Valdes-Santiago et al., 2009).

In general, fungal spores are rather more resistant to different environmental stresses than vegetative cells. However, in the early steps of germination, when they loose their unique spore wall, they become more sensitive to different environmental stresses (Herman and Rine, 1997; Joseph-Strauss et al., 2007). Interestingly, it has been described that polyamines affect positively spore germination in *Glomus mosseae, Rhizopus stolonifer, Botryodiplodia theobromae, Gigaspora rosea*, and *Glomus etunicatum* (Nickerson et al., 1977; El Ghachtouli et al., 1996; Sannazzaro et al., 2004; Cheng et al., 2012). Inhibition of polyamine metabolism gave rise to an inhibition of spore germination and germ tube growth in fungi such as *Uromyces phaseoli* (Galston, 1989). Genes related with polyamine transport have been implicated in the germination process, and it was described that these genes are up-regulated compared to the vegetative state (Ruiz-Herrera, 1994; Dembek et al., 2013). These results suggest a relationship between polyamines and changes in the expression of

genes related to stress. During a study of *S. cerevisiae* sporulation-specific genes, two divergently transcribed genes *DIT1* and *DIT2*, were found to be repressed during vegetative growth via a common negative regulatory element, referred to as NER^{DIT} (Friesen et al., 1997). The authors reported that the spermidine synthase gene was required for complete repression through NER^{DIT}, and cells that could not synthesize spermidine not only failed to support complete repression, but also had modest defects in repression of other genes, suggesting that spermidine could modulate gene expression (Friesen et al., 1998). However, there is not yet a report about gene expression response of fungi to different environmental stresses that reveal information on what is the mechanism through which polyamine are operating in this context.

ROLE OF PHYSIOLOGICAL POLYAMINES ON OSMOTIC STRESS

In silico phylogenetic analyses have revealed that central components of the osmotic, oxidative and cell wall stress signaling pathways are relatively well conserved in fungi (Bahn et al., 2007; Nikolaou et al., 2009). Nevertheless, knowing that polyamines are important in the response to stress, and that their metabolic pathway has been conserved among all living organism; we may suggest that a principal role of polyamines is to promote the restoration of cellular homeostasis allowing survival under stressful conditions. In *S. cerevisiae*, the expression of the major permease for high affinity polyamine import coincided with the osmotic stress imposed by high concentration of NaCl, KCl, or sorbitol (Lee et al., 2002; Aouida et al., 2005). Also in yeast, it was observed that the serine/threonine protein kinases Ptk1p and Ptk2p were involved in the regulation of spermine uptake, and that disruption of *PTK2* gave rise to salt tolerance, while Ptk2p or Sky1p (another serine/threonine kinase that was found in spermine tolerant strains) over-expression, led to increased salt sensitivity (Erez and Kahana, 2001). These and other data clearly reveal that polyamines have a role in osmotic stress response, probably by regulation of the expression of osmotic stress signaling via protein kinases (Auvinen et al., 1992; Shore et al., 1997; Flamigni et al., 1999).

As would be expected, it is known that the lipid composition of the membrane affects the osmotic stability of the yeast plasma membrane (Allakhverdiev et al., 1999). In consequence, it was important to establish if the sensitivity of polyamine mutants to different kinds of stress was due to a defect at the membrane level or to an indirect alteration of other signaling pathways controlled by polyamines. In this sense, data exist showing that osmotic shock in different systems affects the intracellular levels of polyamines. For example, it was reported that under osmotic shock the content of putrescine was increased up to 60-fold in oat, barley, corn, wheat, and wild oat leaves (Flores and Galston, 1982); and the induction of Odc activity under hypo-osmotic stress leading to an increase in the polyamine pool was observed in L1210 mouse leukemia cells (Poulin et al., 1991). The effect of a high osmotic concentration was tested in *U. maydis* mutants affected in the gene encoding spermidine synthase (*spd*), and in double mutants affected also in the ornithine decarboxylase gene (*odc/spd*). Both simple and double mutants showed no significant differences in their growth rate in the absence of salt stress, when compared to the wild type, but growth rate of the same cells treated with 1 M KCl or 0.3 mM SDS was severely inhibited in comparison with the wild type strain. This result demonstrates a role of spermidine in protecting the fungus from different deleterious factors that affect the cell membrane (Valdes-Santiago et al., 2009). When mutants affected in the polyamine oxidase gene (*pao*), and double mutants affected in ornithine decarboxylase and polyamine oxidase (*odc/pao*) were subjected to ionic or osmotic stress (1 M NaCl, 10 mM LiCl, or 1 M sorbitol), only the double mutant displayed a sensitive phenotype, whereas the simple *pao* mutant showed no differences to the wild type strain (Valdes-Santiago et al., 2010).

To understand the effects of polyamine deficiency on the phenotypic alterations suffered by the cell subjected to osmotic stress, it is important to take into consideration the existence of specific mechanisms involved in cation handling, and the possible effect of some pathways that interact with polyamine metabolism. This fact was clearly demonstrated by the comparison of the behavior of *U. maydis S*-adenosylmethionine decarboxylase (*samdc*) and *spd* mutants under stress conditions. Growth rate of both mutants was substantially inhibited by 1 M NaCl, but only the *samdc* mutant was affected by 10 mM LiCl. A fundamental difference between *samdc* and *spd* mutant is that, unlike the wild-type strain, they accumulate high levels of SAM, but *spd* mutants also accumulate SAM. The other metabolite involved in the pathway is dcSAM, which was 46-fold increased in the *spd* mutants (Valdés-Santiago et al., 2012b). The authors explained these differences in the phenotype in relation to their different levels of SAM and dcSAM, which could affect DNA methylation, and other different cellular functions, plus the possibility that sodium and lithium are managed by different transporters.

A further example of the specificity of polyamines in osmotic stress was the observation that in *Synechocystis* sp., a cyanobacterium, salt stress induced an increase in the spermine content, whereas an osmotic stress induced a moderate increase in the total spermidine content (Jantaro et al., 2003). Interestingly, this response was correlated with an increase of arginine decarboxylase mRNA levels, and an increase in the uptake of putrescine and spermidine (Incharoensakdi et al., 2010). In this sense, we may cite that simple or double *U. maydis* polyamine mutants presented a variety of stress sensitivities to osmotic stress (**Figures 2B,D**), compared with wild type and same mutants without stressful agent (**Figure 2A**).

In plants it has been well established that general polyamine biosynthesis is modified under salt stress at transcriptional level [for review see (Mutlu and Bozcuk, 2007; Liu et al., 2008; Alcazar et al., 2010; Gupta et al., 2013)]. However, spermine seems to have a specific role considering that, this is the polyamine reported to be more affected regardless of species, varieties and plant tissue studied. To mention some examples, plants such as *A. thaliana* unable to produce spermine showed hypersensitivity to high levels of NaCl and KCl and the mechanism behind polyamines protectection of salt stress has been studied. Some reports suggest that, polyamines improve ionic equilibrium by modifying the plasma membrane to overcome the osmotic stress since the expression of some genes belonging to signaling pathway related with salt stress were not altered in spermine deficient

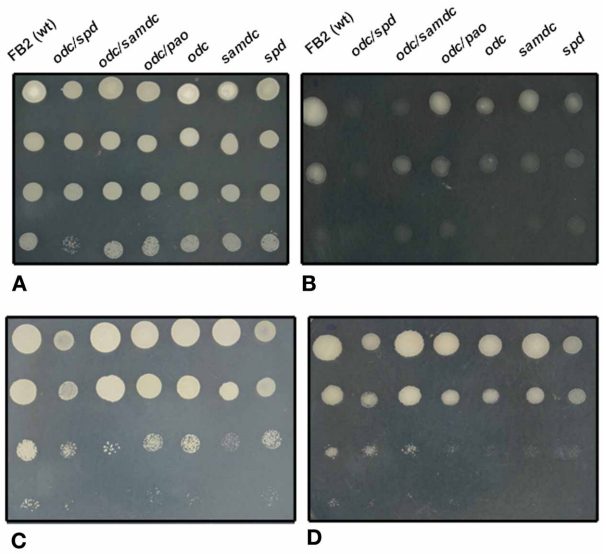

FIGURE 2 | Comparative analysis of different stress conditions on the growth of wild type, and simple and double mutants of *U. maydis* affected in different steps of polyamine metabolism. After depletion of polyamine pools, cell suspensions were decimally diluted and inoculated on solid minimal medium containing 20 mM lysine and 0.1 mM spermidine. After 48 h of incubation at 28°C (except in C), photographs were taken. **(A)**, control; **(B)**, 1.5 M KCl added to the medium; **(C)**, as **(A)**, but incubated at 37°C; **(D)**, 1 M NaCl added to the medium.

mutant. Moreover, spermine seemed to modulate Ca^{2+} permeable channels and change Ca^{2+} allocation restraining the entry of Na^{+}/K^{+} to the cytoplasm (Yamaguchi et al., 2006; Janicka-Russak et al., 2010; Najmeh et al., 2012; Velarde-Buendia et al., 2012). Other reports described that spermidine or putrescine are the polyamines conferring protection under salt stress, although it is difficult to support this conclusion, since putrescine or spermidine can be inter-converted (Quinet et al., 2010; Saleethong et al., 2011). In this respect, mutants are very effective in avoid confused results since they can define whether polyamines are produced by *de novo* synthesis or back-conversion (Valdes-Santiago et al., 2010).

OXIDATIVE STRESS RESPONSE

Fungi, as well as other microorganisms, must deal with the danger of oxidative stress under different scenarios. An important example is the oxidative killing of fungal cells by the host defense mechanisms, and sometimes the ability to proliferate in the host has been correlated with the expression of redox active enzymes such as catalase (Wysong et al., 1998; Moye-Rowley, 2003). Accordingly, it has been concluded that one of the polyamine functions is the cell protection from damage caused by ROS (Rider et al., 2007; Cerrada-Gimenez et al., 2011). In the case of *E. coli*, oxidative stress induces the expression of catalase, hydroperoxide reductase and glutathione reductase (Storz and Imlay, 1999; Chattopadhyay et al., 2003a), and interestingly, putrescine is up-regulated the bacterium in a concentration-dependent manner with the expression level of the transcription factor controlling the genes already mentioned (Kim and Oh, 2000; Tkachenko et al., 2001). In the case of *U. maydis* it was observed that *pao* and *odc* polyamine mutants grown on agar plates where 0.8 mM H_2O_2-containg paper disks were placed, showed halos of inhibition wider in comparison with the wild-type strain; i.e., they were more sensitive than the wild type to the oxidative stress (Valdes-Santiago et al., 2010).

In fungi in general the mechanism regulating their response to oxidative stress has been described to involve the control of oxidant-responsive factors, such as Yap1p at level of cell localization and by regulation of enzyme activity via protein phosphorylation (Moye-Rowley, 2003). When *S. cerevisiae* was affected in the production of spermidine and spermine because of a deletion of the *Samdc* gene; there occurred a loss in cell viability when cultures were incubated under an oxygen atmosphere (Balasundaram et al., 1993).

It has also been shown that yeasts affected in the production of spermidine accumulate ROS, and show an increased sensitive to oxidative damage. In this regard, polyamine-deficiency in *S. cerevisiae* induced accumulation of ROS that led to the development of an apoptotic phenotype (Chattopadhyay et al., 2006b). These authors showed that one of the polyamine functions was the protection to the accumulation of ROS. There is yet to know whether polyamines act by affecting the enzymes involved in the synthesis or degradation of ROS, or by a direct interaction with ROS. Some evidences point out to some hypothetical polyamine-binding sites in proteins involved in these different processes (Watanabe et al., 2012). In this same respect, it has been proposed that spermine acts by direct scavenging of reactive agents (Ha et al., 1998; Fujisawa and Kadoma, 2005), whereas other authors have suggested an inhibition of the activity of NADPH oxidase (Papadakis and Roubelakis-Angelakis, 2005), and a role of polyamines as regulators of the MAPK signaling pathway (Stark et al., 2011).

In plants, a swift accumulation of ROS is presented under stressful conditions (Gill and Tuteja, 2010b; Suzuki et al., 2012); and it has been observed that intracellular ROS produced by polyamine catabolism are essential during development. Thus, it has been described that H_2O_2 generated during their oxidation and back-conversion serve as a signaling molecule correlated with plant defense and stress response (Moschou and Roubelakis-Angelakis, 2011; Pottosin et al., 2012). Accordingly, in *Salvinia natans* response to salinity there occurs an interaction between ROS formation, and the expression of *Adc*, *Samdc*, *Spd*, and *Spm* as well as *Pao* (Tanou et al., 2013).

TEMPERATURE STRESS

Temperature has critical effects on microbial metabolism and cellular composition (Bennett et al., 1992; Feder and Hofmann, 1999; Fargues and Luz, 2000; Gavito and Azcon-Aguilar, 2012). It has been suggested that polyamines are involved in the stabilization of cellular components at high temperatures; for example a *Tapesia yallundae odc* null mutant exhibited temperature-dependent growth: at high temperatures, hyphal elongation was more restricted in comparison to the wild type, and the hyphae were thinner, less melanized, and grew sparsely (Mueller et al., 2001). Similarly, an *S. cerevisiae* mutant unable

to synthesize spermidine or spermine, because it was affected in the gene encoding *S*-adenosylmethionine decarboxylase, was more sensitive to elevated temperatures than the parental strain (Balasundaram et al., 1996). The opposite takes place when polyamines are accumulated. Under these conditions, the cells present resistance to high temperature stress (Cheng et al., 2009). Simple and double *U. maydis* mutants affected in polyamine metabolism also presented a temperature sensitive phenotype in agreement with the reports above mentioned (see **Figure 2C**).

In plants, polyamine alterations have been correlated with temperature changes; under cold treatment putrescine levels were increased together with the expression of *Adc1*, *Adc2*, and *Samdc2* in *A. thaliana* (Urano et al., 2003; Cuevas et al., 2008), while the addition of putrescine had a positive effect over cotton seed subjected to high temperature (Bibi et al., 2010). These results are in agreement with reports on *Arabidopsis thaliana,* where a mutant affected in the gene encoding spermine synthase was hypersensitive to heat shock; whereas an overexpression of spermine synthase-encoding gene conferred thermotolerance to the cell (Sagor et al., 2013). In the same study it was established a direct correlation between spermine content and the expression of heat shock genes and proteins suggesting that there is a control of polyamines at transcriptional and translational levels to induce the protection of plants under temperature stress. It is interesting to notice that although *U. maydis* does not contain spermine, spermidine covers the function to resist temperature stress (**Figure 2C**).

In the same line, it has been suggested that he mechanism behind the sensitivity of polyamine-deficient cells could be related to the stability that polyamines may provide to some cell components (Schuber, 1989).

POLYAMINES IN STRESS PRODUCED BY PATHOGEN-HOST INTERACTIONS

It is well known that during the course of host colonization, fungal pathogens need to overcome a wide range of challenges such as oxidative burst, which results in the production and accumulation of ROS. It has been proposed possible roles for polyamines and polyamine catabolism in plant resistance to pathogen infection (Walters, 2003). In this context, plants would activate polyamine oxidation to produce hydrogen peroxide, which would lead plant defense mechanisms. Yoda et al. (2003) confirmed these data, when they correlated hypersensitive response with the accumulation of polyamines in the apoplast of *Arabidopsis thaliana* infected with *Pseudomonas syringae,* and of rice infected with *Magnaporthe grisea* (Yoda et al., 2003). Expression of *Odc*, *Adc*, and *Samdc* genes in *Theobroma cacao* were induced under different stresses such as, drought and infection with *Phytophthora megakarya,* or the necrosis inducing protein Nep1 from *Fusarium* oxysporum while *Spds* and *Sps* were not changed (Bae et al., 2008). In the same manner, H_2O_2 produced by Paos during hypersensitive response provoke disease tolerance against *Pseudomonas syringae pv* tabaci and *Phytophthora parasitica* var nicotianae (Yoda et al., 2003; Moschou et al., 2009).

Spermine has been proposed as the polyamine that contribute to defense signaling against plant pathogens through the regulation of defense-related genes. In this sense, an *A. thaliana* mutant that overexpressed *Sps* gene, displayed higher resistance to infection with *Pseudomonas viridiflava,* whereas a mutant with low spermine levels showed hyper sensitivity; interestingly the overexpression of *Sps* was accompanied by up-regulation of genes involved in disease resistance protein, as well as several transcription factors (Cona et al., 2006; Kusano et al., 2008; Gonzalez et al., 2011). Likewise, spermine-responsive genes were detected in *A. thaliana* during infection with cucumber mosaic virus, and interestingly, blocking of spermine oxidation abolished induction of these genes (Mitsuya et al., 2009). In general, response from pathogenic organism with distinct strategies to cause disease either necrotrophic or biotrophic, is essentially analogous, as was demonstrated with *Sclerotinia sclerotiorium* and compared with some *Pseudomonas species* or *U. maydis* (Marina et al., 2008; Rodriguez-Kessler et al., 2008; Rodriguez-Kessler and Jimenez-Bremont, 2009). In these two cases what was observed was the induction of polyamines connected with the activation of genes encoding polyamine biosynthesis enzymes. Nevertheless, it is important to mention that changes in plant polyamine metabolism by transgenic strategies allow the modification of plant responses to pathogenic organisms. The expression of yeast *Spe* gene and accumulation of polyamine levels in tomato led to an increasing of sensitivity to the attack by *Botrytis cinerea,* but not by *Alternatia solani* and the normal response to the attack was restored by polyamine inhibitors (Marina et al., 2008; Nambeesan et al., 2012).

From the side of the pathogen, the state of polyamine metabolism affects their interaction with the host. When this *Streptococcus pneumoniae* was affected in polyamine transport, the mutant strain showed attenuation in virulence in a murine model. And it has been reported that human bacterial pathogens use different strategies related with polyamines to infect their hosts (Ware et al., 2006; Di Martino et al., 2013). A polyamine mutant of *Salmonella enterica* serovar *typhimurium*, was unable to invade and survive intracellularly in *Caenorhabditis elegans,* and showed no systemic infection in a mouse model of typhoid fever (Jelsbak et al., 2012). *Francisella tularensis, Yersinia pestis* and *Vibrio cholerae* are other systems in which synthesis and transport of polyamines were found to be important for virulence (Wortham et al., 2010; Russo et al., 2011; Goforth et al., 2013). Regarding fungi, a *spe* mutant of the thermally dimorphic fungus *Penicillium marneffei*, a pathogen of immune compromised persons, showed defects in pathogenesis, conidiogenesis, spore germination, and growth. These results led to suggest that the spermidine biosynthetic may serve as a potential target for combating infections (Kummasook et al., 2013). Agreeing with these ideas, in another pathosytem reviewed by Valdés-Santiago et al. (2012b): *U.maydis*-maize, it was observed that *samdc* mutants of the fungus were avirulent.

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Along this article, evidences indicate multiple roles of polyamines in cell survival during stress. A strategy toward the knowing of the mechanism behind polyamines action should include firstly the clear identification of the specific roles of each polyamine i. e., in *U. maydis* putrescine might be controlling stress response since the mutant (*odc/pao*) was over sensitive to stress, in comparison

to the wild type and and *pao* mutant. Also, spermidine appeared to control differentiation of this fungus, since mutant it was able to carry out a dimorphic transition only when supplied with high concentration of spermidine (Valdes-Santiago et al., 2010). Once the specific roles of each polyamine are known, it will be possible to distinguish the role of other polyamines in the general aspect of cell physiology. This review clearly stresses the fact that changes in polyamine metabolism affect the response of fungi to different types of stress. This fact by itself is an evidence of the importance of polyamines in cell survival.

Although a large advance in our understanding on polyamine metabolism in fungi has taken place in recent years, some aspects are still poorly understood, especially regarding polyamine transport, distribution in the cell, and regulation of metabolism. It is also clear that the role of polyamines in stress-response mechanisms in fungi, and their mode of action have been insufficiently analyzed. We consider that it is necessary to have a global view of the physiology of stress response in fungi that includes polyamines as an important player, possibly using mutants affected in different steps of polyamine metabolism and its regulation, and studies of the relationships of polyamine metabolism with other important metabolic and regulatory processes of the cell. If this expectancy is fulfilled, undoubtedly that fungi will become important models that could help to unravel the mechanism of the protection exerted by polyamines to stress in general.

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Overexpression of *SAMDC1* gene in *Arabidopsis thaliana* increases expression of defense-related genes as well as resistance to *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis*

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It has been previously described that elevation of endogenous spermine levels in Arabidopsis could be achieved by transgenic overexpression of S-Adenosylmethionine decarboxylase (*SAMDC*) or Spermine synthase (*SPMS*). In both cases, spermine accumulation had an impact on the plant transcriptome, with up-regulation of a set of genes enriched in functional categories involved in defense-related processes against both biotic and abiotic stresses. In this work, the response of *SAMDC1*-overexpressing plants against bacterial and oomycete pathogens has been tested. The expression of several pathogen defense-related genes was induced in these plants as well as in wild type plants exposed to an exogenous supply of spermine. *SAMDC1*-overexpressing plants showed an increased tolerance to infection by *Pseudomonas syringae* and by *Hyaloperonospora arabidopsidis*. Both results add more evidence to the hypothesis that spermine plays a key role in plant resistance to biotic stress.

Keywords: biotic stress, spermine, jasmonate, polyamines, stress response and stress tolerance

INTRODUCTION

Polyamines (PAs) constitute a group of low molecular weight aliphatic amines, whose most widespread forms in living organisms are the diamine putrescine (Put), the triamine spermidine (Spd) and the tetraamine spermine (Spm). In plants, PAs have been implicated as key players in growth and development processes, as well as in the response to biotic and abiotic stresses (Kusano et al., 2008; Alcázar et al., 2010; Takahashi and Kakehi, 2010). Intracellular PA levels in plants are mostly regulated by anabolic and catabolic processes, as well as by their conjugation to hydroxycinnamic acids and macromolecules like proteins and DNA.

PA biosynthesis pathway has been well characterized in *A. thaliana* (Alcazar et al., 2006). In this species, PA synthesis is initiated with Put synthesis from aminoacid arginine by the sequential action of arginine decarboxylase (ADC; EC 4.1.1.19), agmatine iminohydrolase (AIH; EC 3.4.3.12), and *N*-carbamoylputrescine amidohydrolase (CPA; EC 3.5.1.53). Spd and Spm are the result of sequential additions of aminopropyl moieties to Put and Spd by the enzymes Spd synthase (SPDS; EC 2.5.1.16) and Spm synthase (SPMS; EC 2.5.1.22), respectively. Decarboxylated S-adenosylmethionine (dcSAM) is used by both enzymes as donor molecule of aminopropyl groups, and is synthesized from the decarboxylation of S-adenosylmethionine (SAM) in a reaction catalyzed by SAM decarboxylase (SAMDC; EC 4.1.1.50). Spd and dcSAM can also form a structural isomer of Spm, known as thermospermine (tSpm), in a reaction catalyzed by tSpm synthase (tSPMS; EC 2.5.1.79). Characterization of the Arabidopsis genome has allowed to identify two genes encoding ADC (*ADC1* and *ADC2*) (Watson and Malmberg, 1996; Watson et al., 1997) and one for each AIH and CPA (Janowitz et al., 2003; Piotrowski et al., 2003). The Arabidopsis genome also carries two genes encoding SPDS (*SPDS1* and *SPDS2*) (Hanzawa et al., 2002), one coding for SPMS (*SPMS)* (Panicot et al., 2002), another one coding for tSPMS (*ACL5*) (Knott et al., 2007; Kakehi et al., 2008), and at least four coding for SAMDC (*SAMDC1-4*) (Urano et al., 2003).

PAs are catabolized through the activity of diamine oxidases (DAO; EC 1.4.3.6) and polyamine oxidases (PAO; EC 1.5.3.3). DAOs display high affinity for diamines, like Put, producing Δ^1-pyrroline, hydrogen peroxide (H_2O_2) and ammonia, while PAOs oxidize secondary amine groups from Spd and Spm leading to the formation of 4-aminobutanal or (3-aminopropyl)-4-aminobutanal, along with 1,3-diaminopropane (DAP) and H_2O_2. PAOs are also able to catalyze the back-conversion of Spm to Spd, producing 3-aminopropanal and H_2O_2. Some PAO isoforms are also involved in back-conversion processes of tSpm to Spm, and Spm to Put, that lead to the production of H_2O_2 (Moschou et al., 2012). At least five genes encoding putative PAOs (Alcazar et al., 2006; Takahashi et al., 2010) and 10 genes encoding putative DAOs (Planas-Portell et al., 2013) are present in the Arabidopsis genome.

PA metabolism is altered in a variety of plant hosts in response to several pathogens (Walters, 2003a,b), suggesting a role for PAs in the biotic defense response. However, the precise mechanism(s) of action by which PAs could exert this defensive role

remains unclear, although some possible mechanisms of action have been proposed. An up-regulation of PA biosynthesis and catabolism has been observed during hypersensitive response (HR) induced by the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* in barley (Cowley and Walters, 2002), as well as in tobacco plants exposed to tobacco mosaic virus (TMV) (Marini et al., 2001). PA catabolism produces H_2O_2, a reactive oxygen species (ROS) that could have an antimicrobial effect as well as participate in host defense mechanisms, including cell wall modifications, or act as a signal molecule triggering processes like HR (Walters, 2003a). PA oxidation has also been observed in compatible interactions between plant hosts and different types of pathogens. Spm accumulates in the apoplast of tobacco plants infected by *Pseudomonas syringae* pv. *tabaci*, and its oxidation by PAO leads to the production of H_2O_2 (Moschou et al., 2009). Moreover, PAO overexpression enhances tobacco tolerance to biotrophic bacteria *P. syringae* as well as to the hemibiotrophic oomycete *Phytophtora parasitica* var *nicotianae* (Moschou et al., 2009). Apoplastic PA accumulation and further oxidation has also been observed in tobacco leaves exposed to the biotrophic bacterium *Pseudomonas viridiflava* (Burkholder) Dowson, restricting bacterial growth in the host (Marina et al., 2008).

A role for Spm in defense signaling has been pointed out in *A. thaliana*, in which exogenous Spm induces a set of genes that are also expressed in response to the cucumber mosaic virus (CMV) infection (Mitsuya et al., 2009). Changes in Spm metabolism and subcellular localization have been associated with plant host responses to pathogenic attack. Induction of acidic pathogenesis-related proteins (PR) observed during TMV infection in tobacco is produced by Spm accumulation in the leaf apoplast, and this induction is not dependent on Salicylic acid (SA) (Yamakawa et al., 1998). Moreover, Spm accumulation, which can also be mimicked by exogenous Spm application on tobacco leaves, triggers a "Spm-signaling pathway" that causes mitochondrial dysfunction by activation of mitogen-activated protein kinases and increase of the expression of a set of HR-specific genes, leading tobacco leaf cells to develop defense responses and HR-like cell death (Takahashi et al., 2003, 2004; Mitsuya et al., 2007).

More recently, the manipulation of PA levels by transgenic approaches and the use of loss or gain-of-function mutations have emerged as new tools to gain knowledge about the role of PAs in plant stress responses (Alcázar et al., 2010; Gill and Tuteja, 2010). Consistent with the hypothesis that Spm could perform a key role in defense signaling, transgenic 35S::*SPMS* Arabidopsis plants accumulate Spm and are more resistant to infection by *P. viridiflava* than the wild type (WT). On the contrary, Spm-deficient *spms* mutant lines are more susceptible to infection (Gonzalez et al., 2011). Comparison of the transcriptomes of Spm-accumulating and Spm-deficient mutants showed that many genes only overexpressed in 35S::*SPMS* lines participate in pathogen perception and defense responses, including several families of disease resistance genes, transcription factors, kinases, and nucleotide- and DNA/RNA-binding proteins (Gonzalez et al., 2011). At the same time, most of those genes appear also induced in other Spm–accumulating lines obtained by overexpression of *SAMDC1* gene (Marco et al., 2011).

In this work we have compared the expression levels of some disease resistance genes between *SAMDC1*-overexpressing lines and WT plants. Furthermore, we have also compared the effect of Spm accumulation on the susceptibility to the bacteria *P. syringae*, and to the oomycete *Hyaloperonospora arabidopsidis*. In both cases, *SAMDC1*-overexpressing lines were more resistant to infection than WT plants.

MATERIALS AND METHODS

PLANT GROWTH CONDITIONS

Infection by the bacterial pathogen *P. syringae* and by the oomycete *H. arabidopsidis* was tested in three Arabidopsis transgenic lines (pBISDCs-S3′, pBISDCs-S9′, pBISDCs-S15) overexpressing the *SAMDC1* gene under the control of *CaMV35S* constitutive promoter (Marco et al., 2011) and in the ecotype Col-0, WT obtained from the Nottingham Arabidopsis Stock Center (University of Nottingham, Loughborough, UK). pBISDCs lines exhibit a similar phenotype to WT plants in terms of growth and development.

Arabidopsis seeds were sown in pots with a 1:1:1 mixture of soil, vermiculite and sand, stratified for 2 days at 4°C, and transferred to growth chambers. Plants were grown in Sanyo MLR-350 (Sanyo Electric Co., Japan) chambers, either under long day conditions (illumination at 23°C for 16 h, darkness at 16°C for 8 h), or short day conditions (illumination at 18°C for 10 h, darkness at 16°C for 14 h), and watered with mild nutrient solution (recipe from Arabidopsis Biological Resource Center, The Ohio State University, USA, handling plants and seeds guide, http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/handling.htm).

Seedlings were also grown on plates under long day conditions. Seeds surface was sterilized by washing in 30% (v/v) commercial bleach, 0.01% (v/v) Triton X-100 by 10 min and rinsed three times with sterile distilled water. Sterile seeds were plated on 4% agar plates containing one half strength MS medium (½MS) (Murashige and Skoog, 1962). When required, seedlings were also grown in ½MS plates supplemented with 0.1, 0.5, and 1 mM of Put, Spd, or Spm. Whole seedling samples were taken after 5 days of growth and immediately frozen in liquid Nitrogen and stored at −80°C for RNA extraction.

P. SYRINGAE INFECTION CONDITIONS AND DISEASE EVALUATION

P. syringae pv. *maculicola* ES4326 and pv. *tomato* DC3000, kindly supplied by Dr. Jürgen Zeier (Julius-von-Sachs-Institute of Biological Sciences, University of Würzburg, Germany) and Dr. John Stavrinides (Department of Botany, University of Toronto, Canada), respectively, were used to infect Arabidopsis WT plants, as well as pBISDCs *SAMDC1*-overexpressing lines. Both bacterial strains were cultivated at 28°C in King's B medium (King et al., 1954). Streptomycin (100 μg/ml) or rifampicin (50 μg/ml) were added to select growth of strains pv. *maculicola* ES4326 and pv. *tomato* DC3000, respectively. For plant inoculation, bacterial cells were grown until cultures reached an OD_{600} of 0.1, collected by centrifugation, washed, and resuspended in 10 mM $MgCl_2$ to a final concentration of 3×10^6 CFU/ml.

Leaves of 15 day-old plants grown in long day conditions were inoculated with *P. syringae* according to Zeier et al. (2004). Briefly, bacterial suspension was inoculated on the abaxial surface of

leaves, using a 1-mL syringe without a needle. Control inoculations were performed with 10 mM $MgCl_2$ pH 7.0. 10 different plants were inoculated with each bacterial strain and disease extension was quantified by two approaches. Image analysis was used to evaluate chlorosis in the diseased plants. Leaves were scanned at 600 dpi and images were processed using the *Image Processing Tool Kit 5.0* (Reindeer Graphics Inc.) and *Photoshop 7.0* softwares (Adobe Systems, Inc) to measure leaf area. Since yellow generates a lighter shade of gray than green, chlorosis-induced yellowing was estimated by the conversion of leaf images to gray tones and the determination of their luminance percentages. On the other hand, severity of infection was also estimated by measuring *in planta* bacterial growth. Foliar extracts were obtained by cutting three leaf discs from each plant with a 0.5 cm-diameter borer, homogenizing them with 10 mM $MgCl_2$. Then, serial dilutions of the extracts thus obtained were plated on King's B agar medium supplemented with the appropriate antibiotic. The number of colony forming units (CFU) was determined after 24 h incubation at 28°C. Both, images and plant extracts were obtained 2 and 3 days after infection. Samples were also harvested, frozen in liquid Nitrogen and stored at −80°C for RNA extraction.

H. ARABIDOPSIDIS INFECTION CONDITIONS AND DISEASE EVALUATION

H. arabidopsidis isolate *Noks1*, was used to infect Col-0 WT Arabidopsis plants by using a dry powder containing pathogen oospores, obtained from previously infected leaves kindly supplied by Dr. Mahmut Tör (Warwick HRI, University of Warwick, UK). Methods for subculturing *H. arabidopsidis* and preparing inoculum for experiments were modified from Tör et al. (2002). Powder was sprinkled on pots containing Arabidopsis seeds, which were then stratified and left to germinate under short day conditions as described above. Presence of sporangiophores on cotyledons was checked daily and cotyledons with abundant sporulation were selected. *H. arabidopsidis* conidiospores were released from infected cotyledons by rinsing infected tissues with distilled water followed by centrifugation. Sedimented conidiospores were resuspended in distilled water, counted with a Neubauer chamber (Hauser Scientific Partnership, HORSHAM, PA 19044) and diluted to a final concentration of 5×10^4 conidiospores/ml.

Inocula consisting of 2 µl of this conidiospores suspension were applied on cotyledons of 14 day-old WT plants and pBIS-DCs transgenic lines. After inoculation, plants were grown under short day conditions and periodically sprinkled with water to maintain moisture. A control batch of plants was inoculated with distilled water and grown in parallel. 10 days after inoculation, 25 leaves were sampled for each line and the developed sporangiophores were counted.

RNA EXTRACTION

Total RNA was extracted from plant tissues using Total Quick RNA Cells and Tissues Kit (Talent SRL, Italy), following the protocol established by manufacturer. RNA was quantified by measuring the absorbance at 260 nm, and their integrity was checked by denaturing agarose gel electrophoresis.

QUANTITATIVE RT-PCR

RNA was treated with RNase free-DNAse (Roche diagnostics, Spain) to remove contaminating genomic DNA. A total of 1 µg of DNA free-total RNA was reverse transcribed to first-strand complementary DNA (cDNA) with random hexamers using SuperScript®III First-Strand Synthesis System 1st (Invitrogen, Spain) according to manufacturer's instructions. Quantitative real time PCR (qRT-PCR) was performed on GeneAmp®5700 Sequence Detection System (PE Applied Biosystems, Japan), using Power SYBR®Green PCR Master Mix (PE Applied Biosystems). 20 µl reactions contained 1 µl of cDNA, 100 nM of each pair of target primers (FW and REV) and 10 µl of SYBR Green PCR Master Mix. PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. Three technical replicates from three independent biological experiments were performed for qRT-PCR analyses. Primers used for real-time PCR are described in **Table 1**. The efficiency of primers and the data were analyzed according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Gene coding for actin-2 (*ACT-2*; *AT3G18780*; An et al., 1996) was used as a reference gene.

Table 1 | Primers used in qRT-PCR analyses.

Gene	AGI locus	Forward (5′–3′)	Reverse (5′–3′)
PR-1	AT2G14610	CCACAAGATTATCTAAGGGGTC	TTCCACTGCATGGGACCTA
PR-2	AT3G57260	CATCCTCGACGTTCCCAGTT	TGTCGGCCTCCGTTTGA
PR-5	AT1G75050	AACGGCGGCGGAGTTC	GCCGCCATCGCCTACTAGA
CYP79F1	AT1G16410	CATCCGTGCCATCACCATAA	CAAATCTGCGTCCCGCTCTCT
WAK1	AT1G21245	TGCTCTCAGGTCAAAAGGCATT	CGCAAAGTAACTCACCAGATGT
FLS2	AT5G46330	CCTGGACCTGTCTCACAACCA	ACGTAAGATTCATCCTTCCGAA
LOX2	AT3G45140	CAACGACAACAAGGATAAGA	CTGGCGACTCATAGAACT
AOC1	AT3G25760	CGTCCCATTCACAAACAACTC	CAGAGACCAGCCGTGATTCC
AOC2	AT3G25780	ACTGGAACGGCGGTTACG	GGCTCCATCGCCTTAGCTT
AOS	AT5G42650	CGGGCGGGTCATCAAG	GCCGTTGGATTTAATCACAGAT
DAD1	AT2G44810	GGAGACGCCGTGGGTTT	GGCGAGTCACGGCTCA
JMT	AT1G19640	CCAACATCACTTACTATATTCAT	GAAGAACTCGCATTACCT
ACT-2	AT3G18780	GATTCAGATGCCCAGAAAGTCTTG	TGGATTCCAGCAGCTTCCAT

STATISTICAL ANALYSES

Data was analyzed by One-Way Analysis of Variance (ANOVA) followed by *post-hoc* comparisons by Tukey's HSD or Dunnet's T3 *t*-test. A probability level < 0.05 was considered statistically significant. Calculations were performed using IBM®SPSS®Statistics v20.0 Software.

RESULTS

pBISDCs TRANSGENIC LINES SHOW CONSTITUTIVE ELEVATED EXPRESSION LEVELS OF DISEASE RESPONSE AND JASMONIC ACID METABOLISM GENES

Previous comparison of the transcriptomes of WT and pBISDCs transgenic lines showed that overexpression of *SAMDC1* gene in Arabidopsis leads to higher Spm levels and to the induction of a set of genes enriched in functional categories involved in defense-related processes against both biotic and abiotic stresses (Marco et al., 2011). Some of these genes were selected and their expression checked by qRT-PCR to confirm the results obtained in transcriptome studies. Expression levels of genes that encode for pathogenesis-related proteins *PR-1* (*AT2G14610*), *PR-2* (*AT3G57260*) and *PR-5* (*AT3G57260*) (Uknes et al., 1992; Van Loon et al., 2006); *CYP79F1* (*AT1G16410*), a cytochrome P450 involved in the biosynthesis of aliphatic glucosinolates (Hansen et al., 2001; Chen et al., 2003); Cell-Wall associated kinase *WAK1* (*AT1G21245*; Verica and He, 2002) as well as the flagellin receptor *FLS2* (*AT5G46330*; Gomez-Gomez and Boller, 2000) were determined by qRT-PCR (**Figure 1A**). Our results demonstrate that all disease response genes tested showed a dramatic increase in mRNA abundance in transgenic lines overexpressing *SAMDC1*. The expression level of these genes was very similar in all the

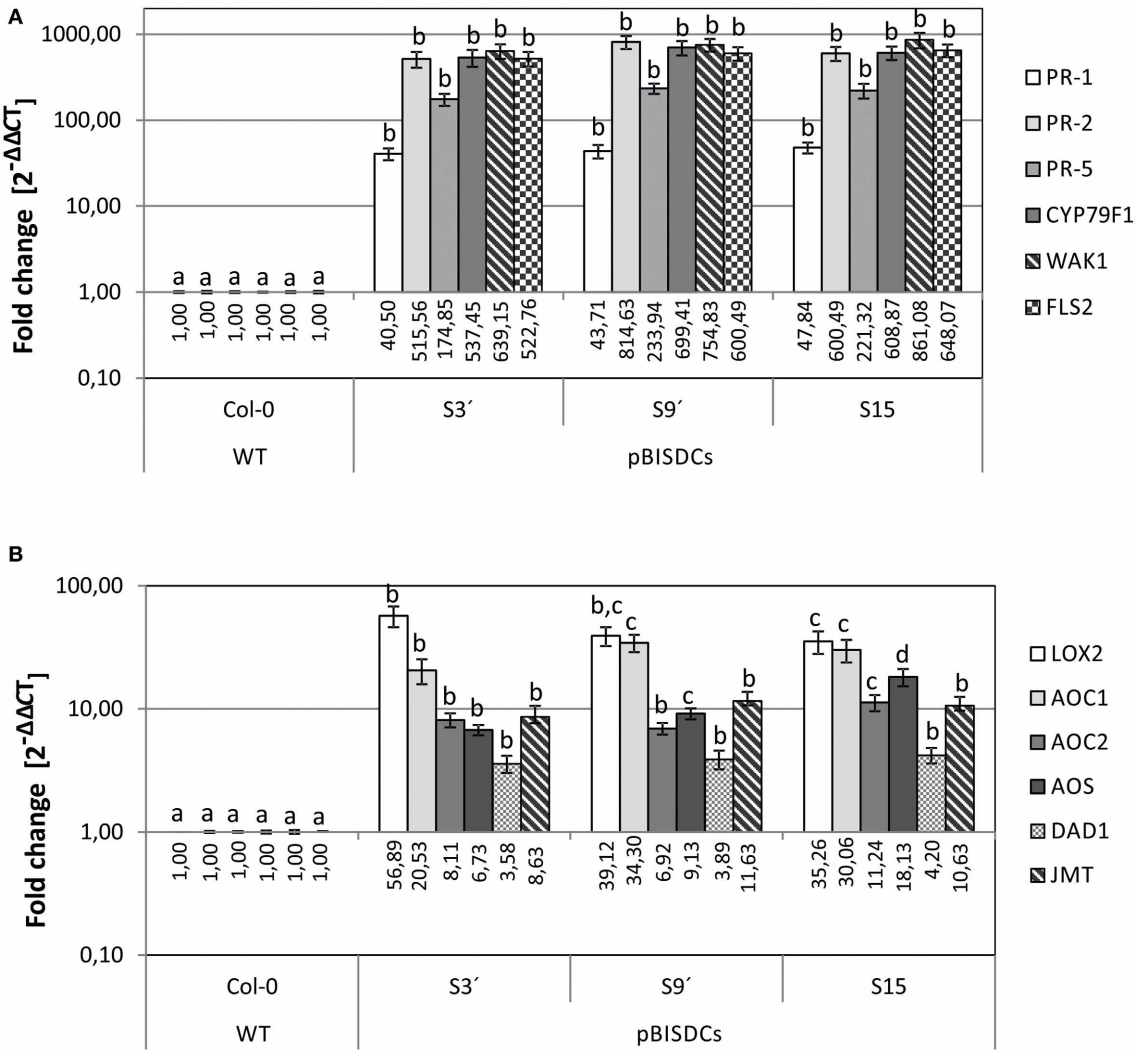

FIGURE 1 | qRT-PCR analysis of biotic stress-related genes in leaves of 4-week old *A. thaliana* WT and pBISDCs transgenic lines overexpressing *SAMDC1* (S3′, S9, and S15). Expression levels were determined for a set of biotic stress defense-related genes **(A)**, as well as jasmonate and methyl-jasmonate biosynthesis genes **(B)**. For each gene, data is expressed as fold change relative to the level measured in WT plants ($2^{-\Delta\Delta CT}$). Graph show the mean of three biological replicates ± standard deviation. Significant differences between plant lines are indicated with letters (ANOVA, Tukey HSD test, $p < 0.05$).

pBISDCs lines selected, ranging from 100 to 800-fold higher than WT, except for *PR-1,* whose expression increased less than 100-fold (**Figure 1A**).

Previous transcriptome analysis showed that another set of overexpressed genes in *SAMDC1*-overexpressing lines were enriched in the category of genes related to jasmonic acid biosynthesis and response (Marco et al., 2011). Expression levels of genes that encode jasmonate (JA) and methyljasmonate (MeJA) biosynthesis enzymes such as Chloroplast lipoxygenase *LOX2* (*AT3G45140*; Bell et al., 1995); Allene oxide cyclase, *AOC1* (*AT3G25760*) and *AOC2* (*AT3G25780*) (Stenzel et al., 2003); Allene oxide synthase *AOS* (*AT5G42650*; Kubigsteltig et al., 1999); chloroplastic phospholipase A1 *DAD1* (*AT2G44810*; Ishiguro et al., 2001), and jasmonate O-methyltransferase *JMT* (*AT1G19640*; Seo et al., 2001), were also tested by qRT-PCR in WT and pBISDCs lines (**Figure 1B**). Higher levels of expression were observed for all JA and MeJA biosynthesis genes in *SAMDC1*-overexpressing lines when compared to WT plants, with *LOX2* showing the most pronounced increase, with a mean of 43.76-fold higher than WT (**Figure 1B**).

Spm TREATMENT RAISES THE EXPRESSION OF DEFENSE-RELATED GENES *PR-1, PR-5,* AS WELL AS JASMONIC ACID BIOSYNTHESIS *AOS* AND *AOC1* GENES

To determine whether the changes in gene expression observed in *SAMDC1*-overexpressing plants (**Figure 1**) were due to their higher Spm levels than WT plants (Marco et al., 2011), the effect of exogenous applied PAs on expression of defense response and JA biosynthesis genes was tested. WT seeds were sown on MS media containing exogenously supplied PAs and expression levels of *PR-1*, *PR-5*, *AOS* and *AOC1* genes were determined by qRT-PCR 5 days after germination. A positive correlation between exogenous Spm concentration and transcript level was observed for the set of defense-related and JA biosynthesis genes analyzed (**Figure 2**). Conversely, exogenous addition of Put or Spd did not produce significant changes in gene expression (**Figure 2**).

OVEREXPRESSION OF *SAMDC1* ENHANCES RESISTANCE TO THE OOMYCETE *H. ARABIDOPSIDIS* AND TO THE BACTERIA *P. SYRINGAE*

The qRT-PCR analyses shown above (**Figure 1**), as well as previous transcriptome analysis of pBISDCs plants (Marco et al., 2011), suggested that *SAMDC1*-overexpressing plants, with elevated Spm levels, have constitutively activated a set of genes related to the defense response of plants to pathogenic microorganisms. Therefore, the response of the transgenic and WT lines against infection by bacterial or oomycete pathogens was studied.

WT and PBISDCs lines were infected with *H. arabidopsidis* isolate *Noks1*. Two-week-old plants were exposed to a suspension containing *H. arabidopsidis* conidiospores and extension of infection was evaluated after 10 days by counting the number of sporangiophores by leaf. The number of sporangiophores by leaf observed in pBISDCs lines was approximately a half of the number obtained in WT plants, suggesting a lower propagation of *H. arabidopsidis* (**Figure 3**).

Additionally, two strains of the bacterial pathogen *P. syringae,* pv. *maculicola* ES4326 and pv. *tomato* DC3000, were also inoculated in 2-week old leaves of WT and pBISDCs transgenic lines. Both strains infect Arabidopsis leaves and cause initial chlorosis followed by appearance of dark spots. Progress of infection was followed by visual estimation of chlorosis appearance (**Figure 4A**) or by quantifying leaf luminance percentage after conversion to grayscale and image analysis (**Figure 4B**). Three days after inoculation, visual symptoms of chlorosis were observed in WT plants inoculated with either *P. syringae* strains (**Figure 4A**) along with an increase in leaf luminance (**Figure 4B**). However, *SAMDC1*-overexpressing lines did not show yellowing symptoms 3 days

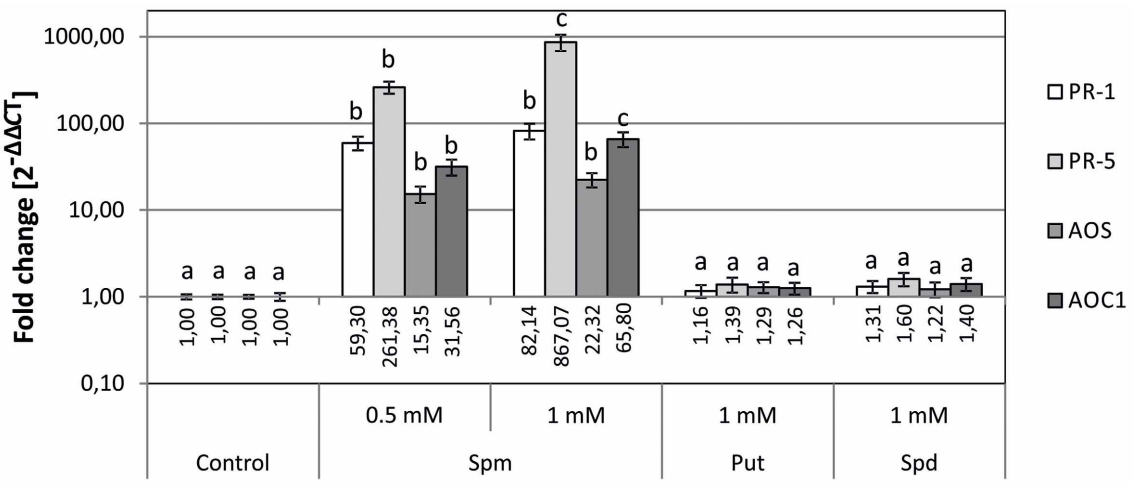

FIGURE 2 | Effects of external polyamine treatment on the expression of biotic stress defense-related genes (*PR-1* and *PR5*) and jasmonate biosynthesis genes (*AOS* and *AOC1*). Plants were grown for 5 days in plates supplemented with Put, Spd, or Spm, as well as in control plates without the amendment of PAs. Expression levels were determined by qRT-PCR. For each gene, data is expressed as fold change relative to the level measured in WT plants in control conditions ($2^{-\Delta\Delta CT}$). Graph show the mean of three biological replicates ± standard deviation. Significant differences between treatments are indicated with letters (ANOVA, Tukey HSD test, $p < 0.05$).

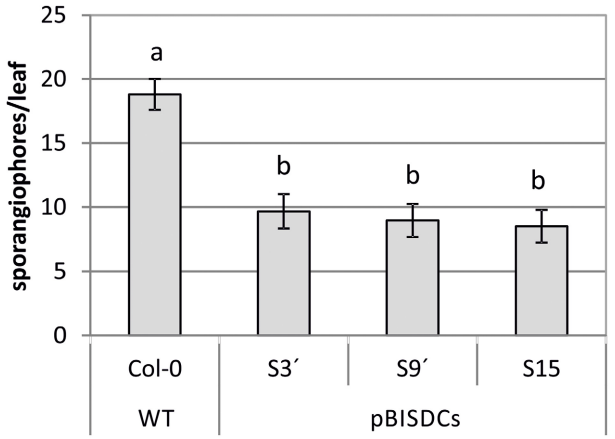

FIGURE 3 | Effects of *H. arabidopsidis* infection in Arabidopsis WT and pBISDCs transgenic lines overexpressing *SAMDC1* (S3′, S9′, and S15). Fourteen-day old plants were inoculated with a conidiosporium suspension of *H. arabidopsidis* isolate *Noks1*. Disease extension was estimated 10 days after inoculation by counting the number of sporangiophores developed in each leaf. Twenty-five leaves were sampled for each line. Graph shows the mean ± standard deviation. Significant differences between plant lines are indicated with letters (ANOVA, Tukey HSD test, $p < 0.05$).

after inoculation (**Figure 4A**), with luminance levels close to control conditions (**Figure 4B**), suggesting at least a delay in infection. *P. syringae* propagation *in planta* was also estimated by the determination of CFU in foliar disks 3 days after inoculation. Compared to WT plants, pBISDCs lines showed a 10-fold reduction in the propagation of both strains of *P. syringae* (**Figure 4C**).

In addition, leaves of plants of each line infected with *P. syringae* pv. *maculicola* or exposed to control inoculum were collected 3 days after infection and expression of defense-related genes *PR-1*, *PR-5*, *CYP79F1*, and *WAK1* was determined by qRT-PCR (**Figure 4D**). A dramatic induction of the expression of the four genes was observed in WT leaves, ranging from 200-fold in the case of *PR-1* or 1000 to 2000-fold in the case of *PR-2*, *PR-5* or *CYP79F1*. In turn, the expression levels of those genes in uninfected leaves of *SAMDC-1* transgenic plants were higher than in uninfected WT plants (**Figure 4D**) and similar to the levels reached in them after infection (**Figure 4D**).

DISCUSSION

Previous studies have shown that overexpression of *SAMDC1* or *SPMS* genes in Arabidopsis leads to plants with higher Spm content than WT plants (Gonzalez et al., 2011; Marco et al., 2011). Transcriptome studies have pointed out that a common set of 233 genes is induced in those Spm-accumulating lines. This set of genes is enriched in functional categories involved in defense-related processes during both biotic and abiotic stresses as well as JA biosynthesis and response (Marco et al., 2011). These results suggest a connection between the increase of Spm levels and the induction of biotic stress responses. The connection among Spm levels and biotic stress responses is confirmed when the set of induced genes found at *SAMDC1*-overexpressing lines is compared to a set of 312 ESTs differentially expressed during Systemic Acquired Resistance (SAR) in Arabidopsis (Maleck et al., 2000). When this comparison is made, a set of 71 common genes emerges, including transcripts coding for pathogenesis-related proteins PR-1, PR-2 as well as the JA biosynthesis enzyme LOX2, which appear overexpressed in both Arabidopsis SAR and pBISDCs transcriptomes (Maleck et al., 2000; Marco et al., 2011).

The gene expression analysis made in the present work confirmed that expression levels of *PR-1*, *PR-2*, and *PR-5* genes, are higher in Spm-accumulating pBISDCs lines than in WT plants (**Figure 1A**), as previously observed by transcriptome studies (Marco et al., 2011). Exogenously applied Spm also produces *PR-1* and *PR-5* induction in WT plants (**Figure 2**), suggesting that their expression levels in pBISDCs lines could be related to the higher Spm levels found in these plants. As expected, those genes are also induced when WT plants are infected by *P. syringae* pv. *maculicola* (**Figure 4D**). Induction of acidic PR proteins in response to abiotic stress and Spm treatment has been previously described in tobacco (Yamakawa et al., 1998). Moreover, *PR-1* induction by Spm in Arabidopsis has been also reported by Mitsuya et al. (2009). Additionally, overexpression of *SPDS* in *Citrus sinensis Osbeck* (sweet orange) leads to plants with higher Spm levels, as well as to the overexpression of genes that code for putative PR proteins, like *PR-4A* and *PR-10A* (Fu et al., 2011; Fu and Liu, 2013).

Our qRT-PCR analysis also confirmed the up-regulation of the defense-related genes *CYP79F1*, *WAK1* and *FLS2* in *SAMDC1*-overexpressing lines (**Figure 1A**). Selection of these genes was made based on previous transcriptome data (Marco et al., 2011) and the different roles played by them during plant pathogenic responses. CYP79F1, a member of the cytochrome P450 (CYP) superfamily, is a key enzyme in the biosynthesis pathway of aliphatic glucosinolates from methionine (Hansen et al., 2001; Chen et al., 2003). Glucosinolates constitute an essential part of plant defense secondary metabolites (Halkier and Gershenzon, 2006). Diverse defense pathways control glucosinolate biosynthesis by activation of different subsets of biosynthetic enzymes (CYP among them), leading to the accumulation of specific glucosinolate profiles (Mikkelsen et al., 2003). In this trend, *CYP79F1* is induced by MeJA (Mikkelsen et al., 2003; Guo et al., 2013) and our results demonstrate that it is also induced by *P. syringae* pv. *maculicola* infection (**Figure 4D**). WAK1 is the most studied member of a five-member family of Cell-Wall associated protein kinases (WAK1-5) (Verica and He, 2002). *WAK1* is induced by *P. syringae* pv. *maculicola* infection and by SA treatment (Schenk et al., 2000). Expression of *WAK1* is required by the plant to survive against lethal accumulation of SA during plant–pathogen interactions (He et al., 1998), and its ectopic overexpression confers resistance to *Botrytis cinerea* (Brutus et al., 2010). *WAK1* is also induced by MeJA and ethylene (Schenk et al., 2000). In addition, another member of WAK family, *WAK2,* appears up-regulated in pBISDCs and Arabidopsis SAR transcriptomes (Maleck et al., 2000; Marco et al., 2011). FLS2 is a receptor kinase essential in the perception of flagellin, a potent elicitor of the defense response to bacterial infection (Gomez-Gomez and Boller, 2000). Flagellin perception initiates a battery of downstream defense pathways that leads to stomatal closure to avoid bacterial invasion, as well as to the activation of mechanisms inhibiting bacterial multiplication in the plant apoplast (Zipfel et al., 2004; Melotto et al., 2006).

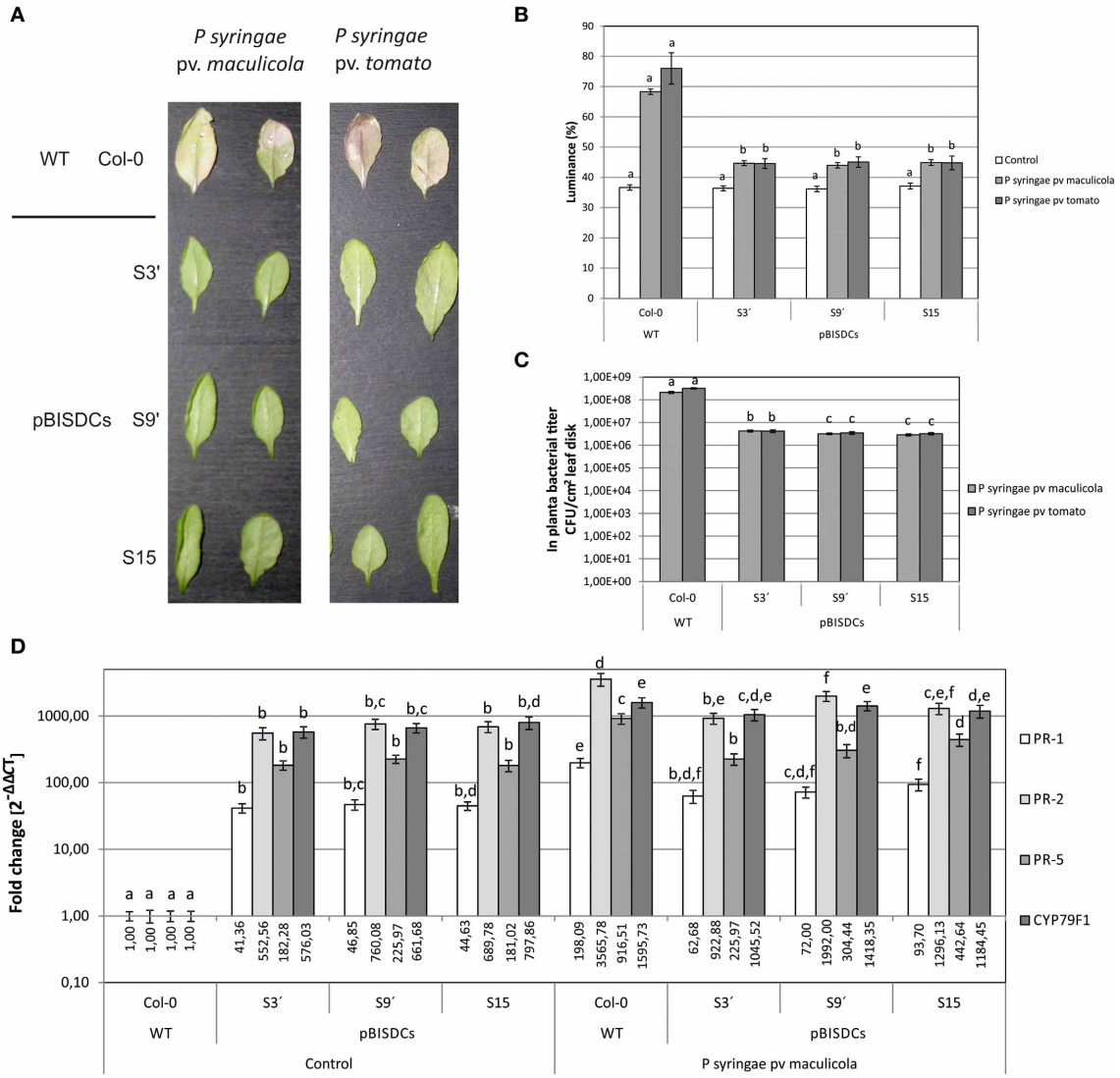

FIGURE 4 | Effects of *P. syringae* infection in Arabidopsis WT and pBISDCs transgenic lines overexpressing *SAMDC1* (S3′, S9, and S15). Leaves of 15 day-old plants were inoculated with a suspension of *P. syringae* strains pv. *maculicola* ES4326 or pv. *tomato* DC3000. Ten different plants for each line were inoculated. Disease extension was evaluated for each strain 3 days after inoculation visually **(A)**, as well as by quantifying leaf luminance percentage by image analysis using the *Image Processing Tool Kit 5.0* and *Photoshop 7.0* softwares **(B)**. Also, propagation of *P. syringae in planta* was estimated by determination of the number of CFU/cm^2 of leaf disk **(C)**. Ten different plants for each line were inoculated. Results show the mean ± standard deviation obtained for each combination of line and bacterial strain. For each condition, significant differences between lines are indicated with letters [ANOVA, Tukey HSD test (luminance data) or Dunnet's T3 test (CFU data), $p < 0.05$]. Plant response to *P. syringae* pv. *maculicola* infection was also studied by comparison of the expression levels for a set of biotic stress defense-related genes 3 days after inoculation with the bacterial strain or control inoculums **(D)**. For each gene, data is expressed as fold change relative to the level measured in WT plants inoculated with control inoculum ($2^{-\Delta\Delta CT}$). Graph show the mean of three biological replicates ± standard deviation. Significant differences between plant lines are indicated with letters (ANOVA, Tukey HSD test, $p < 0.05$).

Furthermore, levels of expression of genes coding for JA and MeJA biosynthesis enzymes, including LOX2, were also checked by qRT-PCR (**Figures 1B**, **2**). Again, Spm was the unique PA able to induce *AOS* and *AOC1* genes by external treatment (**Figure 2**), suggesting that the induction of JA and MeJA biosynthesis genes observed in *SAMDC1*-overexpressing plants (**Figure 1B**) could be promoted by their modified Spm levels. This induction could lead to the rise of JA levels in pBISDCs plants and promote JA-mediated defense mechanisms. It has been previously described that Spm treatment induces JA biosynthesis in lima bean, promoting the production of herbivore-induced volatile terpenoids that attract predatory mites (Ozawa et al., 2009). Previous studies have suggested possible interactions between PAs and JA in disease response. MeJA treatment increases PA levels and renders an improved disease response in barley seedlings exposed to powdery mildew (Walters et al., 2002), as well as in

wheat plants infected with leaf rust (Haggag and Abd-El-Kareem, 2009) or in loquat fruits inoculated with *Colletotrichum acutatum* spores (Cao et al., 2014).

SAMDC1-ovexexpressing plants show an enhanced tolerance when infected by any of the two strains of *P. syringae* assayed (**Figure 4A**) and by the oomycete *H. arabidopsidis* (**Figure 3**), in terms of *in planta* pathogen propagation. Enhanced tolerance to biotrophic bacteria *P. viridiflava* has also been observed in Arabidopsis Spm-accumulating lines obtained by overexpression of *SPMS*, as well as in WT plants treated with exogenous Spm (Gonzalez et al., 2011). In addition, sweet orange *SPDS*-overexpressing plants are also less susceptible to *Xanthomonas axonopodis* pv. *citri,* the bacterial agent that causes citrus canker (Fu et al., 2011).

In summary, qRT-PCR studies conducted in this work confirmed that pBISDCs lines have an up-regulated expression of genes that code for members of the pathogen defense system, as suggested by previous transcriptome studies (Marco et al., 2011). This constitutive activation of the defense-response mechanisms has also a positive impact on the susceptibility of pBISDCs lines against bacterial (**Figure 4**) and oomycete infection (**Figure 3**). Results obtained in this study add more evidence to the role of Spm in plant response to biotic stress, and reinforce the hypothesis that, among the different mechanisms postulated by which Spm could exert their protective action, transcriptional changes of defense genes might play an important role. It remains to be determined which of the changes in gene expression observed in the transcriptome of Spm accumulating plants are the result of the direct action of Spm or the consequence of intricate cross-talking between Spm and other biotic defense-signaling pathways, including JA and MeJA.

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Senescence and programmed cell death in plants: polyamine action mediated by transglutaminase

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Research on polyamines (PAs) in plants laps a long way of about 50 years and many roles have been discovered for these aliphatic cations. PAs regulate cell division, differentiation, organogenesis, reproduction, dormancy-break and senescence, homeostatic adjustments in response to external stimuli and stresses. Nevertheless, the molecular mechanisms of their multiple activities are still matter of research. PAs are present in free and bound forms and interact with several important cell molecules; some of these interactions may occur by covalent linkages catalyzed by transglutaminase (TGase), giving rise to "cationization" or cross-links among specific proteins. Senescence and programmed cell death (PCD) can be delayed by PAs; in order to re-interpret some of these effects and to obtain new insights into their molecular mechanisms, their conjugation has been revised here. The TGase-mediated interactions between proteins and PAs are the main target of this review. After an introduction on the characteristics of this enzyme, on its catalysis and role in PCD in animals, the plant senescence and PCD models in which TGase has been studied, are presented: the corolla of naturally senescing or excised flowers, the leaves senescing, either excised or not, the pollen during self-incompatible pollination, the hypersensitive response and the tuber storage parenchyma during dormancy release. In all the models examined, TGase appears to be involved by a similar molecular mechanism as described during apoptosis in animal cells, even though several substrates are different. Its effect is probably related to the type of PCD, but mostly to the substrate to be modified in order to achieve the specific PCD program. As a cross-linker of PAs and proteins, TGase is an important factor involved in multiple, sometimes controversial, roles of PAs during senescence and PCD.

Keywords: flower corolla, hypersensitive response, leaf, pollen, polyamines, programmed cell death, senescence, transglutaminase

INTRODUCTION

The functions exerted by polyamines (PAs) in plants have been reviewed along the first years of research by several pioneers in the field (Bagni and Serafini-Fracassini, 1974; Smith et al., 1979; Smith, 1985; Friedman et al., 1986; Galston and Kaur-Sawhney, 1987; Bagni, 1989; Evans and Malmberg, 1989; Egea-Cortines and Mizrahi, 1991; Tiburcio et al., 1993) starting from the first paper dealing with the stimulatory effect of the three aliphatic PAs (putrescine, PU; spermidine, SD; spermine, SM) on tuber dormant tissues (Bertossi et al., 1965). More recently, in addition to many excellent reviews, a special issue dedicated to PAs in plants has been published by several scientists of the field, dealing with transport, metabolism, stress tolerance, growth, senescence, unusual PAs, thermospermine, chemoprevention, and conjugated PAs (Various authors, 2010). The general idea is that growth phenomena are regulated by a cohort of environmental and internal factors among which PAs, essential juvenilation growth substances in all living organisms that regulate differentiation, organogenesis, reproduction and cell proliferation in higher plants and algae, as well as senescence, PCD, and homeostatic adjustments in response to external stimuli and stresses.

Polyamines are present in the cells in free and bound form (**Figure 1**). In fact, these polycations are able to form linkages of various types and strength with several molecules. In addition to ionic linkages with negatively charged molecules, interactions may occur by electrostatic linkages, causing conformational stabilization/destabilization of DNA, RNA, chromatin, and proteins. Covalent bonds give rise to the formation of hypusine, insoluble complexes, and "cationization" or formation of cross-links between proteins (e.g., cytoskeleton as in animals), but also with photosynthetic complexes and hydroxy-cinnamic acids, specific of plants. PAs act as free radical scavengers and some of their derivatives might result cytotoxic (**Figure 1**). These multiple aspects

Abbreviations: AZ, abscission zone; ADF, actin-depolymerizing factors; *bis*-PAs, *bis*-(γ-glutamyl)-PAs; CAP, cyclase-associated proteins; DCD, developmental cell death; HR, hypersensitive response; LHCII, light-harvesting complex II; LHC-P, light-harvesting-proteins; *mono*-PAs, *mono*-(γ-glutamyl)-PAs; PAs, polyamines; PCD, programmed cell death; PU, putrescine; ROS, reactive oxygen species; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; SI, self-incompatibility; SD, spermidine; SM, spermine; S-RNase, self-RNase; tTGase or TG2, tissue transglutaminase; TMV, tobacco mosaic virus;TGase, transglutaminase.

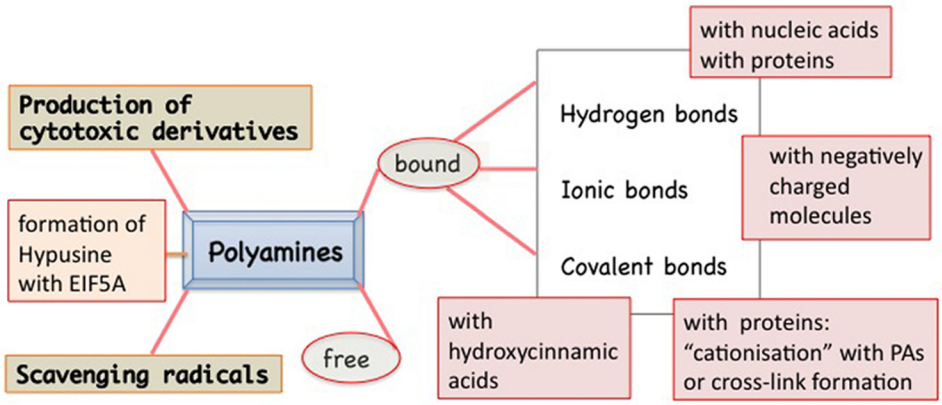

FIGURE 1 | Polyamines (PAs) in plants exist in two main forms: free and bound to many other molecules by different types of bindings: hydrogen type, more or less polarized, ionic type, or covalent one. The covalent linkage catalyzed by transglutaminase to specific proteins is Ca^{2+} dependent. Peculiar of some families of plants is the PA linkage with hydroxycinnamic acids. PAs are also actively metabolized: from PU to SD and SM but also vice versa. In addition, they can give rise to cytotoxic derivatives. PAs can act as free radicals. SD can be metabolized, by removing the aminopropyl group, and linked to a lysyl residue of the EIF5A precursor thus forming a peculiar derivative, the hypusine.

of PAs reflect on their roles in the cell life and several of them are related to the senescence progression and PCD both in animals and in plants. Very recently, a review on PAs and PCD in both organisms has been published, dealing prevalently with the consequences of PA oxidation and their cytotoxic products, but ignoring completely the aspect of PA binding (Moschou and Roubelakis-Angelakis, 2013).

SENESCENCE AND PROGRAMMED CELL DEATH

Programmed cell death (PCD) in plants, like in animals, may be a physiological terminal stage, genetically controlled, of cell differentiation; in some cases the dying cells acquire specific functions (e.g., vascular tissues, fibers) or in contrast cells die after the accomplishment of their role. This kind of PCD could be specifically named developmental cell death (DCD) being related to an internal program of species-specific correct development even though it might also be triggered by adverse environmental factors (Wu et al., 2012). Other types of PCD may occur as the result of external, either biotic or abiotic stimuli, like different environmental signals or pathogen attacks that modify the original cell program. Sometimes, it is difficult to discriminate between programmed and accidental phenomena. In this review, the general term PCD will be used if not otherwise specified. Confusion is also centred on the application of the terms senescence and PCD that may be considered separate, partially overlapping or even identical events (Thomas et al., 2003a; van Doorn and Woltering, 2005; Rogers, 2006). Senescence is not necessarily a stage of PCD; when senescence takes place, it is not a steady state but a gradual evolution of the entire cell and even though sometimes it can be delayed or reversed, usually is preliminary to PCD.

The DCD can involve the co-ordinated death of the cells of an entire vegetative or reproductive organ, or part of it, and might in some cases cause its abscission, preceded by the remobilization of most of the nutrients. DCD can be accompanied by nuclear condensation, membrane blebbing, occasionally DNA fragmentation and cysteine protease activity (Serafini-Fracassini et al., 2002; Ye et al., 2013). At the subcellular level, mitochondria may play a central role, retaining their function during senescence, since respiration continues by alternative oxidase (Vanlerberghe, 2013). Increase of ROS production and protease and nuclease activities have been reported during the leaf senescence (Quirino et al., 2000; Rubinstein, 2000). The cross-talk of nitric oxide and reactive oxygen species in plant PCD (Wang et al., 2013) as well as catabolic and interconversion products of PAs (Moschou and Roubelakis-Angelakis, 2013) have been recently reviewed. In green tissues, chloroplasts swell and redifferentiate into gerontoplasts characterized by the dismantling of thylakoidal membrane; thus, proteins, chlorophylls, lipids as well as nucleic acids are degraded and the photosynthetic activity decreases. Whereas mitochondria catabolize lipids deriving from thylakoids, vacuoles (which represent the lytic compartment of the plant cell) play relevant roles in the autophagic degradative metabolisms, as exemplified for chlorophyll, and finally rupture of tonoplast membrane takes place causing the release of degradative enzymes (van Doorn and Woltering, 2010). Cell walls of some specialized cells, before the cell dies, frequently undergo secondary modifications, such as lignification, suberification, and gelification.

During senescence, the levels of PAs are not constant showing peaks especially at its beginning, but thereafter PAs usually decrease (Galston and Kaur-Sawhney, 1990; Cohen, 1998). This pattern however depends on the type of senescence model, if induced by external factors or natural.

Much data on the PA effect were obtained through the exogenous supply of PAs, PA analogs or from loss of function or mutants in PA metabolism genes. In animal systems, the role of free PAs in apoptosis is still controversial; contrarily to animal cells, plant cells can be less affected by excess PAs, in some plant families by binding them to TCA-soluble conjugates, such as cinnamic acids, or by

storing them in the vacuole (Bagni and Tassoni, 2001). In plants, PAs can allow a prolonged survival of excised organs such as leaves, flowers, and fruits even though, as in animals, some contradictory data are reported (Altman and Bachrach, 1981; Galston and Kaur-Sawhney, 1987; Bagni and Pistocchi, 1989; Legocka and Zajchert, 1999; Lester, 2000; Hanzawa et al., 2000; Mehta et al., 2002; Bagni and Tassoni, 2006; Tassoni et al., 2006; Kusano et al., 2008; Muñiz et al., 2008; Nambeesan et al., 2010; Serafini-Fracassini et al., 2010). Examples are the different types of PCD of excised or senescing leaves and protoplasts (Galston and Kaur-Sawhney, 1990; Besford et al., 1993) or aged leaf disks (Legocka and Zajchert, 1999; Serafini-Fracassini et al., 2010), as well as vessels (Muñiz et al., 2008; Vera-Sirera et al., 2010) incompatible pollen/style system (Del Duca et al., 2010; Gentile et al., 2012) and senescing flowers (Serafini-Fracassini et al., 2002; Bagni and Tassoni, 2006; Tassoni et al., 2006; Della Mea et al., 2007a,b). Thermospermine is a structural isomer of spermine first discovered in thermophilic bacteria (Oshima, 1979). Thermospermine is critical for proper vascular development and xylem cell specification, in preventing premature maturation and death of the xylem vessel elements (Vera-Sirera et al., 2010).

The formation of hydrogen peroxide and cytotoxic products via PA catabolism is considered as one possible mechanism of PA involvement in PCD (Yoda et al., 2003, 2006) and the ability of plants to control stress is related to their capacity to metabolize PAs (Alcazar et al., 2010). In addition to the known functions of PAs in PCD by prevention of membrane damage, retard of nucleic acid and protein degradation, including the chloroplast photosystems, or acting as free radical scavengers (here not described, see the above reviews), PAs could exert their roles also by other mechanisms of action. Thus, in order to re-interpret at least some of the effects of PAs in PCD models above reported and to obtain new insights into their molecular mechanisms, their conjugation to proteins has been revised here.

POST-TRANSLATIONAL MODIFICATION OF PROTEINS: THE TRANSGLUTAMINASE ENZYMES

It is hypothesized that PAs exert some of the above-described effects through a biochemical process of conjugation with proteins. This activity is catalyzed by the enzyme transglutaminase (TGase). The process of transamidation is part of a set of post-translational modifications to which proteins can be subjected and include a number of efficient regulation strategies, such as phosphorylation/dephosphorylation, covalent modification, proteolytic degradation or activation, interaction with partner proteins. At recent, the post-translational modification is identified as one of the most important, rapid, and precise methods by which eukaryotic cells respond to environmental stresses or developmental changes. The covalent linkages of PAs to proteins are catalyzed by the enzyme family of TGase (R-glutaminylpeptide-amine γ-glutamyltransferase; E.C. 2.3.2.13), discovered and studied in animals since many years (Sarkar et al., 1957; Folk, 1980; Lorand et al., 1988). TGases are present in eukaryotic and prokaryotic organisms; in animals they fulfil different enzymic functions as summarized in a book edited by Mehta and Eckert (2005) and also reviewed (Griffin et al., 2002; Lorand and Graham, 2003; Beninati et al., 2009). Transglutaminase 2 (TG2) is the most widely distributed member of the transglutaminase family with almost all cell types in the body; TG2 is an extremely versatile protein exhibiting transamidating, protein disulfide isomerase and guanine, and adenine nucleotide binding and hydrolyzing activities. TG2 can also act as a protein scaffold or linker. This unique protein also undergoes extreme conformational changes and exhibits localization diversity (Gundemir et al., 2012). One of the TGase activities, the transamidation catalysis, consisting in the covalent conjugation of PAs and other amine-donors (among which lysyl-residues) to γ-carboxamide groups of protein endo-glutamine residues (Folk, 1980; Beninati and Folk, 1988), is the activity that has been most extensively studied in plants (reviewed by Del Duca and Serafini-Fracassini, 2005; Serafini-Fracassini and Del Duca, 2008; Del Duca et al., 2014). PU, SD, and SM differ in both their number of positive charges exhibited at the cell physiological pH (2 in PU, 3 in SD, and 4 in SM) and their backbone length (PU: 6.5 Å; SD: 11.12 Å; SM: 14.6 Å). Their two terminal amino groups conjugate to one or two glutamyl residues giving rise to PA derivatives, either *mono*-(γ-glutamyl)-PAs (*mono*-PAs) or *bis*-(γ-glutamyl)-PAs (*bis*-PAs; **Figure 2**). The additional positive charges introduced by protein-bound PAs due to their internal iminic- or free terminal aminic group (*mono*-PAs) may induce protein conformational changes. *Bis*-PA derivatives can form both inter- and intra-molecular cross-links in proteins. The backbone length of the PAs determines the length of the cross-link it forms: *bis*-(γ-glutamyl)-SD (*bis*-SD) bridges, and even more so those involving *bis*-(γ-glutamyl)-SM (*bis*-SM), span greater distances than those formed by *bis*-(γ-glutamyl)-PU (*bis*-PU). The link formed between glutamyl and lysyl residues is much shorter that those involving PAs (**Figure 2**). The binding is highly specific and is probably primarily dependent on the substrate conformation (Griffin et al., 2002). *Mono*-PA production is affected by PA concentration since high levels of PAs saturate the acyl donor residues of the substrate proteins limiting the formation of *bis*-derivatives. In this sense, the levels of PAs have a critical role in the modulation of the number of protein cross-links formed. Since several PA molecules can cross-link more proteins simultaneously, high molecular complexes may form (**Figure 3**). In addition, the free terminal amino group of *mono*-PAs can interact by additional linkages, for example with negatively charged groups of other types of molecules, thus forming heterogeneous complexes (**Figure 3**). The supramolecular nets of linked proteins are resistant to mechanical stress and proteolysis (Martinet et al., 1990) and are observed frequently as product of TGase cross-linking activity.

THE TRANSGLUTAMINASES IN PLANTS

After the discovery of PAs conjugated to proteins in plants about 30 years ago (Serafini-Fracassini and Mossetti, 1985; Mossetti et al., 1987), TGase-like activities were detected to catalyze PA conjugation to proteins (Icekson and Apelbaum, 1987; Serafini-Fracassini et al., 1988). In particular, Mizrahi et al. (1989) observed that PAs delayed senescence in oat and *Petunia* leaves and found PAs strongly bound to proteins of high molecular weight. Further on, the identification of the TGase typical products (namely the PA-derivatives), the stimulation by Ca^{2+} and the inhibition

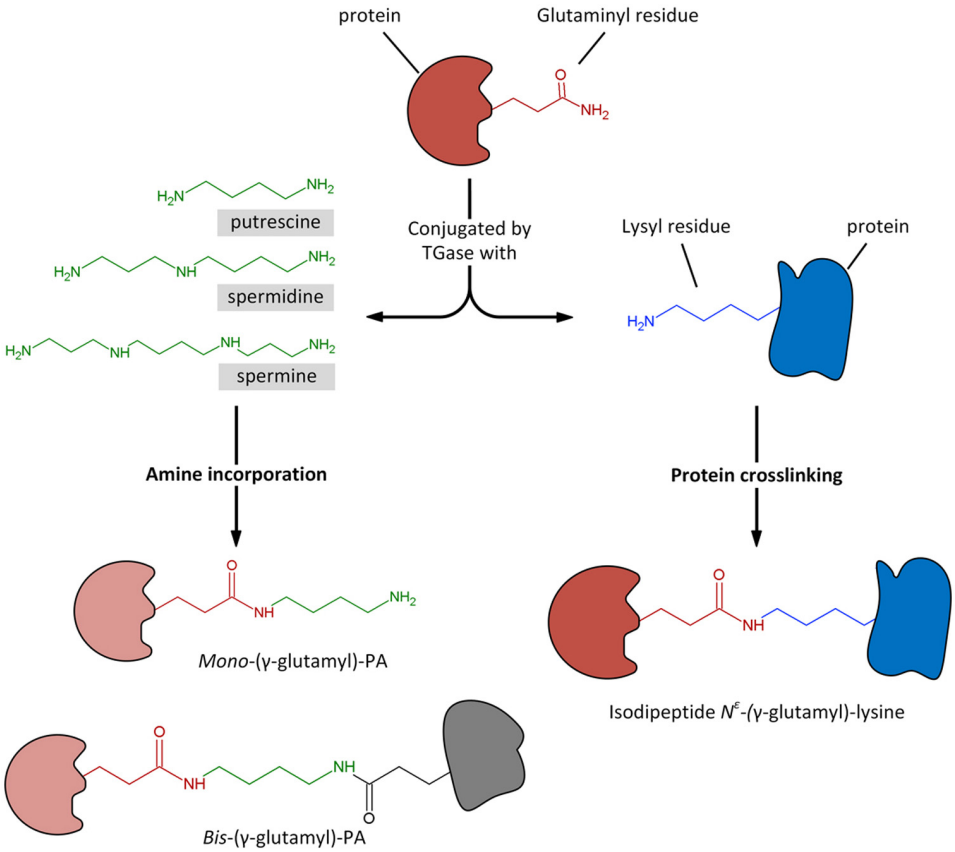

FIGURE 2 | Transglutaminase could catalyze the Ca^{2+}-dependent incorporation of substrates having a primary amino group, as polyamines, to the γ-carboxamide group of a specific protein-bound glutaminyl residue, giving rise to *mono*- and *bis*-(γ-glutamyl)-PAs. Similarly, the enzyme catalyzes the acyl-transfer reaction between the γ-carboxamide group of a specific protein-bound glutaminyl residue and the ϵ-amino group of a distinct protein-bound lysyl residue, giving rise to protein crosslinking by the formation of isodipeptide N^{ϵ}-(γ -glutamyl)-lysine.

by EGTA or EDTA, the immunorecognition by TGase antibodies of animal origin, the inhibition by specific inhibitors, and DTT dependence confirmed the identification of this enzyme (Del Duca et al., 1994, 1995; Lilley et al., 1998; Della Mea et al., 2004a). A family of TGases of different molecular mass are located in various organs of higher plants, such as seeds, pollens, meristems, mature vegetative organs, flowers, and petals. The enzymes are very active in chloroplasts where react to external stimuli, among which light, but they are active also in the cytoplasm, in relationship with cytoskeleton, in cell wall and probably in mitochondria. These topics have been reviewed by Del Duca and Serafini-Fracassini (2005), Serafini-Fracassini and Del Duca (2008), Del Duca et al. (2013b, 2014).

No DNA sequence sharing homology with the well-known animal TGases had been found in the databases of several plants making difficult identifying plant TGases by sequence comparison. A computational analysis has shown the presence in *Arabidopsis thaliana* of only one gene, *AtPng1p,* that contains the TGases catalytic domain with the Cys–His–Asp triad. This gene encodes a putative N-glycanase, active at least *in vitro* in heterologous systems (Diepold et al., 2007; Masahara-Negishi et al., 2012) but its product also acts as a TGase, having a Ca^{2+}- and GTP-dependent transamidase activity and forming glutamyl-PA derivatives (Della Mea et al., 2004b). This was the first plant protein, isolated and characterized at the molecular level, displaying a TGase activity, whose biochemical parameters and 3D structure agree with those typically exhibited by animal TGases. Other TGases of different molecular mass of chloroplasts origin were sequenced (Villalobos et al., 2004; Campos et al., 2013) and the homology of the amino acidic composition of three TGases of *Helianthus tuberosus* meristems with mammal TGases were reported (Beninati et al., 2013).

This review is devoted to the possible role of plant TGase and, consequently, of conjugated PAs, in senescence and PCD. Thus, for other aspects of TGase in plants not strictly related with these subjects, other reviews can provide more information (Del Duca and Serafini-Fracassini, 2005; Serafini-Fracassini and Del Duca, 2008; Della Mea et al., 2009; Serafini-Fracassini et al., 2009; Del Duca et al., 2013b, 2014). One of the possibilities that thermospermine regulates xylem terminal differentiation through the interaction with yet unidentified elements, as suggested by Vera-Sirera et al. (2010) and that also thermospermine is conjugated to proteins by TGase, is a promising hypothesis that cannot be discussed as unfortunately no experimental data are available. Due to the

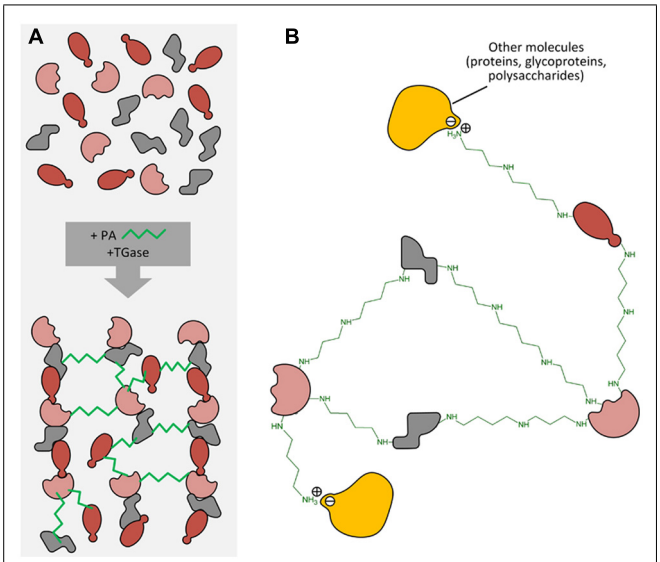

FIGURE 3 | (A) Different protein molecules can form a net by PA linkage catalyzed by transglutaminase. **(B)** PAs can also form other linkages (i.e., ionic ones) with different molecules such as proteins, polysaccharides, phenols, etc., thus forming heterogeneous complexes.

relatively recent and still incomplete data on plant TGases in PCD, references to this subject in animals will be given for comparison and for suggesting possible interpretations.

TRANSGLUTAMINASE IN PROGRAMMED CELL DEATH

One of the most studied mammalian TGase, tissue TGase (tTGase or TG2) is constitutively expressed but has a low activity in growing animal cells and/or in non-stressed cells, whereas it is generally up-regulated in cells undergoing cell death. As an example, TG2 is involved in the formation of apoptotic bodies in which N^{ϵ}(γ-glutamyl)lysine cross-links have been detected. However, due to its multifunctional activity as TGase but also as Ca^{2+}-independent GTPase, it also acts as effector in the prevention of cell death. Overexpression of TG2 in cells leads to increased export of the enzyme to the cell surface and into the extracellular matrix (Griffin and Verderio, 2000; Wang et al., 2012). Activation of the enzyme by increase of cytosolic Ca^{2+} would result in the cross-linking of both intracellular and extracellular proteins leading to stabilization of the dying cell and surrounding matrix thus maintaining both cellular and tissue integrity or remodeling. Expression of the enzyme has been generally correlated to both the level of nuclear fragmentation and to morphological changes of cells undergoing apoptosis. The extensive polymerization into insoluble aggregates of actin, retinoblastoma gene product, and nuclear proteins is a key signal for the initiation of apoptosis (Oliverio et al., 1997). This could be important for preventing the inflammatory responses that would follow the dispersion of the contents of dying cells. However, the evidence that TG2 is likely implicated as a mediator of apoptosis is conflicting (Fesus and Szondy, 2005). It was proposed that the pro-apoptotic or anti-apoptotic effect of TG2 is dependent on the activation pathways and location; nuclear and extracellular TG2 may affect anti-apoptosis while cytosolic TG2 is pro-apoptosis (Milakovic et al., 2004). Intracellular SM and SD are capable of modulation of TG2 expression (Chen and Mehta, 1999). However, the blockage of PA synthesis in different cell types was shown to differently influence TG2 expression by decreasing expression in one cell type and increasing expression in another (McCormack et al., 1994; Wang et al., 1998; Piacentini et al., 2005). It is becoming evident that the multifunctional roles of TG2, both cytosolic and nuclear TG2, in cell death processes (apoptosis and or autophagy) are dependent upon the cell type, stimuli, subcellular localization, and conformational state of the protein. The conformational and functional diversity of TG2 in the context of its role in numerous cellular processes has been recently reviewed by Gundemir et al. (2012); in particular, it has been highlighted how differential localization, conformation and activities of TG2 may distinctly mediate cell death processes.

In plants, an increasing number of reports on TGase in senescence and PCD, studying both reproductive and vegetative organs, suggest a strict correlation of TGase and PA physiological effects. Until now, studies have been mainly focused on senescence and death of the leaf (Sobieszczuk-Nowicka et al., 2007, 2009; Serafini-Fracassini et al., 2010; Sobieszczuk-Nowicka and Legocka, 2014) and of the flower petals (Serafini-Fracassini et al., 2002; Della Mea et al., 2007a,b) or on the growing or abiotic stress-induced dyeing pollen (Iorio et al., 2012b) as well as self-incompatible (SI) pollination system (Del Duca et al., 2010; Gentile et al., 2012) and on the hypersensitive response (IR) to pathogens (Del Duca et al., 2007). The study of these plant models is important not only for basic but also for applied research. Just as an example, freshly cut leaves, which are utilized as food, like lettuce, and freshly cut ornamental flowers have in fact a short shelf life.

THE FLOWER COROLLA PCD

In reproductive organs, various parts undergo DCD. Petals, which are modified leaves, have in general a vexillary role and, once completed this role, they enter senescence and fall; in some cases, e.g., *Nicotiana*, they remain *in situ*, become rigid and papyraceous to protect the initial growth of the ovary. Flower petal senescence and its final death is a highly regulated developmental phase, controlled by hormones and growth factors, like ethylene, cytokinins, abscisic acid (Orzaez et al., 1999; van Doorn and Woltering, 2008; Amasino and Michaels, 2010; Rogers, 2006, 2012) as well as PAs, as above reported. In the long-lived flowers, pollination acts as a signal for senescence, while in the short-lived flowers this event is independent from pollination. Petals are histologically rather homogenous consisting of parenchyma, thin veins and a protecting layer of epidermis.

As a flower corolla model, senescence and death were studied *in planta* or in flowers of *Nicotiana tabacum* excised at different growing stages (Serafini-Fracassini et al., 2002; Della Mea et al., 2007a,b). The senescence of corolla follows a visible acropetal gradient, completed by the death of the entire corolla that concludes with the teeth curling. The stages of maturation, senescence and death were established macroscopically by analysing various morphological parameters. The timing and localization of the most characteristic events were evaluated by biochemical and physiological analyses as well as by cytological observations. Even though

precocious signs can be detectable also before, flower petal senescence was characterized by the appearance at its base of a "ring", named abscission zone (AZ), of dying cells, which detach from each other and blocks the sap transport. This event is concomitant with nuclear blebbing, DNA laddering, cell wall modification, peak of protease activity, decline of protein, water and pigment (anthocyanins, chlorophylls) content, decrease in membrane integrity and increase in Ca^{++}-dependent TGase activity, detected as amount of the protein modified by labeled SM and increase in glutamyl-PA (especially *mono*-PU) production (**Figures 4A,B**). *Bis*-PA derivatives can form both inter- and intra-molecular cross-links in proteins, whereas *mono*-PAs are preferential substrates of PA oxidases. The maximum of TGase activity coincides with the appearance of high polymers immunorecognized by TGase antibody and with the flower senescence (**Figure 4B**, insert). These data could support the hypothesis that the formation of more cross-linkages among proteins possibly increased the dimension and strength of the protein net. This could be relevant for structural substrates, like cytoskeleton, discussed below, or cell walls. Ca^{2+} could exert an important regulatory role of the enzyme activity. It is known that in senescing tissues this cation increases in concentration (Huang et al., 1997; Ma and Berkowitz, 2011), especially because of its release from the vacuole, caused by tonoplast rupture.

The acropetal gradient of tobacco corolla senescence and DCD was temporally preceded by a maximum of TGase activity, which shifted from the proximal to the distal part of the corolla (Della Mea et al., 2007b). This activity modified either the endogenous substrates alone or a specific recombinant mammal TGase exogenous substrate, namely His6-X Press-green fluorescent protein (GFP); the modifications are revealed by changes in their electrophoretic migration, thus of their molecular mass, and the PA glutamyl derivatives produced. The recombinant GFP is a good substrate for TGase, because its electrophoretic shift changes in a similar way after modification by animal and plant TGases.

The TGase protein bands were immunorecognized by three antibodies raised against mammalian, nematode and *Arabidopsis thaliana* TGases. The fact that the antibody raised against *Arabidopsis* TGase recognizes some proteins of *Nicotiana*, also immunodetected by two animal antibodies, and that plant and

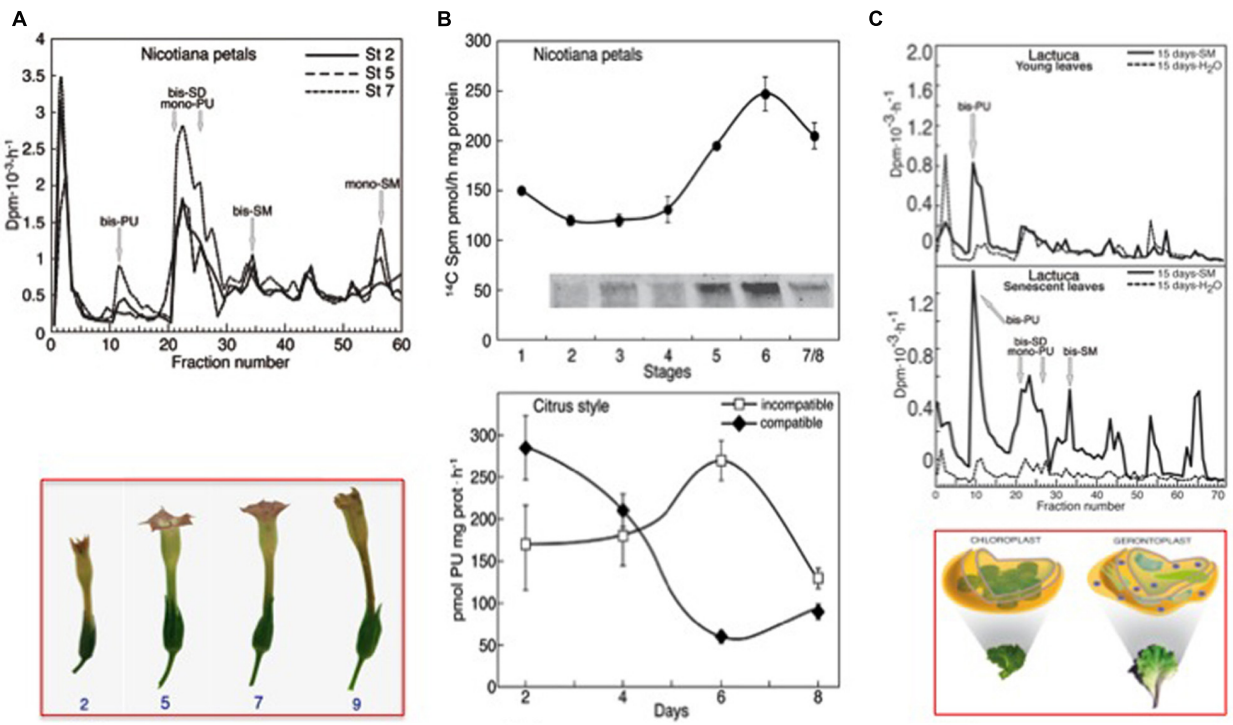

FIGURE 4 | Transglutaminase activity in different senescing or PCD models. (A) Corolla petals of flowers of *Nicotiana tabacum* senescing on the plant, starting from stage 2 (growing) to 7 (senescent), bottom panel. Top panel: glutamyl-PAs were separated by ion-exchange chromatography of extracts of the whole corolla at stages 2, 5, and 7 incubated with [^{3}H] SM as described in **(B)** [modified from Della Mea et al. (2007b)]. **(B)** Comparison of the TGase pattern in two models of PCD. Top panel: petals of *Nicotiana tabacum* during natural corolla life span (stages 1–8). The activity was detected by incubating corolla extracts in the presence of 0.2 mM [^{14}C] SM as tracer and the labelled conjugates produced were either measured by TCA precipitation or, after separation by SDS-PAGE, detected by autoradiography; a detail of the 250 kDa immunorecognized TGase polymers is shown in the insert [modified from Della Mea et al. (2007b)]. Bottom panel: TGase activity in compatible and self-incompatible pollinated styles of *Citrus grandis* at different days of pollination. The activity was detected by incubating style extracts in the presence of [^{3}H]-PU as the tracer (modified from Gentile et al., 2012). At day 6, the incompatible pollen tube growth was blocked. **(C)** Senescing leaves of *Lactuca sativa* compared with the young ones. Glutamyl-PAs were separated by ion-exchange chromatography from extracts of young and senescing leaves, sprayed with SM or water when still on the plant and extracted 15 days after the spray. Extracts were incubated for 60 min with [^{3}H] SM as tracer in the assay mixture containing 5 mM Ca^{2+}. Bottom panel: picture of the *Lactuca* leaves and related plastids [modified from Serafini-Fracassini et al. (2010)].

animal TGases present similar molecular weights and modify GFP in a similar way, would suggest a similarity among these enzymes. However, plant and animal PCD are dissimilar due to the cell structure; in fact, typical plant organelles, such as chloroplasts, vacuoles, and also possibly the cell walls, play a role in the induction or execution of PCD, as reported during the leaf and petal senescence (Quirino et al., 2000; Rubinstein, 2000; Lim et al., 2007). The localization of TGase in the *Nicotiana* petal cells could suggest new and different roles of this enzyme in PCD in addition to those detected in animal cells. As reported by Della Mea et al. (2007b), a 58-kDa band immunodetected by anti-TGase antibodies, representing also the prevalent form in leaves, decreased during corolla life and was present in the soluble, microsomal, plastidial and cell wall fractions. In contrast, the peak location of a 38-kDa band, mainly a plastidial form, moved progressively from basal to distal parts of the corolla, where it was exclusively present. This 38-kDa putative enzyme could match with a TGase isolated from maize (*Zea mays*) thylakoids (Della Mea et al., 2004a) and with a 39-kDa enzyme detected in chloroplasts of *Medicago sativa* (Kuehn et al., 1991). The plastid TGase, stabilizing the photosystems, could favor the efficiency of photosynthesis and indeed sustain the energy requirements of senescence progression. In the soluble fraction a 52-kDa immuno-positive band was decreasing with age and in late senescence a high (>250 kDa) molecular mass band appeared (Della Mea et al., 2007b), suggesting a binding of the enzyme with a cytoplasmic component (possibly cytoskeletal proteins?) or possibly because of enzyme polymerization as suggested in animals (Lorand and Graham, 2003).

TGase activities were detected in different cell compartments (Della Mea et al., 2007b); activity prevailed in the microsome fraction, where it is in general higher in the proximal part of petals, peaking at the corolla opening, and in the plastids, where it shows an increasing trend. Data on the TGase role in chloroplasts are discussed below in the leaf PCD paragraph. In particular stages of senescence, a minor rate of activity was detected also in the cell walls, prevailing in the distal part and progressively increasing as well as in the soluble fraction, where it is present only in the proximal part at senescence. The intracellular TGase, possibly released into the cell wall as in pollen (see below), was hypothesized to co-operate with cell wall strengthening or modification by protein cross-linking, especially either at the basal abscission zone or distally where the teeth curl, outward during differentiation and then refold at the later stages to protect the developing ovary. During these morphological events, cytoskeleton and turgor changes play a major role, but these are presumably supported by cell wall local strengthening. The walls of the corolla parenchyma cells during senescence undergo modifications evidenced by an increased auto-fluorescence, indicative of its suberification/lignificaton (Serafini-Fracassini et al., 2002) and by the rigid/papyraceous-like aspect of the corolla. Relevant cell wall modifications occur also in cells located in the AZ to prevent the release of toxic substances, desiccation, and pathogen attack after corolla abscission; in fact, the tissues around the AZ must be protected by impermeabilization of the scar. Cell wall could be assimilated to extracellular matrix of senescing animal cells, where TGase stabilize the dying cell and surrounding matrix thus maintaining both cellular and tissue integrity or remodeling. Some data on the TGase in plant cell walls are discussed below and revised by Del Duca et al. (2014). Overall, these data suggest a relationship between DCD and TGase, whose roles are probably different, depending on the function and modification of the compartments in which the enzyme is located.

In PCD, mitochondria have a central role and PAs were long ago detected in these organelles (Torrigiani and Serafini-Fracassini, 1980); PAs were shown to have a role also in *Nicotiana* mitochondria function, as suggested for example by the relationship between SM and mitochondria dysfunction involving the SM-signal pathway (Takahashi et al., 2003). Unfortunately, there are no data on TGase in petal mitochondria; the only data available of a covalent binding of PAs to proteins, tentatively via TGase, in plant mitochondria was obtained in potatoes and mung beans (Votyakova et al., 1999).

In order to evaluate the anti-senescence effects of PAs, detached *Nicotiana* flowers were treated with exogenous SM and with an inhibitor of ethylene action, silver thiosulfate; they showed senescence delay, retard DNA fragmentation and vacuole damage, prolong chloroplast viability with visible preservation of chlorophyll content (Serafini-Fracassini et al., 2002). SM taken up was also converted back to PU and SD, found either in free or TCA-soluble form. In *Nicotiana* these conjugates are mainly hydroxycinnamoyl derivatives, which are known to increase during flowering (Martin-Tanguy et al., 1996), but no evidence is reported of their involvement in senescence. The anti-DCD effect could be mediated, at least in part, by SM covalent binding to TGase substrates. PA supply causes the formation of very high molecular mass products, especially in the presence of an excess of PAs, which cannot be separated by electrophoresis, in addition to different protein bands of lower mass. In animals, many protein substrates were detected among which actin, β-tubulin, annexin, fibronectin and core and H1 histones and others, which could easily be involved in PCD (Piacentini et al., 2005). These proteins could also be substrates of *Nicotiana* TGase; currently, those identified in plants are actin and tubulin, and photosystem ones, like LHCII, as well as some cell wall proteins (Serafini-Fracassini and Del Duca, 2008; Del Duca et al., 2014); thus these substrates are located in different cell compartments, exactly like TGase.

THE LEAF SENESCENCE AND PCD

Yellowing of leaves is a visible paradigm of leaf senescence and PCD; therefore, leaf is one of the first and more studied models (Quirino et al., 2000; Lim et al., 2007). Once leaves have completed their role, they generally undergo senescence that results in the coordinated degradation of macromolecules and the subsequent mobilization of components to other parts of the plants. Yellowing is well known to be due to the preferential degradation of chlorophyll over carotenoids. Chloroplasts play a role in leaf senescence; they are involved in sustaining the energy requirements for the progression of senescence and develop into gerontoplasts (**Figure 4C**). Concomitantly with chlorophyll release and degradation, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and the light-harvesting-proteins (LHC-P) are also degraded (Park et al., 2007).

Polyamines are known to be also localized in chloroplasts (Bagni and Serafini-Fracassini, 1974) and are therein both synthesized and oxidized (Torrigiani et al., 1986; Bernet et al., 1999). Correlations among levels of PAs in chloroplasts, where their biosynthesis is controlled by white light, chlorophyll biosynthesis and photosynthetic rate have been observed. PAs are involved in the stabilization of thylakoids, in which organic cations are more efficient than Mg^{2+} in promoting the stacking adjacent thylakoids (Besford et al., 1993; Legocka and Zajchert, 1999; Ioannidis et al., 2009). PAs have been found associated with PSII, particularly with the LHC and operate on the structure and function of the photosynthetic apparatus during photoadaptation and photoprotection against factors such as UV-B, ozone, etc. (Navakoudis et al., 2007). Thus, their binding seems to be relatively strong and related to precise physiological roles. In *Pisum sativum,* it was found that SM stabilized the molecular composition of the membranes by preventing lipid peroxidation (Stoynova et al., 1999).

The first effects observed of exogenous PA application or PA overexpression on leaves were obtained under stress conditions that may cause PCD. Leaves of monocots and dicots under the influence of osmotic stress lose chlorophyll, undergo rapid senescence, and accumulate large amounts of PU (Galston and Kaur-Sawhney, 1990; Cohen, 1998). The exogenous addition of SD or SM inhibited protein degradation, chlorophyll loss and stabilized thylakoid proteins such as D1, D2, cyt f and the large subunit of RuBisCO (Mizrahi et al., 1989; Besford et al., 1993; Duan et al., 2006). Mizrahi and coworkers also found PAs strongly bound to proteins of molecular weight higher than 45 kDa. Their data suggested that binding could be covalent and stimulated the research on TGase in chloroplasts. An indication of the presence of this enzyme in chloroplasts was at first suggested by Cohen et al. (1982) and detected in leaves by Signorini et al. (1991). Later on in isolated chloroplasts of *Helianthus tuberosus*, TGases have been reported to catalyze the conjugation of PAs to both stromal and thylakoid proteins (LHCII, CP29, CP26, CP24, RuBisCO; Del Duca et al., 1994). The LHCII apoproteins are the preferred substrates, being the oligomeric forms of LHCII much more intensely labeled by PAs than monomeric ones and demonstrating that PAs take part in oligomer stabilization through the formation of cross-links (Dondini et al., 2003). The first plastidial TGase has been identified as a 58-kDa form associated with thylakoids (Del Duca et al., 1994; Dondini et al., 2003); in addition, a 39-kDa Ca^{2+}-dependent TGase was found to co-purify with LHCII in mature *Zea mays* chloroplasts (Della Mea et al., 2004a). In mature chloroplasts, TGase is activated by light [as demonstrated by the identification of glutamyl derivatives (Del Duca et al., 1995)], salt stress and kinetin (Margosiak et al., 1990; Del Duca et al., 1994; Dondini et al., 2003; Della Mea et al., 2004b; Sobieszczuk-Nowicka et al., 2007). Separation of thylakoid proteins followed by LC-MS identification of protein complexes, confirmed that *Z. mays* chloroplast TGase forms part of a specific PSII protein complex (Campos et al., 2010).

It has been suggested that remodeling of grana may be possible through overexpression of a TGase and polyaminylation of antenna proteins, and this might play a functional role in the formation of the grana stacks and cause an imbalance between capture and use of light energy (Ioannidis et al., 2009; Ortigosa et al., 2010). A role for TGases in energy production in chloroplasts was recently suggested by analyzing the proton and electron circuit in thylakoids (Ioannidis et al., 2012), also showing that PU stimulates photophosphorylation while SD and SM are efficient stimulators of non-photochemical quenching (Ioannidis and Kotzabasis, 2007).

Polyamines were also found to be involved in the chloroplast development and dismantling. In fact, PAs exogenously added to *Z. mays* leaves during the transformation of etioplast to chloroplast accelerated the enzymatic conversion of protochlorophyllide to chlorophyllide, possibly stabilized through PAs, and the subsequent increased efficiency of photosynthesis (Beigbeder et al., 1995; Andreadakis and Kotzabasis, 1996). The mechanisms by which PAs can affect the assembly of plastidial membrane are partially mediated by their covalent linkage to these membranes via TGases (Sobieszczuk-Nowicka et al., 2007). The general drop in PA levels that occurred during the transformation of etioplast to chloroplast may be a result of the disappearance of a large prolamellar bodies to which PAs are bound for stabilization of its hexagonal structure.

Exogenous PA application or PA overexpression in plant cells also affect chloroplast metabolism during senescence and the forthcoming PCD. Even though it is difficult to compare the induced cell death with the natural one, experiments performed on the first system allow clarifying some events also occurring during natural senescence. Like SM, SD added to cut leaves of barley, senescing in darkness, inhibited the RNase activity, the degradation of chlorophyll and of LHCII protein (Legocka and Zajchert, 1999).

The fact that PAs are also effectively able to retard leaf senescence by their conjugation catalysed by TGase was directly shown in *Lactuca sativa* during induced cell death using leaf disks, or during the normal developmental senescence of leaves (Serafini-Fracassini et al., 2010). In leaf disks, supplied SM caused a delay of chlorophyll decay, an increase of endogenous TGase activity, and a threefold increase in chlorophyll content when supplied together with exogenous TGase. SM was conjugated, via TGase, mainly to 22–30 kDa proteins, a value shared by most of the antenna proteins. When the TGase activity was checked on *Lactuca* leaf left on the plant, it was higher in young leaves in respect to already-senescing ones. In young leaves, TGase was immunodetected in protein SDS gel bands of molecular mass of 77, 58, 39 and 20–24 kDa, close to those detected in chloroplasts of several other leaves (Serafini-Fracassini and Del Duca, 2008). In contrast, higher bands (160 kDa) were found in more senescent leaves (Serafini-Fracassini et al., 2010). A similar form was only detected in very low amount in chloroplast stroma fractions (Dondini et al., 2003).

When young and senescing *Lactuca* leaves, left on the mother plant, were SM-sprayed, an increase in free SM occurred suddenly in the young leaves, whereas over longer periods (15 days) there was an increase in perchloric acid-soluble and -insoluble SM metabolites. In already-senescing leaves, SM prevented degradation mainly of chlorophyll *b*, increased TGase activity and PA–protein conjugates, and maintained the leaf in a visible younger state. SM was conjugated mainly to LHCII by an endogenous

TGase enzyme (co-fractionated with LHC) more significantly in the light, even though its conjugation occurred also in isolated PSI fractions (Serafini-Fracassini et al., 2010), in agreement with the 39 kDa TGase found to co-fractionate with maize LHCII (Della Mea et al., 2004a).

When [^{3}H] SM was spotted as a tracer on leaf epidermis, its conjugation to the natural substrate of the leaf was observed: SM is transferred to chloroplasts and converted into the lower mass PAs, as SD and PU. When SM was sprayed on the young leaves, *mono*- and *bis*-PU and *bis*-SD were immediately produced more efficiently as compared to the control. On the contrary, if the sprayed leaves were left on the plant for additional 15 days and then extracted, only *bis*-PU was produced in higher amount. In SM-treated senescing plants, *mono*- and *bis*-PU and *bis*-SD and some additional derivatives, among which *bis*-SM, were produced in higher amount in respect either to the control or the SM-treated young leaves. Samples collected after 15 days are shown in **Figure 4C** (Serafini-Fracassini et al., 2010).

The protecting effect of SM on chlorophyll degradation could be related to its non-enzymatic binding (Dondini et al., 2003), either as free form but also as *mono*-PU derivative by its free primary amino group. As chlorophyll *b* is also linked to a glutamine of LHCII, this binding could further enhance the complex protein–chlorophyll stability and delay its degradation.

In summary, these data show that TGase activity, which is declining in untreated sample, is stimulated by SM to the level in young leaf; the effect is clearly visible with the endogenous chloroplast substrate and the senescing samples were also very reactive after late excision. The senescence-delaying effects of SM could be mediated by TGase protecting leaves from the decay of their chloroplast photosystem complexes.

In another leaf system, the excised barley leaf maintained in dark condition to cause its senescence, the level of PAs bound to thylakoids changed in senescing leaves: bound PU and SD increased throughout senescence, whereas bound SM decreased (Sobieszczuk-Nowicka et al., 2009). The decrease in bound SM during thylakoid degradation could be related to the breakdown of chloroplasts, degradation of LHCPII as well as other proteins of the chlorophyll *a/b* antenna complexes. An increase in TGase activity was detected by the colorimetric assay by using dimethylcasein as the substrate, an animal substrate frequently used also in plant assays to evaluate the enzyme levels. As the natural substrate is decreasing, the competition between the two substrates could justify the TGase increase. The immunodetection of TGase in thylakoid fraction revealed three bands of 33, 58, and 78 kDa whose intensity increased during senescence, showing a good correlation with the activity detected.

Kinetin supplied to petioles of excised barley leaves retarded senescence and diminished the increase in thylakoid-bound PU and SD and almost completely abolished the decrease of bound SM. These data suggest different roles of PU/SD and SM in thylakoid degradation. Kinetin down-regulated the accumulation of the 58- and 78-kDa TGases and the TGase activity but stimulated the presence of immunodetected thylakoid CP 26 used as a marker for the timing of thylakoid degradation. This could have an impact on the measure of TGase activity. The authors postulate that the formation of covalent bonds between PAs and proteins by TGase is involved in chloroplast senescence. The kinetin-mediated preservation of low TGase levels and activity throughout leaf senescence may represent an important component of the mechanism of kinetin action in the retardation of leaf senescence (Sobieszczuk-Nowicka et al., 2009).

THE POLLEN PCD

The pollen tube is an excellent cell model to study the processes related to stress and cell death. The pollen tube is a cell destined to die as it expires after transporting the sperm cells to the embryo sac. Although this event is crucial to complete the process of fertilization, very little is known about it. Many more information are available on the self-incompatibility (SI) cell death, which is essential to prevent a plant to auto-fertilize thereby allowing plants to interbreed and therefore to increase genetic variability. During SI, the self-pollen is rejected after contact and, eventually, growth in the female tissues (stigma and style), while the non-self-pollen can survive and grow allowing fertilization. The SI process is precisely controlled at genetic level and is extremely selective (Rea and Nasrallah, 2008).

To perform its function, the pollen tube can grow through the stigma and style by "tip growth" (Cole and Fowler, 2006) a process by which secretory vesicles accumulate in the apical growing area and fuse with the plasma membrane releasing what is required for cell growth. A signal transduction system is necessary to allow pollen tubes to grow directionally toward the embryo sac while the cytoskeleton implements the information received from the transduction system and determines the accumulation of secretory vesicles (Šamaj et al., 2006). The oscillating changes of Ca^{2+} concentration at the apex are required to finely adjust the polymerization state of cytoskeletal elements (mainly actin) and consequently to control the flow of organelles and vesicles (Cole and Fowler, 2006). Any physical, chemical, or biological agent (such as SI) capable of altering this delicate mechanism can modify the pollen tube growth up to the dramatic consequence of blocking the process of fertilization.

Currently, little information is available on the relationship between cell death of pollen tubes and TGase/PAs. It is known that a form of extracellular TGase is involved in the apical growth of pollen tubes in apple tree. Since both specific inhibitors and monoclonal antibodies against TGase can block the growth of pollen tubes, TGase may play a role in the construction of the cell wall and in the interaction between pollen tubes and styles during fertilization (Di Sandro et al., 2010). In the pollen of *Malus domestica*, two polypeptides with a mass of 70 and 75 kDa were identified by immunoblotting with monoclonal antibodies against heterologous TGase. These proteins are able to cross-link both actin and tubulin thereby generating a number of products with a higher molecular mass (from 90 to 160 kDa). An additional 55 kDa immunoreactive polypeptide of the cell wall fraction has the same molecular mass as an active TGase extracted from the *Nicotiana* petal cell wall as reported above (Del Duca et al., 2009; Di Sandro et al., 2010).

Three main mechanisms of SI have been characterized in Angiosperms but only two of them operate at the level of pollen tubes, while the third mechanism works primarily at the level of non-germinated pollen grain. In poppy (*Papaver rhoeas*), the SI

response requires a recognition event between specific S proteins of the stigma and pollen (Rudd and Franklin-Tong, 2003). In turn, this event triggers a cascade of Ca^{2+}-dependent signals (many of which are unknown) that inhibit the apical growth of pollen tubes, producing critical changes in the trafficking of organelles and causing the depolymerization of actin filaments (with concomitant formation of actin foci; Thomas et al., 2003b). This process ends with the activation of a caspase-like protease activity. In the Solanaceae, Rosaceae, and Plantaginacee, the SI response is based on the presence of S-RNases, small proteins with RNAse activity that are produced by the pistil and are internalized in the pollen tube by either direct absorption or endocytosis (Wang et al., 2003). This system can be exemplified by the maloideae. In pear, the SI response is based on the internalization of specific proteins, called S-RNase (Liu et al., 2007), through hypothetical endocytotic processes. In incompatible pollen tubes, S-RNases would be released from vacuoles and free up in the cytoplasm where they degrade mRNA (Goldraij et al., 2012). The growth of incompatible pollen tubes would not be blocked exclusively by the degradation of mRNA, as other degradative processes will take part. In pear, the SI response also affects the activity of mitochondria, leading to changes in the production of ROS (reactive oxygen species) and therefore to alterations of the growth process (Wang et al., 2010). In turn, ROS (by affecting the levels of Ca^{2+}) may cause substantial changes to actin filaments (Liu et al., 2007; Wang and Zhang, 2011). Thus, blocking the growth of incompatible pollen tubes requires mechanisms that are partly similar to the SI response of poppy. In the case of pear, the disorganization of actin filaments would lead to the breakdown of vacuoles and to the release of S-RNase in the cytoplasm. Although no information is available on the role of microtubules in the SI response in pear, recent data obtained in apple indicate that microtubules could be critical in the internalization process of S-RNase by driving endocytotic membranes toward the vacuole system in order to release S-RNase in the pollen tube cytoplasm (Meng et al., 2014).

What is the role of PAs and TGase in the above-mentioned processes? TGase has not been characterized in the pollen tube of poppy; therefore, the following assumptions mainly relate to the pollen tube of pear where TGase has been identified and characterized from different points of view (Del Duca et al., 2013a). Data on the possible relationship between TGase and incompatibility were also obtained in *Citrus* (Gentile et al., 2012). In *Citrus*, an increase in the content of either bound or conjugated PAs and of TGase activity occurs during incompatible pollination reaching a peak when the process of SI becomes visible (**Figure 4C**; Gentile et al., 2012). Immediately after, TGase activity decreases. In contrast, during compatible pollination the TGase activity decreases rapidly and then stabilizes approximately on the values of incompatible pollination. In pear, all the TCA-insoluble PAs increase after fertilization while SM and PU are higher in incompatible pollination (Del Duca et al., 2010). These data would be in agreement with a possible increase of TGase as observed in *Citrus*. The increase of TGase activity in the incompatible pollination of pear does not seem dependent on a higher expression of the enzyme (Iorio et al., 2012a) but it is probably related to changes in the concentration of intracellular Ca^{2+} occurring during the SI response (Wang et al., 2010). Since TGase is a Ca^{2+}-dependent enzyme, changes in the concentration of Ca^{2+} as induced by the SI response may significantly alter the enzymatic activity of TGase. A further support for the role of TGase during the SI response comes from the discovery that cytoplasmic TGase of apple pollen is able to post-translationally modify actin and tubulin by conjugating with PAs (Del Duca et al., 1997). Such activity would result in the generation of high-molecular-weight aggregates (Del Duca et al., 2009) capable of altering the dynamic properties of the cytoskeletal filaments, of reducing the affinity of kinesin and myosin and, consequently, of affecting the dynamic activities based on the two motor proteins. Thus, TGase might actively participate in the SI response by playing a critical role in the reorganization of the cytoskeleton (**Figure 5A**). Changes in the Ca^{2+} concentration after the onset of SI response can also modify the functional properties of actin filaments and microtubules (Liu et al., 2007) through the enzymatic activity of TGase. Therefore, the molecular mechanisms of rejection of SI pollen may share common features among different families, such as poppy and pear.

An additional role for TGase during the SI response may also be found in the interaction between TGase and the cell wall. In incompatible pollen tubes of pear, TGase forms a sort of "cap" around the apex of incompatible pollen tubes (Del Duca et al., 2010); as it occurs also in *Citrus*, these data may suggest that TGase increases the rigidity of the apical cell wall, thus counteracting the internal turgor pressure and preventing pollen tubes to grow further. In addition, TGase was also observed to accumulate occasionally as consistent aggregates in the cell wall of pollen tubes (Iorio et al., 2008; Di Sandro et al., 2010; Del Duca et al., 2013a; Del Duca et al., 2014). These aggregates could hypothetically be involved in different processes, such as the modification of specific glycoproteins and polysaccharides of the cell wall (**Figure 5**). Experiments of double immunofluorescence showed co-localization between TGase and other specific cell wall components, including arabinogalactans and pectins (**Figure 5B**; Del Duca et al., 2013a). The mechanism by which TGase is secreted into the cell wall is not known and may require non-canonical mechanisms of secretion, as discussed by Del Duca et al. (2013b). Whatever the process of secretion, TGase could actively participate in changes of the cell wall during the SI response thereby leading to growth arrest of incompatible pollen tubes. However, we cannot exclude that these aggregates are simply the result of an altered secretion process of extracellular TGase due to the SI response.

THE HYPERSENSITIVE RESPONSE

In another type of PCD, during the hypersensitive response induced by tobacco mosaic virus, (TMV) free and conjugated PAs increased their concentration together with their biosynthetic enzymes (Torrigiani et al., 1997). At difference with the mock-inoculated samples, *mono*-PU and *bis*-SD were recovered after TMV-inoculation, which further on increased. A putative 72 kDa-TGase immuno-recognized by AtPng1p polyclonal antibody, increased in TMV-inoculated leaves and in the lesion-enriched areas. TGase activity was found to increase in the intrinsic membrane protein and in cell wall fractions, and it was more persistent in TMV-inoculated leaves (Del Duca et al., 2007). A

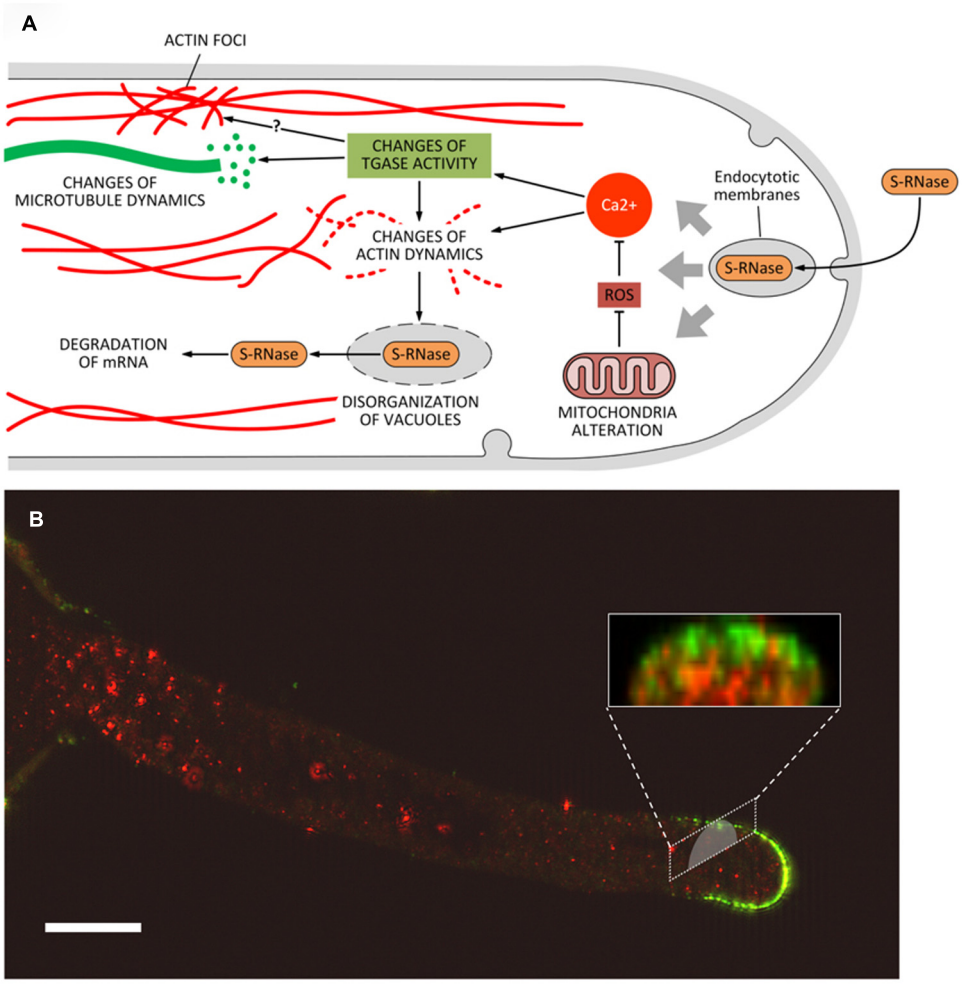

FIGURE 5 | (A) Model of the potential role of transglutaminase (and PAs) during the process of self-incompatibility in pear. S-RNase would be incorporated by endocytosis. This process would trigger a series of subsequent events, including the alteration of mitochondria morphology and consequently the production of ROS. In turn, abnormal levels of ROS might modify the intracellular concentration of Ca^{2+} thus leading to substantial modifications in the structure of actin filaments and/or to interference in the activation/regulation of TGase. As a result, further changes at level of actin (formation of actin foci) and microtubules may also occur. Along with the degradation of mRNA caused by S-RNase released in the cytoplasm, these effects contribute to the death of pollen tubes. **(B)** Distribution of TGase in pollen tubes and relationship with cell wall components. In pollen tubes, TGase (in red) is found in the cytoplasm but also in the cell wall where the enzyme could increase the stiffness of cell wall, contributing to counteract the internal turgor pressure. A further role of TGase during the SI response could be related to the interaction between TGase and specific cell wall components (such as arabinogalactans, in green) as part of the SI response. This interaction may thus prevent the growth of incompatible pollen tubes. Bar: 10 μm. The method for immunolabeling and similar figures can be found in Del Duca et al. (2013a).

possible role in defense by TGase against virus by isolating the infected areas was proposed in agreement with data in mammalian cells where a number of interacting viral and cellular proteins have been found to be modified by TG2, suggesting its novel function in viral pathogenesis (Jeon and Kim, 2006). This role of TG to isolate safe cells from possible damaging organisms recall the isolation of cells from dying ones by isolating their fragments, thus preventing leakage of macromolecules before clearance in the apoptotic bodies (Fesus and Piacentini, 2002).

THE TUBER SENESCENCE AND DEATH

The last example of PCD, probably better defined as DCD, refers to the tubers that at the time of dormancy release become depleted of their storage substances and die when the translocation of nutrients to growing roots and sprouts is completed. In *Helianthus tuberosus,* this organ provides a homogeneous tissue: the medullary parenchyma that stores different compounds like several aminic and glucosidic substances. Cells are characterized by a thin layer of cytoplasm, which includes nuclei, adherent to the thin cell wall, with small nucleoli and a large vacuole. Their non-green plastids are small and contain tubular complexes (**Figure 6**). Even though the winter dormancy of the tuber is not an absolutely stationary period, the large parenchyma cells (arrested in G0 phase) have a slow metabolism, contain very small amounts of growth substances, but a considerable amount of inhibitors. PAs are also present but in insufficient amount to sustain growth and

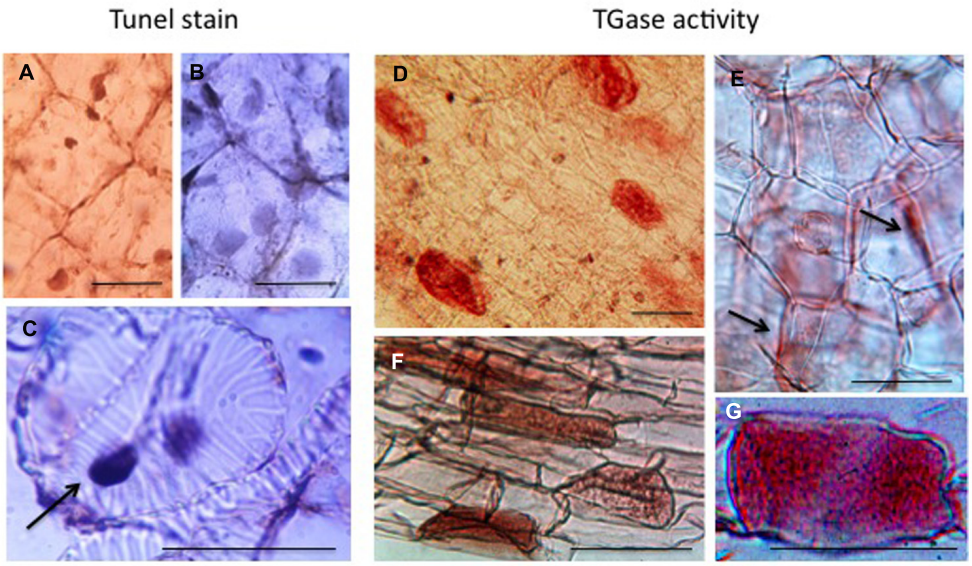

FIGURE 6 | Programmed cell death (PCD) and transglutaminase activity in the storage parenchyma of the tuber of *Helianthus tuberosus* during dormancy release. Scattered cells either parenchymatic **(A)** or tracheary elements undergoing PCD (**C**, arrow) were positive to Tunel staining. Other cells were negative **(B)**. Fresh tuber slices were also incubated with dansyl-cadaverine and after extensive washing, immuno cytochemically recognized by an anti-dansyl antibody. Cells positive to conjugated cadaverine were scattered among others (**D** and **F**). TGase products were found either mainly in the cell walls (**E**, arrows) or in the whole cells (**G**). Bars: 50 μm.

TGase activity is low. For this reason, the dormant parenchyma represents a natural PA-deficient tissue and was utilized to test *in vitro* for the first time the effect of supplied PAs on plant growth (Bertossi et al., 1965). Explants, when put in *in vitro* culture, immediately respond activating many metabolisms, included the biosynthetic and catabolic ones of PAs, their linkage to RNAs and their conjugation by TGase activity especially to very high molecular mass conjugates and to a 18-kDa protein. New TGase enzymes are synthesized either of 58 and 90 kDa but also of higher mass (Serafini-Fracassini et al., 1988; Del Duca et al., 2000). Cells activate the metabolisms of DNA and of different RNAs, and enter the first synchronous amitotic cell cycle continuing to grow, as partially summarized by Bagni and Serafini-Fracassini (1985) and by Tassoni et al. (2010). However, this treatment caused the break of dormancy, which consists of an induced change of the death program of parenchyma cells, many of which become meristematic. On the contrary, during the natural release from dormancy, parenchyma cells are programmed to become totally depleted and die: in fact storage substances decrease. This phase is gradual and long, the system is rather complex involving the development of sprouts and roots and the transfer of nutrients and growth factors therein (Grandi et al., 1992). Unexpectedly, the tuber cells die randomly. In fact, when observed after Tunel staining, parenchyma cells and differentiating tracheids with dark picnotic nuclei are interspersed with cells with normal nuclei (**Figures 6A–C**). Tracheid differentiation could favor the transfer of storage substances.

The amount of bound PU and SM increases during dormancy up to dormancy release and decreases during sprouting in conjunction with a sudden rise of free PAs. Most of the PAs were linked to proteins and possibly are transferred to growing sprouts (Serafini-Fracassini and Mossetti, 1985; Mossetti et al., 1987). At the time of sprouting, a conspicuous increase in protein content of tubers takes place followed by a slow decrease. A 47-kDa protein band increased at the end of dormancy and then decreased during sprouting. Among the high molecular mass bands, a 150-kDa band increased dramatically at dormancy release. Numerous additional bands in the 35–50 kDa range appeared only in this phase (Del Duca et al., 2000). These changes could be related either to the synthesis of degradative enzymes and to released storage substances to be transferred to growing sprouts or to the PCD of tuber parenchyma, which has completed its function.

TGase activity, as measured in tuber cell-free system, increased during dormancy and dropped at sprouting (Grandi et al., 1992). During this phase, this activity was localized scattered only in few parenchyma cells as shown by immuno-cytochemical recognition of conjugated dansyl-cadaverine (**Figure 6D**). This substrate appeared distributed in the whole cell (**Figures 6D–G**) but also in the cell walls (**Figure 6E**). The distribution of these labeled cells recalls the distribution of Tunel positive cells. It can be hypothesized that the same tuber cells, which die randomly, are modified by TGase similarly to the hepatocytes or apoptotic bodies, which are isolated by a net of proteins conjugated by TG2 to protect the surrounding cell from a release of dangerous enzymes (Fesus and Piacentini, 2002).

This example of PCD clarify that it is necessary to use an homogeneous plant material and to verify PCD distribution by morphological methods, otherwise the biochemical data, that provide a mean value of cells in different metabolic stages, might be

misleading. In fact, PAs conjugated by TGase are also present in this PCD system, suggesting that this is a widespread event in PCD.

CONCLUSION

Among the plant models described here, some may be attributed to a type of PCD classified as "vacuolar cell death", others to the so-called "necrotic cell death" or, finally, to a mixture of both according to the classification proposed by van Doorn et al. (2011). During vacuolar cell death, the cell contents are removed by a combination of autophagy-like process and release of hydrolases from collapsed lytic vacuoles. Necrosis is characterized by early rupture of the plasma membrane, shrinkage of the protoplast and absence of vacuolar cell death features. Vacuolar cell death is common during tissue and organ formation and elimination, whereas necrosis is typically found under abiotic stress. However, this classification is based only on morphological observations, not always available; the proper diagnose of vacuolar cell death should be obtained by combining electron microscopy observations together with analysis of autophagic activity of the vacuolar processing enzymes and with changes of the cytoskeleton. Morphological events occurring during vacuolar cell death include the assembly of actin bundles, the breakdown of the nuclear envelope, and even nuclear segmentation. According to van Doorn et al. (2011), vacuolar cell death was previously observed in tracheary elements or in pollen as well as in petals (where we also observed events of DNA laddering and nuclei fragmentation); consequently, vacuolar cell death might occur also in the models here presented. All the characteristics of vacuolar cell death and necrosis can be tracked during the HR cell death; in addition, a mixture of necrotic and vacuolar characteristics also occurs during the SI response, possibly even in the models presented here. Necrosis, no longer considered an unprogrammed process, remains poorly characterized at the biochemical and genetic levels and, although there are no molecular markers, it can be defined as PCD.

The evidence for different TGase forms, some of which are specific for a particular organelle or structure, implies that there must be also different substrates. A role of TGase more frequently observed is linked to the stabilization of structural proteins that, when modified by TGase, are more protected from digestion by proteases. As an example, a cytoplasmic TGase modifies cytoskeletal proteins in pollen, and changes to the cytoskeleton are typical features of PCD.

The effect of TGase is probably related to the type of plant PCD but mostly to the substrate to be modified in order to achieve that specific PCD program. TGase activity increases during natural senescence of the papyraceous petals of *Nicotiana,* whose cell walls become probably lignified or suberified, and during SI pollination, when the pollen apex is covered by a thick cap, or during the PCD induced by hypersensitive response against tobacco mosaic virus, where a defense suberified layer is formed. Perhaps a similar defense role but against the degradative enzymes occurs also in tuber cells. In contrast, TGase activity appears to decrease during natural (developmental) senescence of not-excised old leaves. This difference may depend on changes occurring mostly in the cell wall; in fact, the latter is often involved in PCD, as in tracheids. In contrast, in senescent leaves this modification probably does not occur. Moreover, from our experience, it is critical the age and thus the metabolic stage of the senescent organ examined. In photosynthetic tissues, the stabilizing role of TGase occurs mainly on the proteins of chloroplasts; this event is essential to sustain the photosynthetic activity in order to make the energy available for cell metabolism, also that of senescing leaves or petals, whereas in more senescent ones energy is no more necessary and thus TGase activity declines. In not-excised senescing leaves, senescence is reversible as the addition of PAs stimulated TGase activity and maintained this organ in a state of juvenility. Also in the two models of leaf PCD induced by excision, TGase changed but it could be conditioned by many "accidental" experimental events, like wounding, water availability, dark or light, partial anoxia, effects of different supplied factors (PAs, kinetin, exogenous TGase). Therefore, it is difficult to obtain a clear unambiguous picture.

In all models of plant PCD hitherto examined, TGase appears to be involved in a way to some extent similar to some of those described during apoptosis in animal cells, in particular when TGase catalyzes the posttranslational modification of proteins by transamidation, with consequent formation of cross-links and even large supramolecular nets in which PAs may be involved. However, the role of TG2 in the animal cell death/survival process is extremely complex, and circumstances in which TG2 acts as a prodeath or prosurvival protein is still an area of active investigation (Gundemir et al., 2012). Thus, scarce suggestions can be obtained by animal cell models. Moreover some typical characteristics of animal "apoptosis", a term not used in plants, cannot be detected in plants due to differences at cellular, tissue, and organismic levels, although many properties are shared between animals and plants at molecular level.

Much more information is required to get an overall picture of the role of PAs and TGase during PCD in plant cells. However, it is clear that TGase is involved in all models presented here and that, as a cross-linker of PAs and proteins, it is an important factor involved in multiple, sometimes controversial, roles of PAs during PCD.

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Thermospermine modulates expression of auxin-related genes in *Arabidopsis*

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Thermospermine, a structural isomer of spermine, is widely distributed in the plant kingdom and has been shown to play a role in repressing xylem differentiation by studies of its deficient mutant, *acaulis5* (*acl5*), in *Arabidopsis*. Our results of microarray and real-time PCR analyses revealed that, in addition to a number of genes involved in xylem differentiation, genes related to auxin signaling were up-regulated in *acl5* seedlings. These genes include *MONOPTEROS*, an auxin response factor gene, which acts as a master switch for auxin-dependent procambium formation, and its target genes. Their expression was reduced by exogenous treatment with thermospermine or by transgenic induction of the *ACL5* gene. We examined the effect of synthetic polyamines on the expression of these auxin-related genes and on the vascular phenotype of *acl5*, and found that tetramines containing the NC_3NC_3N chain could mimic the effect of thermospermine but longer polyamines containing the same chain had little or no such effect. We also found that thermospermine had an inhibitory effect on lateral root formation in wild-type seedlings and it was mimicked by synthetic tetramines with the NC_3NC_3N chain. These results suggest the importance of the NC_3NC_3N chain of thermospermine in its action in modulating auxin signaling.

Keywords: *Arabidopsis thaliana*, auxin, lateral root, polyamines, thermospermine, xylem

INTRODUCTION

Polyamines such as spermidine and spermine are abundant in living cells and are involved in a variety of physiological processes including embryogenesis, growth, fruit ripening, and stress responses in plants (Kusano et al., 2007; Alcázar et al., 2010; Takahashi and Kakehi, 2010). Polyamines interact with nucleic acids and membranes and influence many enzymatic reactions. In recent years, attention has been focused on the action of polyamines on the protein synthesis machinery. There is increasing evidence that polyamines stabilize the RNA structure, promote the association of ribosomal subunits, and may affect the rate or efficiency of nascent polypeptide chain elongation (Igarashi and Kashiwagi, 2011). A structural isomer of spermine, thermospermine (**Table 1**), was first discovered in thermophilic bacteria and has also been implicated in the protein synthesis under extreme environmental conditions (Oshima, 2007). The *Arabidopsis acaulis5* (*acl5*) mutant is defective in the synthesis of thermospermine and shows severe dwarf phenotype, indicating that thermospermine is required for stem elongation (Kakehi et al., 2008). Since *ACL5* is predominantly expressed during xylem formation from procambial cells to differentiating xylem vessels (Clay and Nelson, 2005; Muñiz et al., 2008) and *acl5* has over-proliferated xylem vessels (Hanzawa et al., 1997), its dwarf phenotype may be primarily attributed to excess xylem differentiation and thermospermine appears to act as a repressor of xylem differentiation. While exogenous thermospermine can partially but significantly rescue the stem growth of *acl5*, spermine has no such effect (Kakehi et al., 2008). Indeed, *acl5* contains spermine produced by the action of spermine synthase, SPMS, while loss-of-function mutants of *SPMS* show wild-type phenotype under normal growth condition (Imai et al., 2004). We have found that norspermine is also able to rescue the growth of *acl5* (Kakehi et al., 2010). It is noted that the NC_3NC_3N (hereafter expressed as 3-3) arrangement of carbon chains is present in both thermospermine (3-3-4) and norspermine (3-3-3) but not in spermine (3-4-3), suggesting its structural significance for the biological function.

Thermospermine may be widely distributed among the plant kingdom, based on the presence of putative orthologs to *ACL5* in different plant species (Knott et al., 2007; Minguet et al., 2008; Takano et al., 2012). Our analyses of suppressor mutants of *acl5* named *sac* have suggested that thermospermine enhances translation of *SAC51*, a putative basic helix-loop-helix (bHLH) transcription factor gene, by reducing the inhibitory effect of small upstream open reading frames (uORFs) located in its 5′ leader sequence (Imai et al., 2006). A study of the *thickvein* (*tkv*) mutant, which represents another allele of the *ACL5* locus, suggests that the reduction in polar auxin transport is responsible for the vascular phenotype of the mutant (Clay and Nelson, 2005). Muñiz et al. (2008) revealed that cell death occurs before the onset of secondary cell wall formation in the xylem vessels of *acl5*, suggesting a role of *ACL5* for preventing differentiating xylem vessels from premature programmed cell death (Muñiz et al., 2008). More recently, *acl5* mutants were shown to be more sensitive to pathogens than wild-type plants, suggesting a function

Table 1 | Linear polyamines used in this study.

Triamines	**$H_2N(CH_2)_aNH(CH_2)_bNH_2$ a-b**
Norspermidine	3-3
Spermidine	3-4
Tetramines	**$H_2N(CH_2)_aNH(CH_2)_bNH(CH_2)_cNH_2$ a-b-c**
N-(2-Aminoethyl)norspermidine	3-3-2
Norspermine	3-3-3
Thermospermine	3-3-4
N-(5-Aminopentyl)norspermidine	3-3-5
Spermine	3-4-3
Pentamines	**$H_2N(CH_2)_aNH(CH_2)_bNH(CH_2)_cNH(CH_2)_dNH_2$ a-b-c-d**
Homocaldopentamine	3-3-3-4
N,N′-Bis(4-aminobutyl)norspermidine	4-3-3-4

of thermospermine in defense signaling (Marina et al., 2013). However, the precise mode of action of thermospermine remains to be clarified. For comprehensive understanding of the function of thermospermine in plants, we performed microarray and real-time RT-PCR experiments with *acl5* seedlings. Our results revealed that, in addition to a number of genes involved in xylem differentiation, genes related to auxin signaling were up-regulated in *acl5* seedlings. They were reduced by thermospermine and synthetic tetramines containing the 3-3 chain (**Table 1**). These tetramines are further shown to have an inhibitory effect on lateral root formation in wild-type seedlings.

MATERIALS AND METHODS

CHEMICALS

All polyamines used in this study are shown in **Table 1**. Spermidine, spermine, and norspermidine were purchased from Sigma (St. Louis, MO, USA). The other uncommon polyamines were synthesized by the published method (Niitsu et al., 1992). MS salts for plant nutrition were purchased from Wako (Osaka, Japan).

PLANT MATERIALS AND GROWTH CONDITIONS

Arabidopsis thaliana accession Columbia-0 (Col-0) was used as the wild type. The original *acl5-1* mutant in the accession Landsberg *erecta* (L*er*) was backcrossed more than 7 times into Col-0 (Hanzawa et al., 2000). *spms-1* in the Col-0 background has been described previously (Imai et al., 2004). Transgenic *acl5-1* lines carrying the *HS-ACL5* construct and the *SAC51-GUS* construct have been described in Hanzawa et al. (2000) and Imai et al. (2006), respectively. In experiments with *monopteros* (*mp*) in the L*er* background (Berleth and Jürgens, 1993), which were obtained from ABRC, wild-type L*er* and *acl5-1* in L*er* were used as a reference and for crosses. Prior to germination, seeds were surface sterilized with commercial bleach (5% sodium hypochlorite) supplemented with 0.02% (v/v) Triton X-100, and rinsed three times with sterile distilled water. For microarray experiments, seeds were germinated and grown on Murashige and Skoog (MS) agar medium supplemented with 3% sucrose for 7 days under continuous light at 22°C. For the growth in the presence of polyamines, each polyamine was added to the MS agar or liquid medium at 100 μM. For treatment of seedlings with polyamines for 24 h or with heat shock at 37°C for 2 h, seeds were germinated, grown in MS solutions for 7 days under continuous light at 22°C, and incubated as described in figure legends.

RNA EXTRACTION AND REAL-TIME RT-PCR

Total RNA was isolated from whole seedlings according to the SDS-phenol method and reverse-transcribed with oligo-dT primers and PrimeScript™ reverse transcriptase (Takara, Kyoto, Japan) at 42°C for 1 h. The resulting first-strand cDNA was directly used for real-time PCR with target gene-specific primers (**Table 2**). PCR reactions were performed using KAPA SYBR FAST qPCR Kit (KAPA Biosystems, Woburn, MA, USA) and the DNA Engine Opticon2 System (Bio-Rad, Hercules, CA, USA). ACTIN8 (At1g49240) was used as an internal standard in the reactions. Data from three independent biological replicates each with two technical replicates are expressed as means ± *SD*.

MICROARRAY ANALYSIS

Microarray experiments were performed in three independent biological replicates using the Agilent *Arabidopsis* 3 oligo microarray (Agilent Technologies, Wilmington, DE, USA) in Hokkaido System Science (Sapporo, Japan). RNA quality was confirmed by gel electrophoresis as well as by OD260/280 nm ratios. The cRNA probes from wild-type and *acl5-1* seedlings were labeled with Cy3 and Cy5 dyes, respectively, with a Low RNA Fluorescent Linear Amplification Kit (Agilent). After hybridization in Agilent SureHyb chambers, slides were washed according to the manufacturer's instructions and scanned using an Agilent G2505C Microarray Scanner. The scanned images were processed with Feature Extraction software 10.10 (Agilent). The microarray data were submitted to the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2333. The data were subjected to LOWESS normalization and statistical significance was tested by an unpaired *t*-test with GeneSpring GX software (Agilent). $P < 0.05$ were considered to be statistically significant. Gene ontology (GO) analysis was performed using the AgriGO online tool (Zhou et al., 2010) (http://bioinfo.cau.edu.cn/agriGO/analysis.php).

MICROSCOPY

For observation of xylem development, seedlings were fixed in ethanol/acetic acid (6:1) overnight, incubated twice in 100% ethanol for 30 min, once in 70% ethanol for 30 min, and cleared with chloral hydrate:glycerol:water mixture (8:1:2, w:v:v) overnight (Kakehi et al., 2010). Samples were mounted on a microscope slide and examined under differential interference contrast microscopy.

POLYAMINE ANALYSIS

Polyamines were extracted by grinding 0.5 g fresh weight of the aerial parts of agar-grown seedlings in liquid nitrogen,

Table 2 | Primer sequences of the genes used for RT-PCR analysis.

Gene name	AGI code	Forward primer sequence	Reverse primer sequence
ACL5	At5g19530	ACCGTTAACCAGCGATGCTTT	CCGTTAACTCTCTCTTTGATTC
XTH3	At3g25050	GTGTTTTTGTAGTAACGTTATGG	GGTTGGATTGAACCAAAGCAA
VND6	At5g62380	ATGGAAAGTCTCGCACACAT	CTCTTCCACATAACTCTTGG
VND7	At1g71930	CGATGCATCAATATGGCAAC	AGGGAAGCATCCAAGAGAAT
CNA	At1g52150	GGTATTTGCTGATTCATGAGC	ATGGTTTACACTTGACAGAGC
ATHB8	At4g32880	AGCGTTTCAGCTAGCTTTTGAG	CAGTTGAGGAACATGAAGCAGA
MP/ARF5	At1g19850	GATGATCCATGGGAAGAGTT	TAAGATCGTTAATGCCTGCG
TMO5L1	At1g68810	CACCACCAAAACGGATAAAG	CGTTTTGAGACGCATAGCTT
PIN1	At1g73590	CTTAGCACTGCGGTGATATT	TTGCTGAGCTCCTACTTAAG
PIN6	At1g77110	CTATCGTACAGGCTGCTCTA	CTCCTCAAGAACAACTCTTA
YUC2	At4g13260	ATGTGGCTAAAGGGAGTGAA	AACTTGCCAAATCGAAACCC
IRT1	At4g19690	GTCTAATCACTCTAGCCATTGA	TGTATACTCAGCCTGGAGGA
PXY/TDR	At5g61480	CGGTTACATTGCACCAGAAT	GCTTGTACACAACAACGCAA
SAC51	At5g64340	AATTGCCAGGCTGAGTACTT	GACCGACCTACTATATCCTT
ACT8	At1g49240	GTGAGCCAGATCTTCATTCGTC	TCTCTTGCTCGTAGTCGACAG

suspending the powder in 2.5 ml of 5% (w/v) perchloric acid, and incubating on ice for 1 h. After centrifugation at 15,000 g for 30 min, 2 ml of the supernatant was filtered through syringe filter of 0.2 μm pore size, neutralized by 1 ml 2 N NaOH, incubated with 10 μl of benzoyl chloride for 20 min, mixed with 2 ml saturated NaCl and then added to 2 ml of diethyl ether. After vigorous shaking and centrifugation at 3000 g for 10 min, the ether layer was evaporated under vacuum and the residue was suspended in 50 μl of methanol. The benzoylated sample was analyzed using reverse phase HPLC system equipped with TSKgel ODS-80Ts column (Toso, Tokyo, Japan). The elution was performed with 42% (v/v) acetonitrile at a flow rate of 0.5 ml/min for 50 min and monitored by measuring its UV absorbance at 254 nm.

RESULTS

MICROARRAY ANALYSIS OF *acL5-1*

To examine the effect of thermospermine deficiency on gene expression profiles in *Arabidopsis*, we performed microarray experiments with RNA samples from 7-day-old *acl5-1* and wild-type seedlings. The results from three independent experiments identified 173 genes whose transcript level was reproducibly increased more than two-fold in *acl5-1* (**Supplemental Table 1**). GO analysis of these 173 genes revealed significant enrichment of the genes encoding cell wall-related proteins (**Supplemental Table 2**). These include enzymes involved in cell-wall carbohydrate metabolism such as glycosyl hydrolase family proteins, laccases, pectate lyases, and peroxidases. Representative genes are listed in **Table 3**. Expression of the genes related to proteolysis such as those encoding serine proteases of the subtilisin family (i.e., *SBT1.1* and *SBT5.2*) and cysteine peptidases (*XCP1* and *XCP2*) were also increased in *acl5-1* seedlings. *XCP1* has been implicated in the developmental cell death associated with lignified xylem vessel differentiation (Funk et al., 2002). The data also confirmed previous findings that *ACL5* and *SAMDC4/BUD2* are up-regulated in *acl5-1*, indicating a negative feedback regulation of these polyamine biosynthetic genes by thermospermine (Kakehi et al., 2008). *SAMDC4/BUD2* is one of the four genes encoding S-adenosylmethionine decarboxylase, which produces decarboxylated S-adenosylmethionine for the synthesis of higher polyamines (Ge et al., 2006), and has been implicated specifically in the synthesis of thermospermine (Kakehi et al., 2010). With regard to transcription factors, members of the NAC-domain protein gene family (*VND1*, *VND2*, *VND6*, *VND7*, and *XND1*), and those of the class III homeodomain leucine-zipper (HD-ZIP III) gene family (*ATHB8*, *PHB*, and *CNA*), which are all involved in the regulation of vascular differentiation, were up-regulated in *acl5-1*. This is consistent with previous reports that showed up-regulation of the members of these gene families in *acl5* mutants (Imai et al., 2006; Kakehi et al., 2008; Muñiz et al., 2008). The mutant also showed increased transcript levels of *MYB46*, a key player in the regulation of secondary wall biosynthesis in fibers and vessels in stems (Zhong et al., 2007). We further found that the transcript level of *MONOPTEROS* (*MP*)/*AUXIN RESPONSE FACTOR5* (*ARF5*), which encodes an auxin-responsive transcription factor (Hardtke and Berleth, 1998), was higher in *acl5-1* than in the wild type. In addition, *TARGET OF MP 5* (*TMO5*), which encodes a bHLH transcription factor and has been shown to be a direct target of *MP/ARF5* (Schlereth et al., 2010), and *TMO5-LIKE1* (*TMO5L1*) were also up-regulated in *acl5-1*. As for signaling molecules, *PHLOEM INTERCALATED WITH XYLEM/TDIF RECEPTOR* (*PXY/TDR*), which encodes a receptor kinase and acts in the promotion of cambial cell proliferation and in the suppression of its differentiation into xylem (Fisher and Turner, 2007; Hirakawa et al., 2008), was up-regulated in *acl5-1*.

On the other hand, our results revealed only 14 genes that were down-regulated more than two-fold in *acl5-1* (**Supplemental Table 3**). Only two genes, *IRT1*, and *ZIP8*, which are highly homologous to each other and code for a metal transporter (Vert et al., 2002), showed a more than five-fold reduced expression in the mutant.

RESPONSE OF THE SELECTED GENES TO THERMOSPERMINE

XYLOGLUCAN ENDOTRANSGLYCOSYLASE/HYDROLASE3 (XTH3), *VND6*, *VND7*, *ATHB8*, *PXY/TDR*, and *IRT1* were

Table 3 | List of representative genes up-regulated in *acl5-1* seedlings.

Functional category	AGI Code	Annotation	Fold change ± SE
Polyamine metabolism	At5g18930	S-adenosylmethionine decarboxylase 4 (SAMDC4/BUD2)	21.3 ± 2.4
	At5g19530	Thermospermine synthase/ACAULIS 5 (ACL5)	11.6 ± 1.4
Cell-wall metabolism	At3g25050	Xyloglucan endotransglycosylase/hyrdolase 3 (XTH3)	38.5 ± 3.4
	At2g22620	Rhamnogalacturonate lyase	17.2 ± 5.5
	At1g77790	Glycosyl hydrolase family 17/Endo-1,3-beta-glucanase	14.7 ± 2.7
	At5g08370	Alpha-galactosidase 2 (AGAL2)	13.5 ± 3.6
	At2g46570	Laccase 6 (LAC6)	12.5 ± 2.4
	At3g19620	Glycosyl hydrolase family 3/Beta-xylosidase A	10.2 ± 1.4
	At2g46760	D-arabinono-1,4-lactone oxidase family protein	7.8 ± 2.2
	At1g56710	Glycosyl hydrolase family 28/Polygalacturonase	7.4 ± 1.8
	At3g27400	Pectate lyase	7.0 ± 0.6
	At5g51890	Peroxidase involved in TE lignification	5.9 ± 0.9
	At2g34790	FAD-binding domain-containing protein (MEE23/EDA28)	5.2 ± 1.4
	At5g17420	Cellulose synthase/IRREGULAR XYLEM 3 (IRX3)	5.1 ± 0.8
Proteolysis	At5g22860	Serine carboxypeptidase S28	25.0 ± 2.5
	At1g01900	Subtilisin-like serine protease (SBT1.1)	9.7 ± 2.4
	At1g20160	Subtilisin-like serine protease (SBT5.2)	8.7 ± 0.6
	At2g04160	Subtilisin-like protease (AIR3)	6.0 ± 1.3
	At4g35350	Xylem cysteine endopeptidase 1 (XCP1)	5.4 ± 0.5
	At1g20850	Xylem cysteine endopeptidase 2 (XCP2)	3.9 ± 1.1
Transcription	At2g18060	Vascular-related NAC domain protein 1 (VND1)	16.4 ± 4.0
	At4g36160	Vascular-related NAC domain protein 2 (VND2)	12.0 ± 1.9
	At5g62380	Vascular-related NAC domain protein 6 (VND6)	5.9 ± 0.7
	At4g32880	Class III homeodomain-leucine zipper protein (ATHB8)	5.8 ± 1.5
	At1g68810	Basic helix-loop-helix (bHLH) family protein (TMO5L1)	5.7 ± 1.8
	At5g64530	Xylem NAC domain 1 (XND1)	5.5 ± 1.3
	At1g53160	Squamosa promoter-binding protein-like 4 (SPL4)	5.5 ± 2.5
	At1g19850	Auxin response factor 5/MONOPTEROS (MP/ARF5)	5.1 ± 2.1
	At5g12870	Myb protein (MYB46)	4.7 ± 0.7
	At4g13480	Myb protein (MYB79)	4.4 ± 1.0
	At1g71930	Vascular-related NAC domain protein 7 (VND7)	4.2 ± 1.4
	At3g25710	Basic helix-loop-helix (bHLH) family protein (TMO5)	4.0 ± 0.9
	At2g34710	Class III homeodomain-leucine zipper protein (PHB)	3.8 ± 1.3
	At1g52150	Class III homeodomain-leucine zipper protein (CNA/ATHB15)	2.8 ± 1.2
Signal transduction	At5g61480	Leucine-rich repeat transmembrane kinase (PXY/TDR)	5.0 ± 1.9
	At2g01950	Leucine-rich repeat transmembrane kinase (BRL2/VH1)	3.0 ± 1.1
Hormone-related	At1g77110	Auxin transport protein/PIN-FORMED 6 (PIN6)	10.7 ± 2.0
	At5g55250	IAA carboxylmethyltransferase (IAMT1)	5.7 ± 1.8
	At3g53450	Cytokinine-activating enzyme/LONELY GUY 4 (LOG4)	5.0 ± 1.8
	At4g13260	Flavin-containing monooxygenase/YUCCA2 (YUC2)	4.8 ± 0.6
	At2g26700	Protein kinase/PINOID2 (PID2)	4.0 ± 1.5
	At1g73590	Auxin transport protein/PIN-FORMED 1 (PIN1)	3.0 ± 0.7
Transport	At4g02700	Sulfate transporter (SULTR3;2)	6.8 ± 2.2
	At1g77380	Amino acid permease (AAP3)	4.4 ± 1.0
	At2g21050	Amino acid permease/LIKE AUXIN RESISTANT 2 (LAX2)	3.7 ± 1.2

selected for further analysis and their transcript levels were validated by real-time RT-PCR. Our results confirmed that all of these genes except *IRT1* were up-regulated in *acl5-1* seedlings compared to wild-type seedlings (**Figure 1**).

We then examined the effect of exogenous application of thermospermine on the expression of these up-regulated genes in *acl5-1*. Treatment of 7-day-old *acl5-1* seedlings with 100 μM thermospermine for 24 h led to a marked reduction in the transcript

levels of these genes except for *VND7* (**Figure 2A**). When grown in MS solutions with 100 μM thermospermine for 7 days, the *acl5-1* seedlings showed a drastic reduction in the transcript levels of all of the genes tested, compared to those grown without thermospermine and those grown with 100 μM spermine (**Figure 2A**). Similar responses of these genes were also observed in wild-type seedlings (**Figure 2B**).

The effect of thermospermine on gene expression was also examined using transgenic *acl5-1* plants carrying a heat-shock inducible *ACL5* (*HS-ACL5*) cDNA. A previous study has shown that the dwarf phenotype of *acl5-1* is complemented in a heat-shock dependent manner by using this transgenic system (Hanzawa et al., 2000). Similar to the case of exogenous treatment for 24 h, transcript levels of all of the genes examined showed a reduction at 24 h after heat-shock treatment for 2 h (**Figure 2C**).

On the other hand, we confirmed that *IRT1* expression was reduced in *acl5-1* seedlings (**Figure 1**). However, *IRT1* expression was increased neither by treatment with thermospermine in *acl5-1* and wild-type seedlings nor by heat shock in transgenic *acl5-1* plants carrying the *HS-ACL5* (not shown).

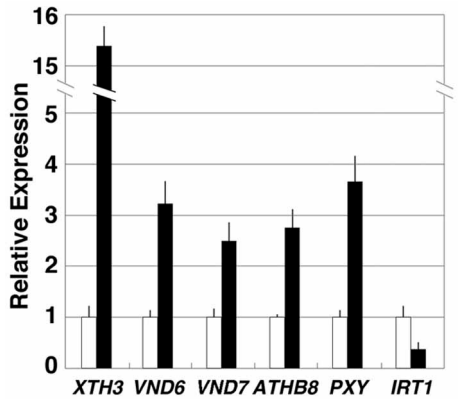

FIGURE 1 | Expression levels of selected genes altered in *acl5-1*. Total RNA was prepared from 7-day-old wild-type (Col-0) and *acl5-1* seedlings, and analyzed by quantitative real-time RT-PCR. All transcript levels in *acl5-1* (black bars) are relative to those in the wild type (white bars). *ACTIN8* transcripts were used as internal control. Error bars represent the SE ($n = 3$).

THERMOSPERMINE NEGATIVELY REGULATES AUXIN-RELATED GENES

We have recently found that 2,4-D and its derivatives enhance xylem formation in *acl5-1* but not in the wild type, suggesting repressive control of auxin-induced xylem formation by thermospermine (Yoshimoto et al., 2012). Auxin-induced procambium formation is mediated by *MP/ARF5* (Przemeck et al., 1996; Hardtke and Berleth, 1998), which was listed as an up-regulated gene in *acl5-1* (**Table 3**). In addition, genes related to auxin biosynthesis and transport such as *PIN-FORMED1* (*PIN1*), *PIN6*, and *YUCCA2* (*YUC2)* were also up-regulated in *acl5-1* (**Table 3**). *PIN1* and *PIN6* encode an auxin efflux carrier (Petrásek et al., 2006), while *YUC2* encodes a flavin monooxygenase essential for auxin biosynthesis (Cheng et al., 2006). We thus examined the effect of thermospermine on the expression of these genes by real-time RT-PCR. All of these genes showed higher levels of expression in *acl5-1* than in the wild type and were down-regulated by thermospermine in both *acl5-1* and wild-type seedlings (**Figure 3A**). We also confirmed that transcript levels of all of these genes, except for *YUC2*, were up-regulated by treatment with 100 μM 2,4-D for 24 h in both wild-type and *acl5-1* seedlings (**Figure 3B**). As reported previously (Hanzawa et al., 2000; Kakehi et al., 2008), *ACL5* expression is down-regulated by thermospermine (**Figure 3A**) and up-regulated by

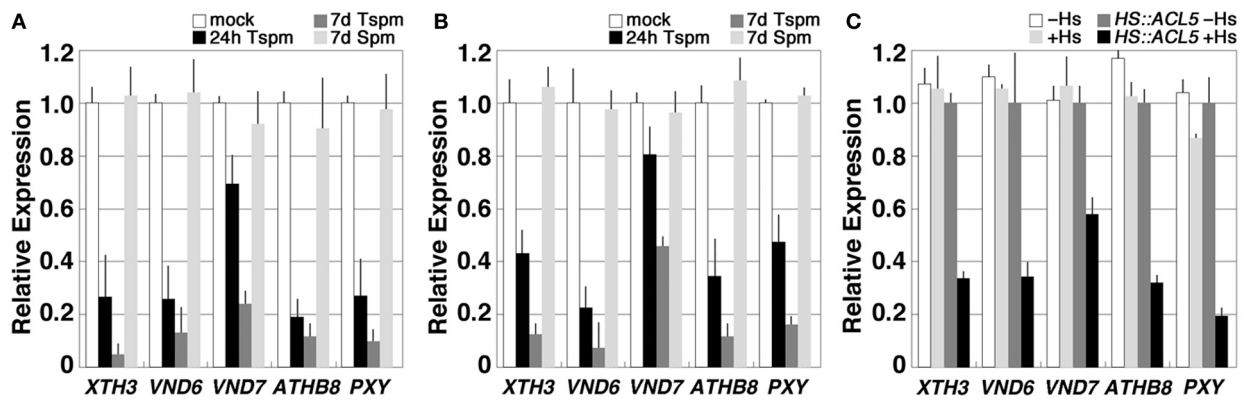

FIGURE 2 | Thermospermine down-regulates the genes that are up-regulated in *acl5-1*. (A,B) Effect of exogenous thermospermine and spermine in *acl5-1* **(A)** and wild-type **(B)** seedlings on the expression of the genes up-regulated in *acl5-1*. Transcript levels in seedlings grown for 7 days in MS solutions and incubated for 24 h in MS plus 100 μM thermospermine (black bars) and those in seedlings grown for 7 days in MS solutions supplemented with thermospermine (dark gray bars) or spermine (light gray bars) are shown relative to those in seedlings grown for 7 days in MS solutions with no polyamines (white bars). **(C)** Effect of endogenously-induced thermospermine on the expression of the genes up-regulated in *acl5-1*. Transcript levels in *acl5-1* seedlings heat-shocked at 37°C for 2 h followed by 24-h culture at 22°C (light gray bars), those in transgenic *acl5-1* seedlings carrying the *HS-ACL5* construct untreated (dark gray bars) and those in transgenic *acl5-1* seedlings carrying the *HS-ACL5* construct heat-shocked at 37°C for 2 h followed by 24-h culture at 22°C (black bars), are shown relative to those in *acl5-1* seedlings untreated (white bars). Error bars represent the SE ($n = 3$).

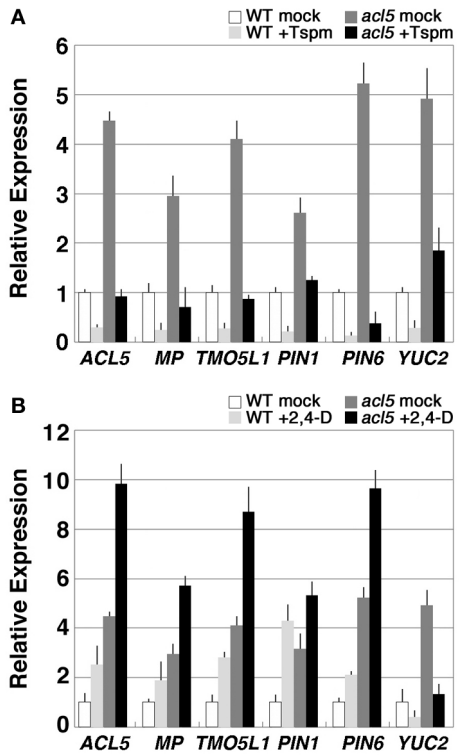

FIGURE 3 | Thermospermine down-regulates the genes that are involved in auxin biosynthesis, transport, and signaling in *acl5-1*. (A) Effect of thermospermine on the expression of auxin-related genes. Transcript levels in wild-type seedlings treated for 24 h with 100 μM thermospermine (light gray bars), those in *acl5-1* seedlings treated with mock (dark gray bars) and those in *acl5-1* seedlings treated with thermospermine (black bars), are shown relative to those in wild-type seedlings treated with mock (white bars). **(B)** Effect of 2,4-D on the expression of auxin-related genes. Transcript levels in wild-type seedlings treated for 24 h with 100 μM 2,4-D (light gray bars), those in *acl5-1* seedlings treated with mock (dark gray bars) and those in *acl5-1* seedlings treated with 2,4-D (black bars), are shown relative to those in wild-type seedlings treated with mock (white bars). Error bars represent the SE ($n = 3$).

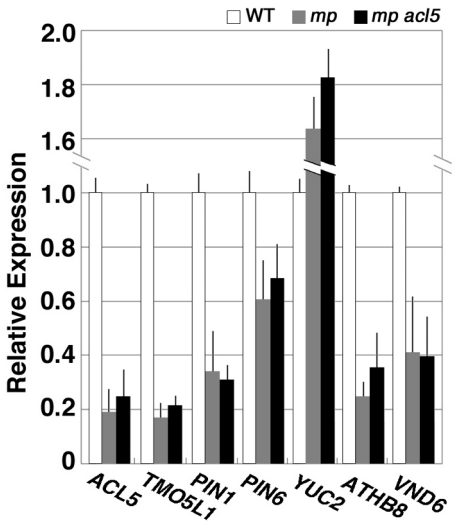

FIGURE 4 | Expression of thermospermine-responsive genes in the *mp* mutant. Total RNA was prepared from seedlings of the L*er* wild type (white bars), *mp* (gray bars), and *mp acl5-1* (black bars) grown for 7 days in MS agar plates. All transcript levels are relative to those in wild-type seedlings. Error bars represent the SE ($n = 3$).

2,4-D (**Figure 3B**). Transcript levels of these genes were also increased in flowering shoots of *acl5-1* compared to those of the wild type, and down-regulated by heat shock in transgenic *acl5-1* plants carrying the *HS-ACL5* (not shown).

We further examined expression of these genes in *mp*, a seedling-lethal allele of *MP/ARF5* that lacks basal body structures such as hypocotyl and root meristem (Berleth and Jürgens, 1993). Transcript levels of all of the auxin-related genes examined except for *YUC2* were reduced in *mp* seedlings and were not restored to wild-type levels in *mp acl5-1* seedlings (**Figure 4**), which are morphologically indistinguishable from *mp*.

ARTIFICIAL TETRAMINES CAN MIMIC THE EFFECT OF THERMOSPERMINE

We next examined the effect of 24-h treatment with synthetic polyamines on *ACL5*, *VND6*, and *MP/ARF5* in *acl5-1 spms-1* seedlings, which produce no tetramines. In addition to thermospermine and norspermine, tetramines of 3-3-2 and 3-3-5 (**Table 1**) significantly repressed the expression of these genes, while homocaldopentamine (3-3-3-4) and the 4-3-3-4 pentamine had little or no effect on their expression (**Figure 5A**). Effect of these synthetic polyamines on xylem development was also examined under microscopy. The *acl5-1 spms-1* seedlings were grown in the presence of each polyamine for 7 days. As is the case with thermospermine and norspermine (Kakehi et al., 2010), 3-3-2 and 3-3-5 tetramines drastically reduced excess accumulation of lignin but homocaldopentamine and the 4-3-3-4 pentamine had little or no reduction in *acl5-1 spms-1* (**Figure 5B**). We confirmed that these higher polyamines were absorbed by the root and transported to the shoot by detecting the content of polyamines in the aerial part of the seedlings with HPLC (**Figure 5C**).

Although our microarray experiments did not identify *SAC51* as a gene down-regulated in *acl5-1*, a previous study revealed that the *SAC51* transcript level is increased by thermospermine probably at least in part due to its stabilization (Kakehi et al., 2008). Transgenic *acl5-1* seedlings carrying the *HS-ACL5* construct described above showed an approximately 2-fold increase in the *SAC51* transcript level at 24 h after heat-shock treatment for 2 h (**Figure 6A**). Furthermore, the GUS reporter activity under the control of the *SAC51* promoter and its 5′ leader sequence is increased by treatment for 24 h with thermospermine and norspermine in transgenic *acl5-1* seedlings (Kakehi et al., 2010). We therefore examined the continuing effect of synthetic polyamines on *SAC51*. Transgenic *acl5-1* seedlings carrying the GUS reporter gene under the control of the *SAC51* promoter and its 5′ leader sequence were grown for 7 days in the presence of each polyamine. The results revealed that the GUS activity was increased by thermospermine, norspermine, 3-3-2, and 3-3-5 tetramines, but unaffected by spermidine, norspermidine, spermine, homocaldopentamine, and the 4-3-3-4 pentamine (**Figure 6B**).

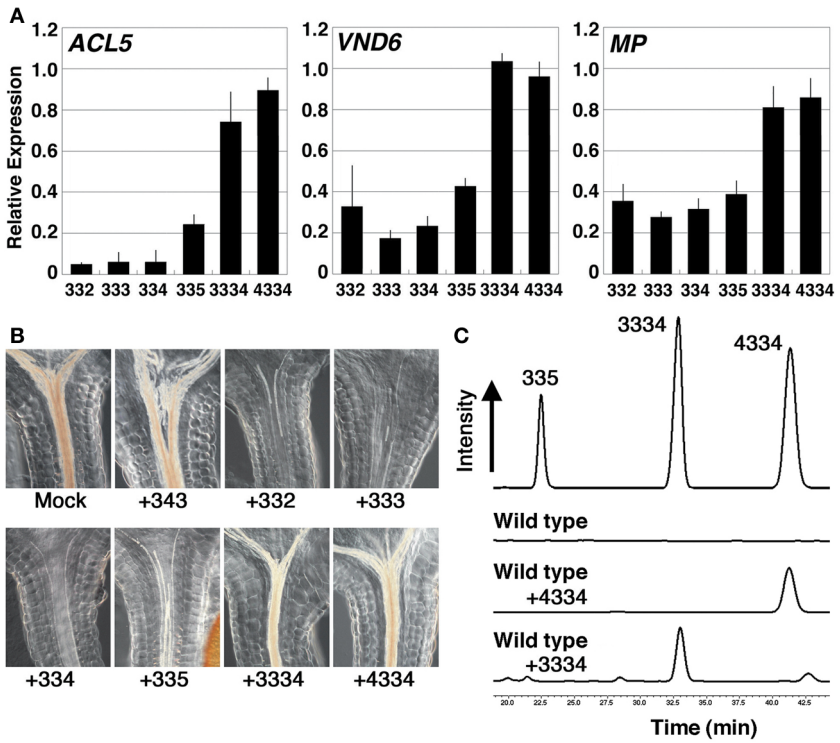

FIGURE 5 | Effect of polyamines in the *acl5-1 spms-1* double mutant. (A) Effect of polyamines on the expression of *ACL5, VND6,* and *MP*. Total RNA was prepared from *acl5-1 spms-1* seedlings grown for 7 days in MS solutions and treated for 24 h in MS plus 100 μM each polyamine. Numbers correspond to those of the carbon shown in **Table 1**. All transcript levels are relative to those in mock-treated *acl5-1 spms-1* seedlings. Error bars represent the SE ($n = 3$). **(B)** Effect of polyamines on xylem development. *acl5-1 spms-1* seedlings were grown for 7 days in MS solutions supplemented with 100 μM each polyamine. **(C)** HPLC separations of benzoylated-polyamines from the aerial part of wild-type seedlings. Seedlings were grown for 7 days in MS agar plates supplemented with homocaldopentamine (C3C3C3C4), N,N′-bis(4-aminobutyl)norspermidine (C4C3C3C4) or no polyamines. The elution pattern of C3C3C5, C3C3C3C4 and C4C3C3C4 polyamines is shown above as a reference.

TETRAMINES WITH THE 3-3 CHAIN REPRESS LATERAL ROOT FORMATION

In the course of the study, we found that exogenous treatment with thermospermine has an inhibitory effect on lateral root formation in both wild-type and *acl5-1* seedlings. We then examined whether or not synthetic polyamines have the same effect as thermospermine in wild-type seedlings. The results revealed that lateral root formation was also severely repressed by norspermine, 3-3-2, and 3-3-5 tetramines, but not by spermidine, norspermidine, spermine, homocaldopentamine, and the 4-3-3-4 pentamine (**Figure 7**).

DISCUSSION

In bacteria, yeast, and animal cells, effects of polyamines on cell growth were mainly studied at the level of mRNA translation, because polyamines exist to a large extent as RNA-polyamine complexes in cells (Igarashi and Kashiwagi, 2006). A group of genes whose expression is enhanced by polyamines at the level of translation is referred to as a "polyamine modulon." The polyamine modulon includes transcription factors and kinases that in turn activate gene expression of other proteins (Igarashi and Kashiwagi, 2011). In plants, only few genes have been identified as polyamine-responsive genes. In *Arabidopsis*, spermine up-regulates expression of transcription factor genes such as *WRKY40* and *bZIP60*. However, since amine oxidase inhibitors cancel this response, it may be triggered by hydrogen peroxide derived from oxidative degradation of spermine (Mitsuya et al., 2009). On the other hand, thermospermine has been shown to negatively regulate expression of *ACL5* and the members of the HD-ZIP III gene family such as *ATHB8* and *PHB* (Kakehi et al., 2008). In agreement with the phenotype of *acl5-1*, which shows excess differentiation of lignified xylem cells, a number of genes involved in the regulation of xylem differentiation and those involved in secondary cell wall formation were up-regulated in *acl5-1* seedlings and down-regulated by exogenously supplied and endogenously induced thermospermine. Amongst them was included *MP/ARF5*. *MP/ARF5* encodes a transcription factor of the ARF family, which is activated by auxin-dependent degradation of its interacting repressor, *BODENLOS/INDOLE ACETIC ACID-INDUCED PROTEIN 12* (*BDL/IAA12*), and acts as a master regulator for the establishment of vascular and body patterns in embryonic and post-embryonic development (Berleth and Jürgens, 1993; Przemeck et al., 1996; Hardtke and Berleth, 1998; Mattsson et al., 2003; Hardtke et al., 2004; Weijers et al., 2005). Mutations in *MP/ARF5* interfere with the formation of vascular strands and with the initiation of the body axis in the early embryo (Przemeck et al., 1996). In recent studies, such transcription factors as *ATHB8* and *TMO5* have been identified as a direct

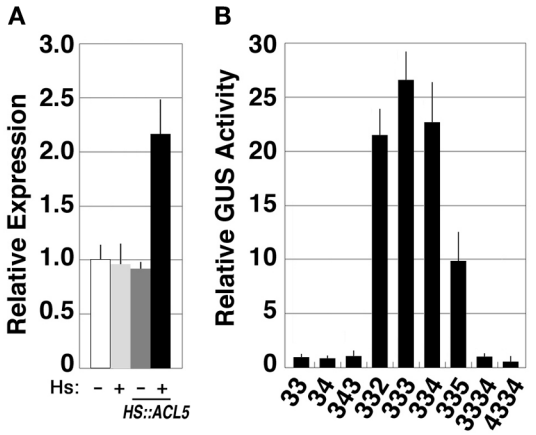

FIGURE 6 | Responses of *SAC51* to polyamines in *acl5-1*. (A) Effect of endogenously-induced thermospermine on *SAC51* expression. Transcript levels in *acl5-1* seedlings heat-shocked at 37°C for 2 h followed by 24-h culture at 22°C (light gray bars), those in transgenic *acl5-1* seedlings carrying the *HS-ACL5* construct untreated (dark gray bars) and those heat-shocked at 37°C for 2 h followed by 24-h culture at 22°C (black bars), are shown relative to those in *acl5-1* seedlings untreated (white bars). **(B)** Effect of polyamines on *SAC51-GUS* expression. Transgenic *acl5-1* seedlings carrying the GUS gene fused to the *SAC51* promoter and its 5′ leader sequence were grown for 7 days in MS solutions supplemented with each polyamine. Numbers correspond to those of the carbon shown in **Table 1**. The activity is shown relative to that of mock-treated seedlings. Error bars represent the SE ($n = 3$).

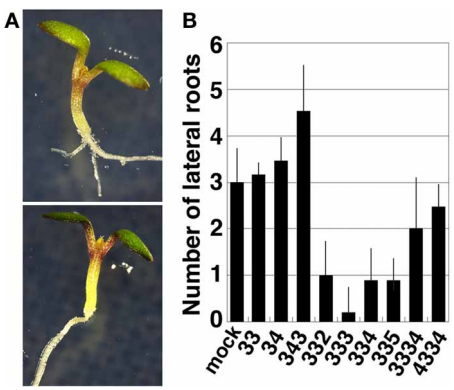

FIGURE 7 | Effect of polyamines on lateral root formation. (A) A wild-type seedling grown for 5 days in MS solutions (upper panel) and that supplemented with 100 μM thermospermine (lower panel). **(B)** Number of lateral roots in wild-type seedlings grown for 5 days in MS solutions supplemented with 100 μM each polyamine. Numbers correspond to those of the carbon shown in **Table 1**. Error bars represent the SE ($n = 10$).

target of *MP/ARF5* (Donner et al., 2009; Schlereth et al., 2010). Thus, auxin-induced expression of *ATHB8* found in earlier studies (Baima et al., 1995) appears to be mediated by *MP/ARF5*. *MP/ARF5* is also suggested to play a pivotal role in a positive feedback loop for auxin canalization by stimulating expression of *PIN1* (Wenzel et al., 2007). We confirmed that transcript levels of these genes were also increased in *acl5-1* and decreased by thermospermine. This is possibly due to the effect of *MP/ARF5* expression. In fact, they were reduced in *mp* seedlings. Based on these results together with previous phenotypic analyses of *acl5* alleles (Hanzawa et al., 1997; Clay and Nelson, 2005; Imai et al., 2006; Kakehi et al., 2008; Muñiz et al., 2008) and a chemical biology approach revealing that that 2,4-D derivatives enhance the excess xylem phenotype of *acl5-1* but have no effect on wild-type xylems (Yoshimoto et al., 2012), it is possible that thermospermine plays an opposing role to auxin in xylem differentiation through negative regulation of the expression of *MP/ARF5*.

On the other hand, a previous study has shown that knockdown of the HD-ZIP III genes by transgenic overexpression of microRNA *miR165*, which targets all the five HD-ZIP III genes in *Arabidopsis*, results in the reduced expression of *ACL5* and a subset of the genes related to vascular formation including *XCP1*, *XCP2* and *IRX3* (Zhou et al., 2007). These transcript levels were increased along with the *acl5-1* mutant transcript level in *acl5-1* seedlings (**Table 3**). Quadruple mutations of the five HD-ZIP III genes cause the lack of metaxylem development and little or no expression of *ACL5* in the root tissue (Carlsbecker et al., 2010). These results indicate that the HD-ZIP III transcription factors directly or indirectly activate the *ACL5* expression. It is also noted that *ACL5* expression was reduced in *mp* (**Figure 4**), suggesting that *ACL5* expression follows the onset of vascular formation by *MP*. Although the *ATHB8* expression is under the direct control of *MP/ARF5*, *ATHB8* is postulated to play a role in stabilizing procambium precursor cells to narrow regions against perturbations in auxin flow (Donner et al., 2009). Thus, the role of *ATHB8* might be virtually performed by thermospermine. In vascular formation, undifferentiated stem cells become procambium precursor cells, procambial cells, xylem precursor cells, and eventually three types of xylem cells: vessels, fibers, and parenchyma cells (Lehesranta et al., 2010). While *MP/ARF5* and *ATHB8* act in the early steps of this process, downstream thermospermine might participate in a negative feedback loop that fine-tunes *MP* expression. A previous study suggests that *ACL5* functions in preventing xylem precursors from premature cell death before secondary cell wall formation based on the observation that metaxylem vessels and xylem fibers are absent in *acl5* mutants (Muñiz et al., 2008). Taking into account the vast effect of thermospermine on the expression of such key regulators as *MP/ARF5*, HD-ZIP III, and *VND* genes, it is apparent that thermospermine plays a major inhibitory role against auxin during the process of continuing vascular formation.

Interestingly, we found that exogenous thermospermine strongly represses lateral root formation in wild-type seedlings. Since auxin controls lateral root development through multiple auxin-signaling modules, among which MP/ARF5 is one of major regulators (Lavenus et al., 2013), the inhibitory effect of thermospermine on lateral root formation may also be attributed to the reduced expression of auxin-related genes.

Our microarray study did not identify the regulatory genes whose expression is up-regulated by thermospermine. Given the fact that polyamines have enhancing effects on translation of specific mRNAs, a bHLH transcription factor gene *SAC51* whose translation is enhanced by thermospermine is a most probable candidate for a mediator of thermospermine signaling that negatively regulates expression of *MP/ARF5* and/or other regulatory

genes for xylem development. The *Arabidopsis* genome has three genes homologous to *SAC51*, all of which contain conserved multiple uORFs within their long 5′ leader regions. These transcription factors might also be up-regulated at the level of translation by thermospermine. On the other hand, two metal transporter genes, *IRT1*, and *ZIP8*, showed reduced expression in *acl5-1* mutants. So far, the causal relationship between thermospermine deficiency and the function of these metal transporters remains unknown and needs further investigation.

The results of the experiments using artificial polyamines revealed that, in addition to norspermine, 3-3-2 and 3-3-5 tetramines are also potent substitutes for thermospermine. Although the efficiency of import and/or transport of polyamines may depend on their structure and size, we confirmed that linear pentamines were absorbed by the root and transported to the shoot but were not able to replace thermospermine in terms of cellular functions. Thus, we suggest that the tetramines containing the core structure of 3-3 are biologically active and they may be useful as novel plant growth regulators against auxin for the control of xylem differentiation and lateral root formation. Since *ACL5* orthologs are present in unicellular algaes (Knott et al., 2007), the original role of thermospermine may be unrelated to cell differentiation in multicellular organisms. It is of interest to speculate that, while retaining the mode of action at the molecular level, thermospermine may have been recruited as a part of a negative feedback control in auxin-induced proliferation of xylem cells, which are programmed to die, during the evolution of vascular systems in land plants. Future work should be done with non-vascular plants to address the original function of thermospermine.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00094/abstract

Supplemental Table 1 | List of the genes up-regulated in *acl5-1* seedlings.

Supplemental Table 2 | GO analysis of the gene up-regulated in *acl5-1* seedlings.

Supplemental Table 3 | List of the genes down-regulated in *acl5-1* seedlings.

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Impact of 1-methylcyclopropene and controlled atmosphere storage on polyamine and 4-aminobutyrate levels in "Empire" apple fruit

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1-Methylcyclopropene (1-MCP) delays ethylene-meditated ripening of apple (*Malus domestica* Borkh.) fruit during controlled atmosphere (CA) storage. Here, we tested the hypothesis that 1-MCP and CA storage enhances the levels of polyamines (PAs) and 4-aminobutyrate (GABA) in apple fruit. A 46-week experiment was conducted with "Empire" apple using a split-plot design with four treatment replicates and 3°C, 2.5 kPa O_2, and 0.03 or 2.5 kPa CO_2 with or without 1 μL L^{-1} 1-MCP. Total PA levels were not elevated by the 1-MCP treatment. Examination of the individual PAs revealed that: (i) total putrescine levels tended to be lower with 1-MCP regardless of the CO_2 level, and while this was mostly at the expense of free putrescine, large transient increases in soluble conjugated putrescine were also evident; (ii) total spermidine levels tended to be lower with 1-MCP, particularly at 2.5 kPa CO_2, and this was mostly at the expense of soluble conjugated spermidine; (iii) total spermine levels at 2.5 kPa CO_2 tended to be lower with 1-MCP, and this was mostly at the expense of both soluble and insoluble conjugated spermine; and (iv) total spermidine and spermine levels at 0.03 kPa were relatively unaffected, compared to 2.5 kPa CO_2, but transient increases in free spermidine and spermine were evident. These findings might be due to changes in the conversion of putrescine into higher PAs and the interconversion of free and conjugated forms in apple fruit, rather than altered *S*-adenosylmethionine availability. Regardless of 1-MCP and CO_2 treatments, the availability of glutamate showed a transient peak initially, probably due to protein degradation, and this was followed by a steady decline over the remainder of the storage period which coincided with linear accumulation of GABA. This pattern has been attributed to the stimulation of glutamate decarboxylase activity and inhibition of GABA catabolism, rather than a contribution of PAs to GABA production.

Keywords: abiotic stress, apple fruit, controlled atmosphere storage, 4-aminobutyrate (GABA), 1-methylcyclopropene (1-MCP), high performance liquid chromatography, polyamines

INTRODUCTION

Over the last decade, 1-methylcyclopropene (1-MCP) has been adopted by the apple industry as a means of delaying ethylene-mediated fruit ripening and senescence, especially in combination with storage under controlled atmosphere (CA) conditions (i.e., 0–3°C, 2–2.5 kPa O_2, 2–4 kPa CO_2; DeEll et al., 2008; Fawbush et al., 2008; Watkins, 2008). 1-MCP inhibits ethylene binding and production in apple fruit held at ambient or chilling temperature, and reduces the expression of genes responsible for ethylene biosynthesis (Dal Cin et al., 2006; Pang et al., 2006; Vilaplana et al., 2007; **Figure 1**). Under commercial CA conditions, these findings are accompanied by a decline in the level of 1-aminocyclopropane-1-carboxylic acid (ACC), and the level of its precursor *S*-adenosylmethionine (SAM) is not directly linked to the rate of ethylene production and does not appear to be limiting (Bulens et al., 2012).

S-adenosylmethionine is essential for conversion of putrescine (Put) into the higher polyamines (PAs) spermidine (Spd) and spermine (Spm), as well the production of ethylene (**Figure 1**). During the ripening of a bulky fruit such as tomato, there is an inverse relationship between the production of higher PAs and ethylene (Lasanajak et al., 2014), 1-MCP treatment inhibits autocatalytic ethylene production without affecting SAM levels (Van de Poel et al., 2013), and decreased levels of ethylene in RNAi-1-aminocyclpropane-1-carboxylate synthase fruits enhance PA levels and upregulate PA biosynthesis genes (Gupta et al., 2013). Research with non-bulky fruits such as rice grain also suggests interaction between PAs and ethylene in regulating plant growth and in response to environmental stress (Chen et al., 2013). Notably, there is a transient increase in the level of Put only, and no changes in the level of Spd, Spm, or total PAs in apple fruit stored at 24°C, even though the expression of only one of two SAM decarboxylases (SAMDCs) is repressed (Pang et al., 2006).

During CA storage 4-aminobutyrate (GABA) accumulates in apple fruit (Deewatthanawong and Watkins, 2010; Lee et al., 2012; Trobacher et al., 2013a), but it is uncertain whether this

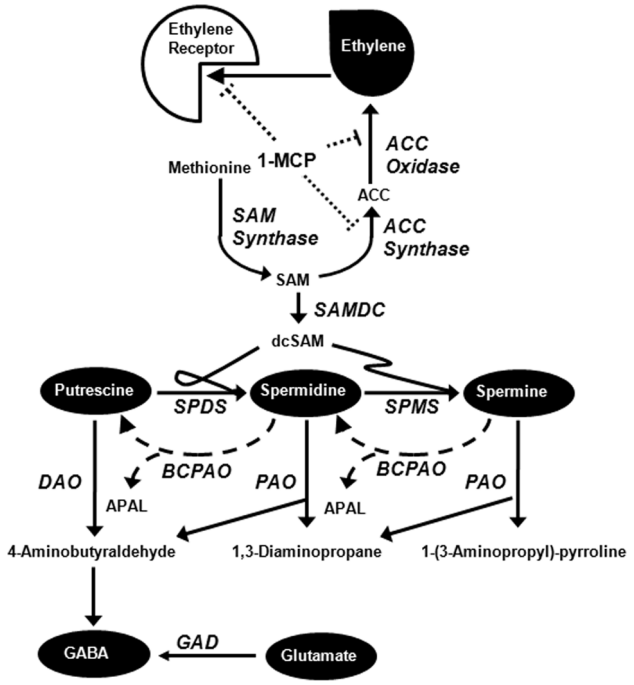

FIGURE 1 | Simplified diagram of metabolic relationships among ethylene, polyamines, and GABA [adapted from Shelp et al. (2012c)]. 1-MCP inhibits ethylene binding to its receptor and the expression of both ACC synthase and ACC oxidase (see text for detailed explanation). Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; APAL, 3-aminopropionaldehyde; BCPAO, back-conversion polyamine oxidase; dcSAM, decarboxylated *S*-adenosylmethionine; DAO, diamine oxidase; GABA, 4-aminobutyrate; GAD, glutamate decarboxylase; 1-MCP, 1-methylcyclopropene; PAO, polyamine oxidase; SAM, *S*-adenosylmethionine; SAMDC, SAM decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase.

GABA is derived from PAs or glutamate (Shelp et al., 2012c; **Figure 1**). Notably, the levels of both PAs and GABA can be stimulated by abiotic stresses such as chilling, O_2 deficiency and elevated CO_2, conditions that are the basis of CA storage (Alcázar et al., 2010; Bitrián et al., 2012; Shelp et al., 2012a,b,c; Trobacher et al., 2013a).

Here, we tested the hypothesis that 1-MCP and CA storage enhances the levels of PAs and GABA in apple fruit imposed by abiotic stresses associated with CA storage. Since 1-MCP has been shown to increase the incidence of CO_2-induced physiological injury in "Empire" fruit during CA storage (Watkins, 2006; Fawbush et al., 2008; Jung and Watkins, 2011), we utilized two levels of CO_2 (2.5 and 0.03 kPa).

MATERIALS AND METHODS

APPLE SOURCE AND CONTROLLED ATMOSPHERE STORAGE TRIALS

Sixteen boxes (~20 kg each) of apple (*Malus domestica* Borkh. cv. Empire) fruit were harvested in the morning from a commercial orchard within 15 km of Simcoe, Ontario on 29 September 2011, and immediately transported to our post-harvest facility at the University of Guelph. On arrival, four fruit were randomly chosen, frozen as quickly as is practical in liquid N_2 and stored at −80°C for future metabolite analysis. Then the boxes were divided into two treatments without or with 1 μL L^{-1} 1-MCP (SmartFresh^{SM}, AgroFresh Inc., Spring House, PA, supplied in sealed polybags) and stored at approximately 23°C for 1 day.

A split-plot design was used to eliminate the possibility of chamber effects. Fruit were stored in two random chambers that were set at 3°C; within each chamber, two rooms were supplied with 2.5 kPa O_2 and either 2.5 kPa or 0.03 kPa CO_2, giving a total of four rooms, each containing fruit treated without or with 1-MCP. The four rooms in each chamber were treated as biological replicates in this nested design.

About 90 fruit were stored in individual boxes in the CA rooms, which were sealed and flushed with 99 kPa N_2. When the O_2 concentration declined to 2.5 kPa, N_2 flushing was replaced with CO_2 until 2.5 or 0.03 kPa CO_2 was achieved. Four fruit were collected from each box at eight different time points over the storage period (17 days, and 4, 8, 10, 14, 23, 33, and 46 weeks) following harvest, frozen in liquid N_2 and stored at −80°C prior to metabolite analysis. Fruit were also collected for assessment of fruit quality, but these data will be reported elsewhere.

METABOLITE COMPOSITION OF WHOLE APPLE FRUIT

Polyamine analysis

For each treatment replicate, four whole frozen apples were cryogenically pulverized, taking care to ensure that the apple tissue did not thaw during the procedure (Trobacher et al., 2013a). PAs were essentially extracted and analyzed as described by Smith and Davies (1985) and Shiozaki et al. (2000). Briefly, 100 mg of fine frozen powder (comprised of 25 mg from each of the four apple subsamples in each treatment replicate) was homogenized in cold 5% perchloric acid (PCA, 100 mg mL^{-1}), placed on ice for 30 min and then spiked with 10 μL of the internal standard 1,6 hexanediamine (0.25 nmol μL^{-1}; Fisher Scientific, Whitby, ON, Canada). The sample was centrifuged for 20 min at 14,000 *g* at room temperature, and the supernatant was transferred to a 2-mL microfuge tube and maintained on ice for 30 min. The pellet was extracted again in 1 mL of 5% PCA and treated as above. For analysis of free and conjugated soluble polyamines, the two supernatants were combined and dried in vacuo at 60°C or under a stream of filtered air at room temperature, then redissolved in 0.4 mL of 5% PCA. One half of this extract was transferred to a 5-mL amber reacti-vial and derivatized for analysis of free PAs as described below. The remaining 0.2 mL was hydrolyzed with HCl as described below before derivatization. The soluble conjugated soluble polyamines were estimated as the difference between the hydrolyzed and original supernatants.

The final pellet was resuspended in 0.2 mL of 1 M NaOH and centrifuged for 5 min at 10,000 *g*. The supernatant was transferred to a 2-mL amber Eppendorf tube and hydrolyzed with 0.2 mL of 12 M HCl at room temperature for 18 h. The sample was then dried as above, resuspended in 0.2 mL of 5% PCA and derivatized for analysis of conjugated insoluble polyamines.

A 0.6 mL aliquot of dansyl chloride (Sigma Aldrich, Oakville, ON, Canada) in acetone (7.5 mg mL^{-1} acetone) was added to a 0.2-mL aliquot of the various PCA extracts. Then 0.3 mL of a

saturated sodium carbonate solution was added with brief vortexing to give a pH of 10. The mixture was placed in a 60°C waterbath for 30 min (Gennaro et al., 1988). A 0.1-mL aliquot of proline (0.1 g mL^{-1}; Sigma Aldrich) was added to the solution to remove excess dansyl chloride, and then the mixture was incubated in the dark for 15 min at room temperature. Dansylated PAs were extracted from the mixture by adding 0.5 mL toluene (Fisher Scientific, Whitby, ON, Canada) and vortexing for 1 min. Then, 0.4 mL was taken from the organic layer and dried under filtered air for 30 min. The dry residue was dissolved in 0.2 mL methanol and stored at 4°C in the dark for up to 2 weeks.

Dansylated PAs were passed through 0.45 um syringe filter and 20-μL aliquots injected onto a reverse-phase column (Agilent Zorbax ODS 5 μm, 4.6 mm × 150 mm) linked to an Agilent 1100 HPLC system (Allan and Shelp, 2006). Initially, 60% methanol was provided for 4 min at a flow rate of 1.5 mL min^{-1}, followed by a linear increase to 95% methanol over 10 min and then 95% methanol for a further 5 min. The PAs were quantified with a fluorescence detector set at excitation and emission wavelengths of 254 and 500 nm, respectively (Gennaro et al., 1988). Although other peaks might appear on the chromatogram, they eluted before all dansylated PAs of interest, allowing for good resolution of Put (RT = 12.6 min), 1,6 hexanediamine (RT = 13.8 min), Spd (RT = 16.4 min), and Spm (RT = 18.5 min). Each treatment replicate was considered to be the average of the four subsamples.

The linearity of the standard curves up to 120 pmol for Put, Spd, Spm and 1,6 hexanediamine (Sigma Aldrich, Oakville, ON, Canada) was not affected by the addition of frozen apple powder tissue to the extraction procedure. The recovery of the internal standard across apple samples was approximately 60%; all samples were corrected for the actual loss during preparation. Furthermore, reverse-phase HPLC analysis of the PA standards and apple samples showed baseline separation of all PAs of interest. For routine analysis, a suite of external standards (80 pmol each) was run every sixth sample. The detection limit for the overall method was approximately 0.10 nmol g^{-1} fresh mass (FM).

Amino acid analysis

The amino acid composition of each apple was determined essentially as described previously (Allan and Shelp, 2006). Briefly, 1 g of the fine frozen powder was ground in four volumes of 30 g L^{-1} sulfosalicylic acid using a chilled mortar and pestle and fine silica sand, and 1.0 mL of the solution was centrifuged. The supernatant was adjusted to neutrality with NaOH, and then passed through a 0.45-μm syringe filter prior to on-line derivatization with *o*-phthalaldehyde. Aliquots (0.5 μL) of the supernatant were analyzed by reverse-phase HPLC. Each treatment replicate was considered to be the average of the four subsamples analyzed and each mean was the average of four treatment replicates. A suite of external amino acid standards (125 pmol each) derived from protein hydrolysate and the individual amino acids GABA, asparagine and glutamine were run every sixth sample.

Statistical analysis of data

All statistical analyses were conducted using SAS 9.2 at the $\alpha = 0.05$ level (SAS Institute Inc., Cary, NC, USA). Replicate room effects were analyzed using analysis of variances (ANOVAs; proc mixed method), which partitioned variance into the fixed effects (1-MCP, temperature, CO_2, storage time) and their interactions, and the random effect of chambers. In cases where interactions were significant ($P \leq 0.05$), treatment means were compared within weeks (slice option) over the period from 17 days to 46 weeks using a Fisher's protected least significant difference test. For experiments where interactions were significant ($P \leq 0.05$), data were pooled across repeated measures to determine differences among treatments and over storage period using the Tukey's test. All data were arcsine square root transformed to ensure a normal distribution of variance and the treatment means were back-transformed for presentation.

RESULTS

Total apple PAs consisted of free, soluble conjugated, and insoluble conjugated forms, with free and soluble conjugated forms being at much higher concentrations than the insoluble conjugated forms (**Figures 2A–D**). The concentrations of all forms fluctuated considerably during the 1-MCP treatment and the initial collection periods at 17 days and 4 weeks, but were much steadier from 8 to 46 weeks. Therefore, statistical analysis of the main effects and interactions was conducted for the entire treatment period, whereas detailed comparisons between the 1-MCP treatments were made over the 8–46 week period. Furthermore, the total PAs were comprised primarily of total Put and total Spd, followed by total Spm (**Figures 3–5**, panel A). The concentrations of individual PAs consisted mainly of the free form, followed closely by the soluble conjugated form, and more distantly by the insoluble conjugated form (**Figures 3–5**, panels B–D). Notably, the concentrations of the free forms tended to fluctuate less than the conjugated forms.

The concentrations of total PAs showed significant storage time, CO_2 and 1-MCP main effects, as well as storage time × CO_2, 1-MCP × CO_2, and storage time × CO_2 × 1-MCP interactions ($P \leq 0.05$, ANOVA table not shown). During the period from 8 to 46 weeks, the total PA concentrations in 1-MCP-treated fruit at 2.5 kPa CO_2 were, with the exception of 8 and 23 weeks, 33–61% of those in control fruit (i.e., not treated with 1-MCP), whereas at 0.03 kPa CO_2 they were 29–39% of those at 33 and 46 weeks (**Figure 2A**). The concentrations of total free PAs showed significant storage time and CO_2 main effects, as well as storage time × 1-MCP, and storage time × 1-MCP × CO_2 interactions ($P \leq 0.05$, ANOVA table not shown). At 2.5 kPa CO_2 the total free PA concentrations in 1-MCP-treated fruit were 49–51% of those in control fruit at 23 and 33 weeks, whereas at 0.03 kPa CO_2 they were, with the exception of weeks 23 and 33, 27–52% of those in control fruit (**Figure 2B**). The concentrations of total soluble conjugated PAs showed significant CO_2 and 1-MCP main effects, as well as CO_2 × 1-MCP, storage time × CO_2 × 1-MCP interactions ($P \leq 0.05$, ANOVA table not shown). At 2.5 kPa CO_2, the concentrations of total soluble conjugated PAs in 1-MCP-treated fruit were similar to those in control fruit, whereas at 0.03 kPa CO_2 they were 6% of that in the control at 33 weeks (**Figure 2C**). The concentrations of total insoluble conjugated PAs showed significant CO_2 and 1-MCP main effects, and storage time × 1-MCP, CO_2 × 1-MCP interactions ($P \leq 0.05$, ANOVA table not shown).

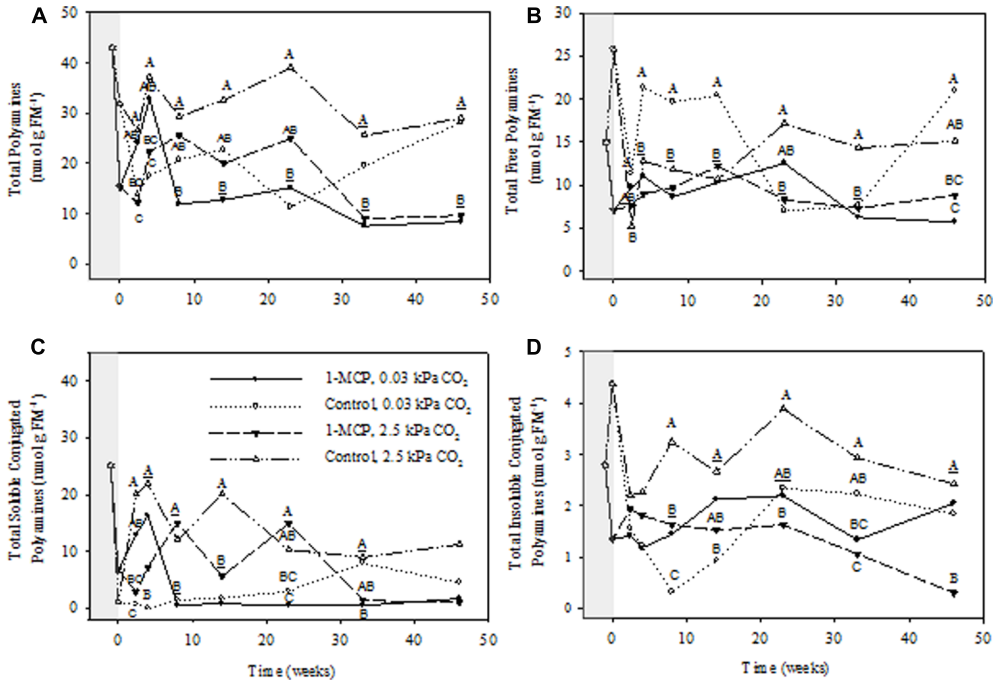

FIGURE 2 | Effects of 1-MCP and elevated CO_2 on total (A), total free (B), total soluble conjugated (C), and total insoluble conjugated (D) polyamines in "Empire" fruit during controlled atmosphere storage. With the exception of the beginning and end of 1-MCP treatment (−1 day to 0 week, the shaded area), all data are mean estimates of four storage replicates. Different letter groupings indicate significant differences between treatments within weeks ($P \leq 0.05$). Underlined letters indicate a shared letter for overlapping data; where letters are absent at a time point, there were no significant differences.

At 2.5 kPa CO_2 the concentrations of total insoluble conjugated PA concentrations in 1-MCP- treated fruit were, with the exception of week 14, 13–57% of those in control fruit, whereas at 0.03 kPa CO_2 they were 130–350% greater at 8 and 14 weeks (**Figure 2D**).

The concentrations of total Put displayed significant CO_2 main effects, but no interactions ($P \leq 0.05$, ANOVA table not shown). The total Put concentrations at 2.5 and 0.03 kPa CO_2 in 1-MCP-treated fruit were 41–44% and 32–37% of those in control fruit at 8, 33, and 46 weeks and at 8, 14, and 33 weeks, respectively (**Figure 3A**). There were significant storage time and CO_2 main effects on the concentrations of free Put, as well as storage time × CO_2 × 1-MCP interaction ($P \leq 0.05$, ANOVA table not shown). At 2.5 kPa CO_2 the free Put concentrations in 1-MCP-treated fruit were 25–44% of those in control fruit at 23, 33, and 46 weeks, whereas at 0.03 kPa CO_2 they were, with the exception of week 23, 13–40% of the controls (**Figure 3B**). The concentrations of soluble conjugated Put showed significant 1-MCP and CO_2 main effects, as well as storage time × 1-MCP and storage time × CO_2 × 1-MCP interactions ($P \leq 0.05$, ANOVA table not shown). At 2.5 kPa CO_2 the concentrations of soluble conjugated Put in 1-MCP-treated fruit were 200% greater than those in control fruit at 23 weeks, whereas at 0.03 kPa CO_2 there were no significant differences among treatments (**Figure 3C**). The concentrations of insoluble conjugated Put showed significant CO_2 main effects, as well as storage time × CO_2, storage time × 1-MCP, CO_2 × 1-MCP and storage time × CO_2 × 1-MCP interactions ($P \leq 0.05$, ANOVA table not shown). At 2.5 kPa CO_2 the concentrations of insoluble conjugated Put in 1-MCP-treated fruit were 3–20% of those in control fruit at 33 and 46 weeks, whereas at 0.03 kPa CO_2 they were 490–1420% greater than controls at 8 and 14 weeks (**Figure 3D**).

The total Spd concentrations displayed significant storage time, CO_2 and 1-MCP main effects, as well as storage time × CO_2, storage time × 1-MCP, and CO_2 × 1-MCP interactions ($P \leq 0.05$, ANOVA table not shown). At 2.5 kPa CO_2 total Spd concentrations in 1-MCP-treated fruit were 25–52% of those in control fruit at 14, 33, and 46 weeks, whereas at 0.03 kPa CO_2 there were no significant differences between treatments (**Figure 4A**). Free Spd concentrations showed significant storage time, CO_2, and 1-MCP main effects, as well as storage time × CO_2, storage time × 1-MCP, and storage time × CO_2 × 1-MCP interactions ($P \leq 0.05$, ANOVA table not shown). At 2.5 kPa CO_2 free Spd concentrations in 1-MCP-treated fruit were 121% greater than those in control fruit at 14 weeks, whereas at 0.03 kPa CO_2 they were 230–350% greater at 8, 23, and 33 weeks and 49% of the control at 46 weeks (**Figure 4B**). The concentrations of soluble conjugated Spd displayed CO_2 and 1-MCP main effects, and storage time × CO_2, CO_2 × 1-MCP and storage time × CO_2 × 1-MCP interactions ($P \leq 0.05$, ANOVA table not shown). At 2.5 kPa CO_2 the concentrations of soluble conjugated Spd in 1-MCP-treated fruit never exceeded 4% of those at 14, 33, and 46 weeks of storage, whereas at 0.03 kPa CO_2, they were undetectable at 33 weeks of storage (**Figure 4C**). The concentrations of insoluble conjugated Spd showed significant storage time and 1-MCP

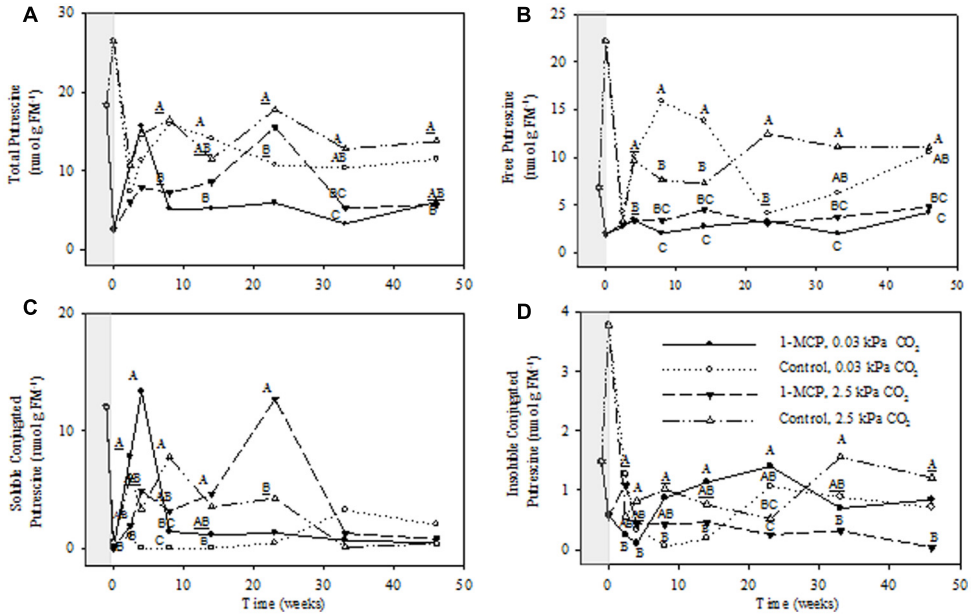

FIGURE 3 | Effects of 1-MCP and elevated CO_2 on total (A), free (B), soluble conjugated (C), and insoluble conjugated (D) putrescine in "Empire" fruit during controlled atmosphere storage. With the exception of the beginning and end of 1-MCP treatment (−1 day to 0 week, the shaded area), all data are mean estimates of four storage replicates. Different letter groupings indicate significant differences between treatments within weeks ($P \leq 0.05$). Underlined letters indicate a shared letter for overlapping data; where letters are absent at a time point, there were no significant differences.

main effects and storage time × 1-MCP interactions ($P \leq 0.05$, ANOVA table not shown). At 2.5 and 0.03 kPa CO_2, there were no significant differences between the 1-MCP treatments (**Figure 4D**).

The concentrations of total Spm showed significant storage time and CO_2 main effects, as well as CO_2 × 1-MCP and storage time × CO_2 × 1-MCP interactions ($P \leq 0.05$, ANOVA table not shown). At 2.5 kPa CO_2 total Spm concentrations in 1-MCP-treated fruit were 140% greater than those that in control fruit at 8 weeks, but 34% of the control at 23 weeks (**Figure 5A**). There were no differences between treatments at 0.03 kPa CO_2. The concentration of free Spm showed significant storage time and 1-MCP main effects ($P \leq 0.05$, ANOVA table not shown). At 2.5 kPa CO_2 there was no significant differences between the treatments, whereas at 0.03 kPa CO_2 the free Spm concentration was 260% greater in 1-MCP-treated fruit than that in control fruit at 23 weeks (**Figure 5B**). There were significant CO_2 and 1-MCP main effects, as well as storage time × CO_2, CO_2 × 1-MCP and storage time × CO_2 × 1-MCP interactions, for concentrations of soluble conjugated Spm ($P \leq 0.05$, ANOVA table not shown). At 2.5 kPa CO_2 the concentration of soluble conjugated Spm in 1-MCP-treated fruit was much greater than that in control fruit at 8 weeks, but less than 3% of those at 14 and 23 weeks, whereas at 0.03 kPa CO_2 there were no differences between 1-MCP treatments (**Figure 5C**). The concentrations of insoluble conjugated Spm showed significant CO_2 and 1-MCP main effects, as well as storage time × CO_2, CO_2 × 1-MCP interactions ($P \leq 0.05$, ANOVA table not shown). At 2.5 kPa CO_2 the concentrations of insoluble conjugated Spm in 1-MCP-treated fruit never exceeded 18% of those in control fruit over the 8- to 46-week period; however, at 0.03 kPa CO_2 the concentrations were 280–1250% greater at 8, 14, and 23 weeks (**Figure 5D**).

Regardless of 1-MCP and CO_2 treatments GABA accumulated in a linear fashion over the storage period (**Figure 6A**), whereas glutamate declined after an initial rise (**Figure 6B**). Notably, fruit receiving 2.5 kPa CO_2 and 1-MCP accumulated twice as much GABA (~2 nmol g^{-1} FM $week^{-1}$) as fruit receiving 0.03 kPa CO_2 only, accounting for approximately one half of the decline in glutamate over the same period. The GABA concentrations displayed significant storage time, CO_2 and 1-MCP main effects, as well as storage time × 1-MCP interactions ($P \leq 0.05$, ANOVA table not shown). At 2.5 and 0.03 kPa CO_2 the GABA concentrations in 1-MCP-treated fruit were 19 and 25% greater, respectively, than those in control fruit at 33 weeks (**Figure 6A**). Glutamate showed significant storage time, CO_2 and 1-MCP main effects, as well as storage time × 1-MCP, CO_2 × 1-MCP interactions ($P \leq 0.05$, ANOVA table not shown). At 2.5 kPa CO_2 glutamate concentrations in 1-MCP-treated fruit were 34–38% greater than those in control fruit at 14 and 23 weeks, whereas at 0.03 kPa CO_2 they were 8–57% greater than those in control fruit over the storage period (**Figure 6B**).

DISCUSSION

Both PAs and GABA appear to function in various physiological processes such as stress responses and growth and development (Alcázar et al., 2010; Fincato et al., 2012; Moschou et al., 2012; Shelp et al., 2012b,c). The presence and interconversion of free forms of Put, Spd and Spm have been the focus of PA research, but soluble (i.e., bound to small molecules such as phenolic acids)

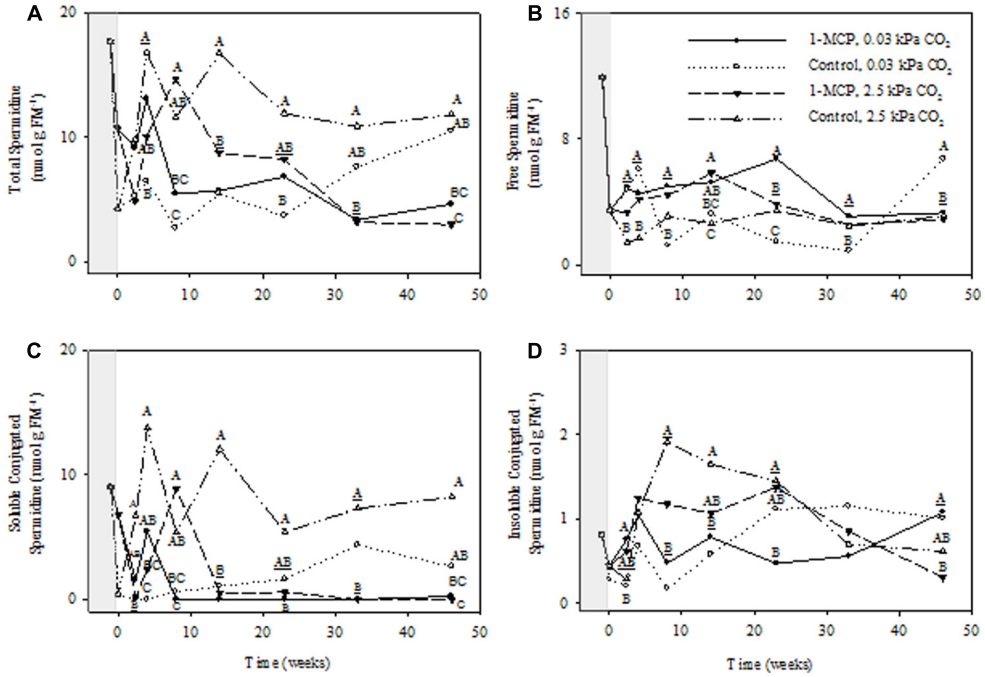

FIGURE 4 | Effects of 1-MCP and elevated CO_2 on total (A), free (B), soluble conjugated (C), and insoluble conjugated (D) spermidine in "Empire" fruit during controlled atmosphere storage. With the exception of the beginning and end of 1-MCP treatment (−1 day to 0 week, the shaded area), all data are mean estimates of four storage replicates. Different letter groupings indicate significant differences between treatments within weeks ($P \leq 0.05$). Underlined letters indicate a shared letter for overlapping data; where letters are absent at a time point, there were no significant differences.

and insoluble (i.e., bound to macromolecules such as nucleic acids and proteins) conjugated forms also exist. The ratios between free and conjugated PAs vary among plant species (Bagni and Tassoni, 2001), and some evidence exists for interconversion between free and conjugated PAs (Bassard et al., 2010), which could influence our interpretation of the impact of 1-MCP and CA on PAs and possibly GABA in apple fruit (**Figure 1**). Interestingly, it has been suggested that the stress hormone abscisic acid contributes to the conversion of conjugated forms of PA to the free forms (Ben Hassine et al., 2009).

In the present study, the concentrations of free and conjugated forms of PAs were determined in ripening 1-MCP-treated "Empire" apple fruit over a 46-week storage period under CA conditions. Initially, there were marked fluctuations in the concentrations of these PAs as the fruit acclimated to the shift in environmental conditions. Steady-state levels were evident after approximately 8 weeks of storage. It is well known that 1-MCP treatment should inhibit the autocatalytic production of ethylene in apple fruit (Pang et al., 2006; DeEll et al., 2008; Fawbush et al., 2008; Jung and Watkins, 2011), reduce the expression of genes involved in ethylene biosynthesis (Dal Cin et al., 2006; Pang et al., 2006; Vilaplana et al., 2007), and decrease the level of ACC (Bulens et al., 2012). However, we found no evidence for elevated levels of total PAs, Put, Spd, or Spm during the steady-state period (8–46 weeks) in response to 1-MCP treatment; indeed the levels tended to be lower regardless of CO_2 (**Figures 2–5**), suggesting that the availability of SAM for PA biosynthesis in apple fruit was not influenced by the 1-MCP treatment. These findings are consistent with previous studies of ripening apple and tomato fruits, which suggest that the requirement for SAM in PA biosynthesis is not limited by the requirement in ethylene biosynthesis (Van de Poel et al., 2013; Lasanajak et al., 2014), and suggest an unknown biochemical or transcriptional mechanism, possibly altering the PA biosynthetic rates from glutamate (Alcázar et al., 2010; Majumdar et al., 2013), was responsible for the lower total PA levels in 1-MCP-treated fruit under CA storage. However, these findings are at odds with the generally accepted view that PAs, especially Spd and Spm, accumulate in respond to abiotic stress (Groppa and Benavides, 2008; Bassard et al., 2010; Shelp et al., 2012c), which could be due to a variety of reasons: that view is generally based on free PA levels, rather than total PAs; apple fruit experience multiple stresses during CA storage; and, ripening apple fruit, like tomato fruit (Mattoo et al., 2010), are at a terminal developmental stage.

1-Methylcyclopropene application changed the levels of individual PAs and the relative proportions of free and conjugated forms. Total Put levels tended to be lower with 1-MCP regardless of the CO_2 level, and while this was mostly at the expense of free Put, large transient increases in soluble conjugated Put were also evident (**Figure 3**). Total Spd levels tended to be lower with 1-MCP, particularly at 2.5 kPa CO_2, and this was mostly at the expense of soluble conjugated Spd (**Figure 4**). Total Spm levels at 2.5 kPa CO_2 tended to be lower with 1-MCP, and this was mostly at the expense of both soluble and insoluble conjugated Spm (**Figure 5**). Overall, total Spd and Spm levels at 0.03 kPa were relatively unaffected, compared to 2.5 kPa CO_2, but transient

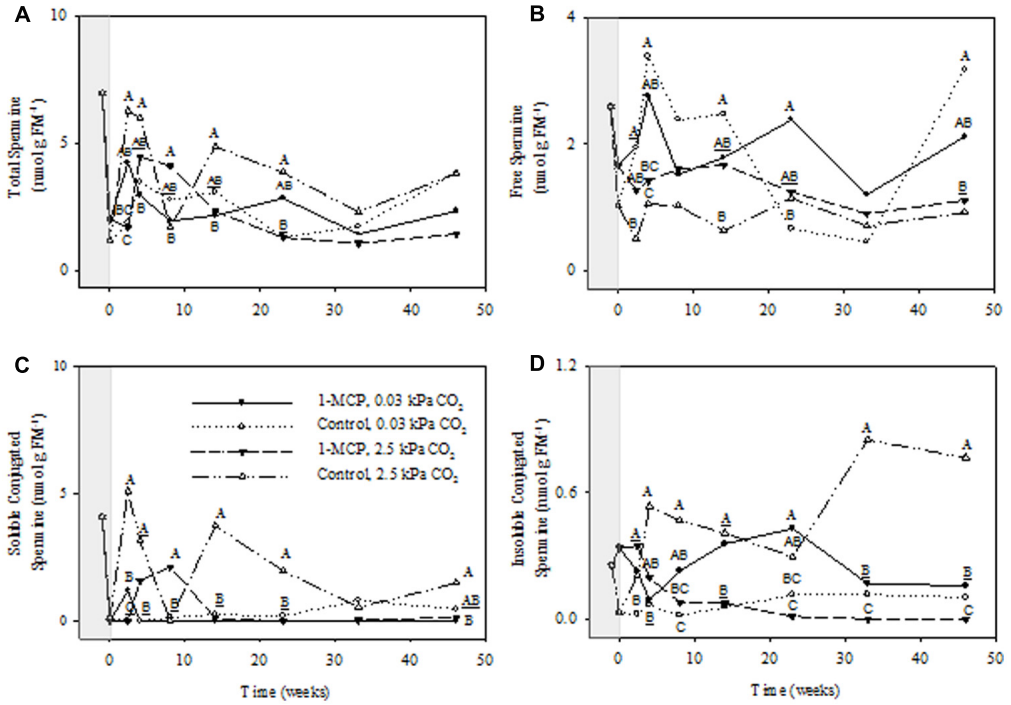

FIGURE 5 | Effects of 1-MCP and elevated CO_2 on total (A), free (B), soluble conjugated (C), and insoluble conjugated (D) spermine in "Empire" fruit during controlled atmosphere storage. With the exception of the beginning and end of 1-MCP treatment (−1 day to 0 week, the shaded area), all data are mean estimates of four storage replicates. Different letter groupings indicate significant differences between treatments within weeks ($P \leq 0.05$). Underlined letters indicate a shared letter for overlapping data; where letters are absent at a time point, there were no significant differences.

increases in free Spd and Spm were evident. Thus, 1-MCP treatment reduced the accumulation of individual PAs with elevated CO_2 under CA conditions; however, there was a general decrease and increase in the ratio of free:conjugated forms for Put and Spd plus Spm, respectively. The accumulation of PAs was unaffected under low CO_2, but there was some evidence for accumulation of conjugated Spd and Spm. Since total PA levels declined with 1-MCP (**Figure 2**), this differential response of Put and Spd plus Spm in the partitioning between free and conjugated forms could not be attributed to altered SAM availability. The response might be attributed, at least in part, to changes in the conversion and back-conversion of PAs, and the interconversion of free and conjugated forms in apple fruit, although the activities of polyamine oxidases (PAOs) could be limited by the low O_2 status of intact apple fruit stored under CA conditions (Ho et al., 2011; Shelp et al., 2012c).

Glutamate serves a precursor for biosynthesis of both GABA and PAs, raising the possibility that GABA in CA-stored apple fruit can be derived directly from glutamate or indirectly from free Put or free Spd (**Figure 1**). Regardless of 1-MCP and CO_2 treatments, the availability of glutamate showed a transient peak initially, probably due to protein degradation (Magné et al., 1997; Sugimoto et al., 2011), and this was followed by a steady decline over the remainder of the storage period which coincided with accumulating GABA (**Figure 6**). This pattern could be attributed due to the stimulation of glutamate decarboxylase (GAD) activities via bound Ca^{2+}/calmodulin or lower cytosolic pH (Shelp et al., 2012a; Trobacher et al., 2013b), and product inhibition of GABA transaminase activity (Clark et al., 2009) due to the restricted activity of succinic semialdehyde dehydrogenase activity under O_2 deficiency (Busch and Fromm, 1999; Shelp et al., 1999). GABA accumulation was lowest at 0.03 kPa CO_2 and noticeably greater with either 1-MCP or 2.5 kPa CO_2. These findings are consistent with previous research showing that GABA accumulates in tissues of "Empire" apple fruit during CA storage with elevated CO_2 and is then catabolized when the fruit are transferred to ambient conditions (Deewatthanawong and Watkins, 2010; Lee et al., 2012; Trobacher et al., 2013a). Relatively higher levels of free Put and Spd than free Spm are also consistent with studies of other species and tissues (Mattoo et al., 2010). Interestingly, the changing levels of Spd seemed to be more highly correlated than those of Put with the rate of GABA production regardless of the 1-MCP and CO_2 treatments, suggesting that Spd could be in steady-state equilibrium with GABA. It has been argued that changes in O_2 availability and cellular redox balance in apple fruit stored under CA conditions would directly influence the activity of 4-aminobutyraldehyde dehydrogenase, as well as diamine oxidases (DAO), thereby restricting GABA formation from both Spd and Put (Shelp et al., 2012c). Unfortunately, pool sizes do not indicate the flux through a pathway, mutants of the metabolic routes for GABA and PAs are not readily available for apple, and radiolabelled precursors cannot be supplied to intact apples without perturbing the internal gaseous environment, which would most certainly affect GABA formation from glutamate. Useful

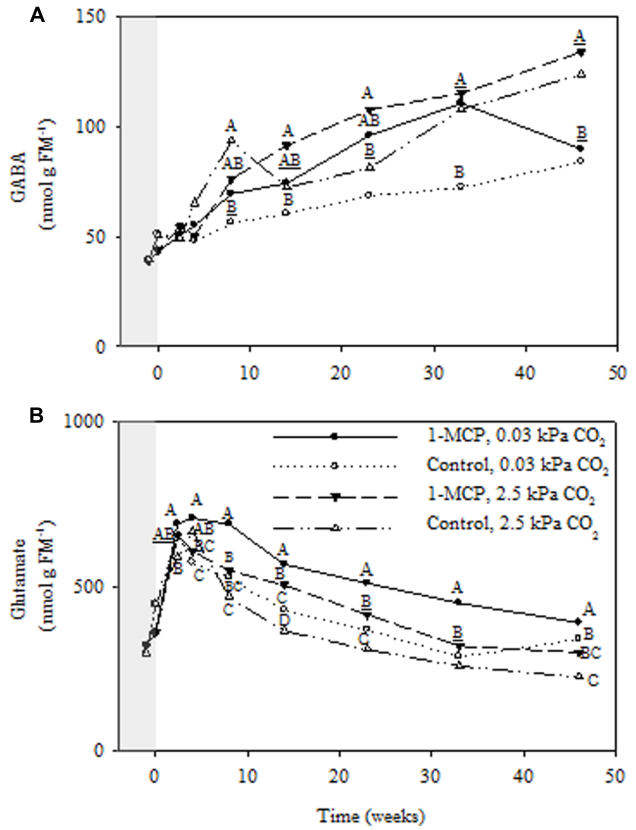

FIGURE 6 | Effects of 1-MCP and elevated CO_2 on GABA (A) and glutamate (B) levels in "Empire" fruit during controlled atmosphere storage. With the exception of the beginning and end of 1-MCP treatment (−1 day to 0 week, the shaded area), all data are mean estimates of four storage replicates. Different letter groupings indicate significant differences between treatments within weeks ($P \leq 0.05$). Underlined letters indicate a shared letter for overlapping data; where letters are absent at a time point, there were no significant differences.

information about the relative contributions of glutamate and PAs to GABA production in intact apple fruit could be gained via a combination of metabolite and gene transcript analyses. Recently, use of the DAO inhibitor aminoguanidine suggested that approximately 30% of the GABA accumulated in fava bean seed germinating under hypoxia is derived from PAs (Yang et al., 2013). Notably, the free levels of the three PAs, especially Put, increased, unlike the case reported here, and this was accompanied by some loss of GAD activity, which was attributed to growth inhibition.

While uncertainty continues regarding the relationship between PA and ethylene biosynthesis in fruits, especially those exposed to multiple abiotic stresses, our research indicates that the requirement for SAM in PA biosynthesis in CA-stored apple fruit was probably not limited by the requirement in ethylene biosynthesis. Moreover, the differential response of PA partitioning between free and conjugated forms could be attributed to changes in the interconversion of free and conjugated forms and the conversion and back-conversion of PAs. Also, the data could be interpreted as preliminary evidence for a relationship between Spd level and GABA production, but it is argued that the biochemical reactions involved would be limited by the *in vivo* O_2 level, and that elevated GAD activity and product inhibition of GABA transaminase activity would be responsible for GABA accumulation in these fruit.

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Polyamines in chemiosmosis *in vivo*: a cunning mechanism for the regulation of ATP synthesis during growth and stress

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Polyamines (PAs) are low molecular weight amines that occur in every living organism. The three main PAs (putrescine, spermidine, and spermine) are involved in several important biochemical processes covered in recent reviews. As rule of thumb, increase of the cellular titer of PAs in plants is related to cell growth and cell tolerance to abiotic and biotic stress. In the present contribution, we describe recent findings from plant bioenergetics that bring to light a previously unrecognized dynamic behavior of the PA pool. Traditionally, PAs are described by many authors as organic polycations, when in fact they are bases that can be found in a charged or uncharged form. Although uncharged forms represent less than 0.1% of the total pool, we propose that their physiological role could be crucial in chemiosmosis. This process describes the formation of a PA gradient across membranes within seconds and is difficult to be tested *in vivo* in plants due to the relatively small molecular weight of PAs and the speed of the process. We tested the hypothesis that PAs act as permeable buffers in intact leaves by using recent advances *in vivo* probing. We found that an increase of PAs increases the electric component ($\Delta\psi$) and decreases the ΔpH component of the proton motive force. These findings reveal an important modulation of the energy production process and photoprotection of the chloroplast by PAs. We explain in detail the theory behind PA pumping and ion trapping in acidic compartments (such as the lumen in chloroplasts) and how this regulatory process could improve either the photochemical efficiency of the photosynthetic apparatus and increase the synthesis of ATP or fine tune antenna regulation and make the plant more tolerant to stress.

Keywords: ATP synthesis, proton motive force, chloroplast, photosynthesis, polyamines, putrescine, stress

CHEMIOSMOSIS

Organisms need ATP for many cellular processes such as translation, metabolite production, proliferation and stress response. Most ATP (95%) is produced by chemiosmosis (i.e., the movement of ions across a selectively permeable membrane, down their electrochemical gradient), therefore this synthesis is the most important process for cell physiology (Mitchell 1961,1978). Not surprisingly, partial or full inhibition of chemiosmosis leads to disease or death in animals and plants. Hence, any factor (protein or solute) that increases or more generally speaking, modulates ATP synthesis is of exceptional biological significance. In this contribution, we will discuss the role of polyamines (PAs) in chemiosmotic ATP synthesis based on findings from plant bioenergetics. The chemiosmotic hypothesis states that ATP synthesis in respiring cells comes from the electrochemical gradient across membranes such as the inner membranes of mitochondria and chloroplasts (Kramer et al., 2004). In other words, energization of a single membrane simultaneously and continuously powers many ATP synthases. Usually in biochemistry, an enzyme converts a substrate into a product, but in chemiosmosis the situation is slightly more complex. Hence, for the purpose of this review it is important to clarify basic features of the chemiosmotic mechanism before the role of PAs is described. The chemiosmotic mechanism in plants, animals and microbes has three conserved features: (i) an electron transport chain that supports vectorial release of protons (proton producers), (ii) a coupling membrane or "energized" membrane (cristae membrane in mitochondria, thylakoid membrane in chloroplasts, and plasma membrane in bacteria), (iii) transmembrane proton motive ATPases that are vectorially embedded in the membrane (proton consumers). The following scheme (**Figure 1A**) illustrates the sequence of events in classical chemiosmosis.

A chemiosmotic unit (a membrane that houses many proton producers and many ATPases) functions as a battery and as long as it is charged phosphorylates ADP. This battery can be seen as a huge enzymatic complex that uses an electrochemical gradient also called proton motive force (*pmf*) to produce ATP. *Pmf* is a combination of two gradients across the membrane: a concentration proton gradient (ΔpH) and an electrical gradient ($\Delta\psi$). In simpler terms, electron carriers and related enzymes in the membrane produce protons that are released on one side of the membrane and decrease the pH of this compartment (e.g., lumen of thylakoids). Consequently, protons will diffuse from an area of high proton concentration (lumen) to an area of lower proton concentration (stroma). The main efflux path for protons is the ATPase, which in turn uses protons' free energy to phosphorylate ADP. Important factors for the amplitude of *pmf* are the proton release rate, the conductivity of the ATPase to protons and the ionic strength. In plants, *pmf* is established both in mitochondria and chloroplasts. Next, we will describe why *pmf* in

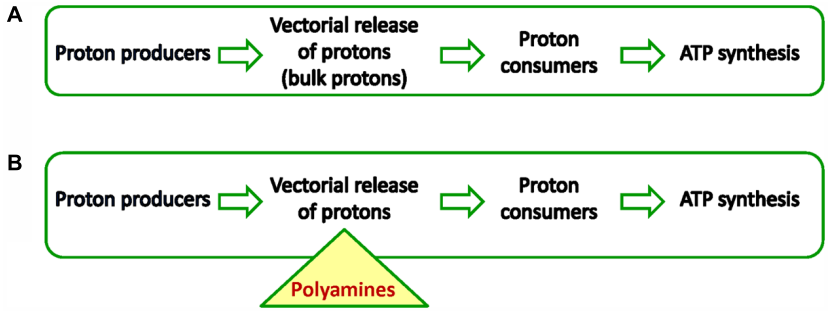

FIGURE 1 | (A) Chemiosmosis in all cells powers ATP synthesis by forming a proton motive force. Important for the establishment of *pmf* is a membrane the so-called coupling membrane. Proton producers are usually enzymes of the respiratory chain or photosynthetic subcomplexes. Proton consumers are usually proton-driven ATPases. **(B)** New chemiosmosis. PAs buffer acidic compartment and energize the membrane that houses ATPases. The triangle shows the central point of the PAs role in chemiosmosis. In other words PAs act as intermediates receiving protons from producers and deliver them to consumers.

chloroplasts has a more complex and important role than in mitochondria. Noteworthily, in plant science data derive both from *in vitro* and *in vivo* measurements. In other disciplines, most data in particular for ATPases come from *in vitro* experiments. Thus chemiosmosis *in vivo* is better understood and described in plants.

Pmf IN PLANT CHLOROPLASTS

Proton motive force in chloroplasts produces energy and regulates photoprotection. Thus light-driven transthylakoid *pmf* plays several essential roles in plant physiology (Kramer et al., 2004). More particularly both the ΔpH and Δψ components of *pmf* contribute to ATP synthesis at the CF_O–CF_1 ATP synthase, in a thermodynamically equivalent fashion (Kramer et al., 2003). In addition, the ΔpH component of *pmf* is a key signal for initiating photoprotection. This photoprotection mechanism the so-called energization quenching (qE), is a process that harmlessly dissipates the excess absorbed light energy as heat (Li et al., 2000; Pascal et al., 2005; Ruban et al., 2007). Acidification of the lumen also controls photosynthetic electron transfer by slowing the rate of plastoquinol oxidation at the cytochrome b_6f complex (Hope, 2000; Takizawa et al., 2007), preventing the accumulation of highly reducing species within photosystem I (Kramer and Evans, 2011).

Parsing of the thylakoid *pmf* into ΔpH and Δψ components has been observed in thylakoids (Cruz et al., 2001) and in intact leaves (Avenson et al., 2004) and was proposed to constitute an important fine-tuning mechanism for photosynthesis (Avenson et al., 2005). "Under optimal conditions, when down-regulation is not needed, a large fraction of *pmf* can be stored as Δψ, leading to moderate lumen pH and low qE, even at high *pmf* (and thus high rates of ATP synthesis). In contrast, under environmental stresses–e.g., high light, low CO_2/O_2, when photoprotection is advantageous–*pmf* can be predominantly stored as ΔpH, maximizing lumen acidification for a given *pmf*" (Ioannidis et al., 2012).

The mechanism by which thylakoid *pmf* is partitioned *in vivo* into Δψ and ΔpH remained until recently unclear, but *in vitro* experiments and modeling have established that at least three factors are critical (Cruz et al., 2001; Avenson et al., 2005): (i) the capacitance of the thylakoid membrane, (ii) the ionic composition of the stroma and lumen, and (iii) the proton-buffering capacity of the lumen.

We proposed that Δψ/ΔpH control involves biological weak bases, such as PAs, which occur normally in chloroplasts and can act as "permeant buffers," specifically dissipating the ΔpH component and thus favoring Δψ (Ioannidis et al., 2012). Because the titer of these weak bases can be regulated by the organism (by synthesis, degradation, transport, conjugation, and covalent binding to proteins), this can constitute a key-mechanism for the adjustment of the ΔpH/Δψ ratio in the short (seconds) and long term (hours to days) conditions. In the following section, we explain the role of PAs in the chemiosmotic scheme. The classical scheme is expanded in order to accommodate the mode of action of PAs (**Figure 1B**).

According to Williams (1978), Mitchell also tried to incorporate data from amines (Ort et al., 1976) under similar experimental conditions to ours (Ioannidis et al., 2006) and broke the rules of chemiosmosis, expanding his theory. By that time it was not clear that amines could be used by nature in chemiosmosis, and were used as a tool to study phosphorylation. Even 10 years later Slater reviewed the numerous studies on the nature of the intermediate between the redox reaction and ATP synthesis concluding that the matter was still open (Slater, 1987). In light of recent data, biogenic amines (i.e., PAs) seem to play the role of an intermediate *in vivo,* this matter is currently being better understood.

We expand on chemiosmosis once again by introducing natural amines that their existence in thylakoids is well established and their molecular role is getting better understood. Moreover the intermediate is not obligatory for ATP synthesis as one may assume. Thus, ATP synthesis can occur *in vitro* without PAs. However, the intermediate (i.e., PAs) increases the efficiency of ATP synthesis and allows regulation (Ioannidis et al., 2006, 2012).

THE ROLE OF POLYAMINES IN CHEMIOSMOSIS

In the past, PAs were seen by researchers as cations. This is correct to some extent, but underestimates the importance of their free forms (uncharged bases). Can free forms that are less than 0.1%

of the total PA pool play a significant role in cell physiology? A widely known but rather overlooked chemical property of the PA pool is the dynamic equilibrium between the neutral base and its protonated forms. This simple property greatly increases the complexity of the PA mode of action because of ion trapping phenomena that appear when (i) a membrane barrier (for instance the thylakoid membrane) is present and (ii) a basic compartment become more acidic (acid jump) or more generally speaking a ΔpH difference is established across a membrane. PA trapping will be explained in detail below.

A first attempt to differentiate between the effects that are due to the cationic character of PAs and those that are due to the chemical equilibrium of the free and charged forms was done in isolated thylakoids (Ioannidis et al., 2006). Cationic (coulombic) effects can induce *in vitro* stacking of thylakoids, increase photochemical efficiency of PSII and increase LEF similarly to divalent inorganic cations. Moreover, PAs can stimulate ATP synthesis in isolated thylakoids from 15 to 70% (Ioannidis and Kotzabasis, 2007). Spermine (Spm) marginally stimulated photophosphorylation (∼30%) at a very low concentration (∼100 μM), whereas putrescine (Put) greatly stimulated phosphorylation (∼70%) at about 1000 μM (Ioannidis et al., 2006). These effects are due to the buffering role of PAs and therefore cannot be mimicked by inorganic cations. In addition, recently we verified that PAs participate in the modulation of *pmf* in thylakoids *in vivo* (Ioannidis et al., 2012). Stimulation of ATP synthesis by low molecular weight amines like imidazole, methylamine, and ammonia was formerly reported and occurs through ion trapping (Giersch and Meyer, 1984; Pick and Weiss, 1988). The term "ion trapping" is used to describe the build-up of a higher concentration of a chemical across a cell membrane due to the pK value of the chemical and difference of pH across the membrane. This results in the accumulation of basic chemicals (such as amines) in acidic compartments (such as the thylakoid lumen). In theory for a $\Delta pH = 2$, amines inside the acidic vesicle will be 100 times more than the amines outside. PAs have relatively high pK (>7.5) and are ideal molecules for trapping phenomena in basic transitions (e.g., from pH 7 down to pH 5). The ion trapping mechanism was formerly theoretically described and experimentally demonstrated in thylakoid membranes for Put (Guarino and Cohen, 1979a,b). When chloroplasts are incubated in darkness with Put, the diamine is expected to be equally distributed at both sides of the thylakoid membrane ($C_{in} = C_{out}$) (Schuldiner et al., 1972). The amine in each compartment (in lumen or in stroma) is in a complex dynamic equilibrium that is demonstrated below (see equilibria 1, 2, and 3). One should pay attention to the second type of equilibrium between the permeant uncharged amine in the lumen and that in the stroma (**Figure 2**). Upon illumination protons are released in thylakoid lumen and shift the equilibrium No1 to the right. In the lumen charged amines are produced with a parallel depletion of uncharged forms (Ioannidis et al., 2006). This disequilibrium forces a rapid influx of uncharged diamines from the stroma via equilibrium No2 (let us for a moment assume that the membrane is impermeable to charged Put).

The ΔpH value defines the extent of trapping and the internal concentration of Put (C_{in}) increases so much that the ratio C_{in}/C_{out} can be increased 500- to 3000-fold (Guarino and Cohen, 1979a,b). This gradient of Put buffers protons in the lumen, but interestingly, it does not change the total cationic charge in the lumen more than it is already increased due to proton release (Ioannidis et al., 2006). Finally, steady state ATP synthesis is stimulated. A possible cause is that the rate of proton transfer along a net of hydrogen bonds can be faster than the rate of proton transfer in water at pH 7 (Williams, 1978). Furthermore, buffering of the lumen pH by PAs keeps conditions at a more moderate pH and inhibits overacidification which in turn would hinder electron transport and photophosphorylation (Kramer et al., 2004).

The assumption that thylakoids are impermeable to charged Put is an oversimplification. It is known that Cl^- channels of the thylakoid membrane open at 30 mV (Schoenknecht et al., 1988) and the influx of Cl^- (counter-ions) is expected to neutralize amine molecules and allow its efflux in the stroma (Sigalat et al., 1988). The voltage dependency of those channels may act as a safety valve sensor that hinders Put overaccumulation in the lumen and simultaneously is able to fine tune membrane potential in values sufficient for ATP synthesis (Ioannidis et al., 2006). To summarize, the data (Ioannidis et al., 2006, 2012) reveal an unknown dynamic behavior of the Put pool (dual role). We suggest that in the dark, the lumen has a relatively low concentration of Put, and in the light the lumen has a higher concentration of Put (**Figure 3**).

POLYAMINE IMPLICATIONS IN STRESS PHYSIOLOGY WITH RESPECT TO CHEMIOSMOSIS

During the last years, plant research has focused on the role of PAs in the defense of plants against a series of environmental stress conditions (Galston, 2001); such as temperature (Hummel et al., 2004; Sfakianaki et al., 2006), salinity (Maiale et al., 2004; Demetriou et al., 2007), enhanced atmospheric ozone (Navakoudis et al., 2003) or UV-B radiation (Lütz et al., 2005; Sfichi et al., 2008). At present it is well established that many types of abiotic and biotic stress lead to an increase in the PA titer of plants and particularly of leaves. In plants, salt and osmotic stress were some of the first examples of the great increase of the PA titer (Richards and Coleman, 1952; Flores and Galston, 1984). Many reviews cover the topic (Alcázar et al., 2010a,b; Marco et al., 2011). In addition, a promising field of research is H_2O_2 production during stress via PA oxidases (for recent advances see Moschou and Roubelakis-Angelakis, 2011 and Moschou et al., 2008). However, the role of PAs during stress is not well understood.

New chemiosmosis may help to elucidate the role of PAs during stress. Below we consider only two cases (salt and osmotic stress) but the concept could be adopted with some modifications in other stress cases as well. "For example in *Arabidopsis* grown under high salt stress, photosynthesis would likely need to operate under conditions where the ionic strength inside the plastid is high. In this case, *pmf* storage would be heavily biased toward ΔpH formation (Robinson et al., 1983; Sacksteder and Kramer, 2000; Cruz et al., 2001). Consequently, energy dissipation would be more easily and strongly induced at low and moderate light intensities, severely limiting the productivity and growth of the plant, even if water and CO_2 were not limiting factors. Thus,

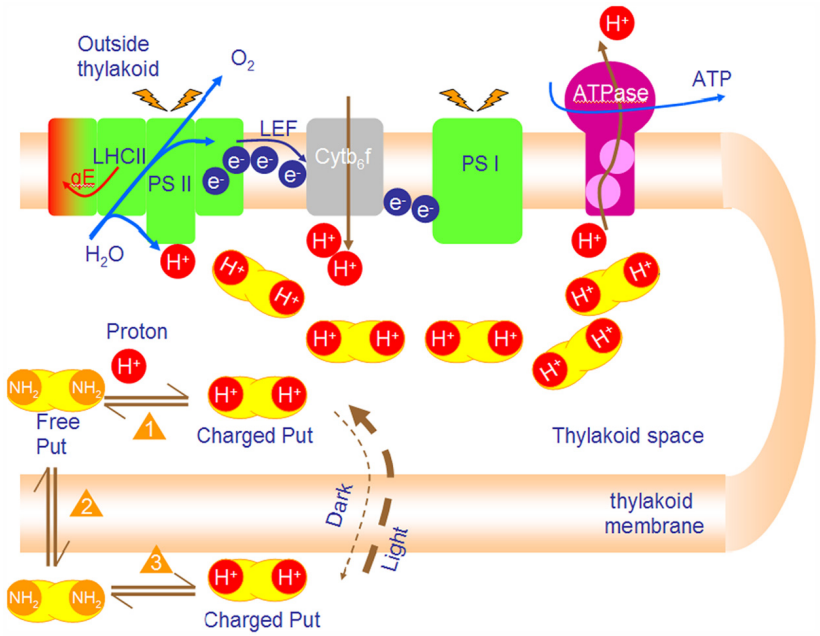

FIGURE 2 | PAs accumulate in the lumen and buffer the lumen pH during photophosphorylation. Photosynthetic reactions produce protons that are vectorially released in the lumen. Lumen acidification shifts equilibrium 1 to the right (production of charged Put in lumen). Depletion of free putrescine urges new free Put to diffuse from stroma into lumen (Le Chatelier principle equilibrium 2). Finally, free Put in stroma is replaced by charged Put in stroma which is ionized (equilibrium 3). The net result of this process (i.e., new poise of the 3 equilibria) is ion trapping. That is the accumulation of Put in lumen up to 100 times. The final concentration of Put depends mainly on ΔpH and counterion concentration (such as Cl^-).

the accumulation of Put observed in plants grown under high salt stress (Alcázar et al., 2006; Bagni et al., 2006) and particularly in *Arabidopsis* through *adc2* induction (Urano et al., 2004) could serve to increase buffering solutes, rebalancing *pmf* toward $\Delta\psi$ and optimizing the regulation of energy transduction. In line with this view, blocking this up-regulation of Put during salt stress, e.g., in the *adc-2-1* mutant of *Arabidopsis*, leads to increased sensitivity to salt stress, which is restored upon addition of Put (Kasinathan and Wingler, 2004; Urano et al., 2004), whereas over-expressing *adc* increases tolerance to drought (Capell et al., 2004)" (Ioannidis et al., 2012). Similarly, under osmotic stress the plant faces a decrease in relative water content. This decrease in water content

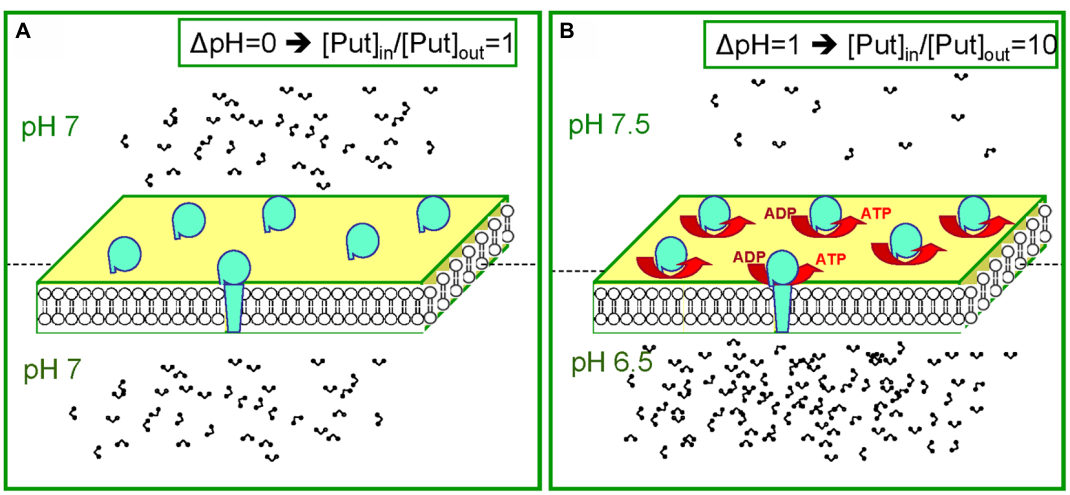

FIGURE 3 | The dual behavior of the Put pool during dark and light. (A) Equal distribution of protons leads to equal distribution of PAs (for example during the dark). **(B)** Establishment of a ΔpH between the two compartments leads to unequal distribution of PAs. Note in b that for ΔpH = 1 ten times more Put will accumulate in the acidic compartment.

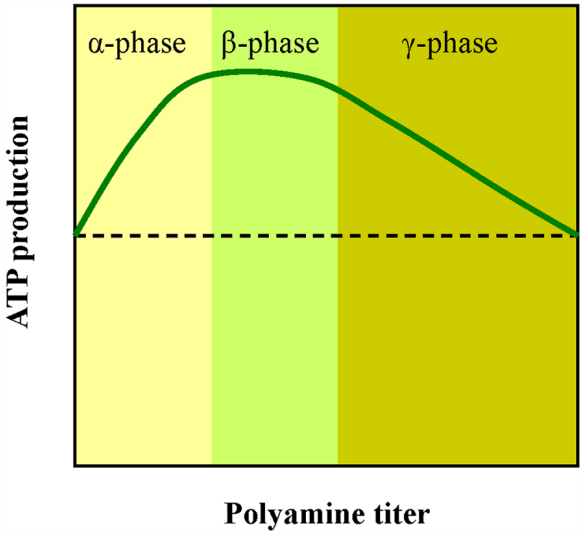

FIGURE 4 | A simplified scheme showing the effect of PAs on ATP production by thylakoids. Low doses of PAs stimulate photophosphorylation (α phase). Higher doses lead to reduction of ATP synthesis (γ phase). The x axis is qualitative because in all three PAs (Put, Spd, and Spm) the peak value corresponds to different concentration although the shape of the curve is similar.

is also evident in chloroplasts. It is well established that under conditions of water stress arginine decarboxylase (ADC) which is located in thylakoid membranes of chloroplasts (Borrell et al., 1995) is significantly upregulated, i.e., 2–60-fold increase (Flores and Galston, 1982). For recent works of ADC up-regulation upon stress see Alcázar et al. (2010a,b). This increase of Put titer is part of the protective response of the plant to osmotic stress. Artificial increase of Put titer in leaf disk 1 h before the stress significantly protects the photosynthetic apparatus (Kotakis et al., 2014). In all cases data from leaf discs should be examined with caution. In addition, the role of other organelles such as the vacuole that contain most of the water reserves in the plant cell could be important. Hence, the titer of PAs in each compartment of the cell (e.g., chloroplasts, mitochondria, vacuole, nucleus) should be estimated both under physiological conditions and under stress. In this capacity, new protocols and methods should be used solving problems that derive from the properties of PAs (i.e., high pKs and rapid penetration of membrane barriers). PAs will accumulate *in vivo* in every cellular compartment/organelle that is more acidic than the surrounding microenviroment (the driving force is their high pK as explained before in the ion trapping) and will be depleted rapidly (within seconds) upon grinding of the tissue.

POLYAMINE IMPLICATIONS IN GROWTH

PAs are described in early papers as growth factors however, it is not clear why the increase of PAs stimulates growth and cell division, while inhibition of PA synthesis hinders division and growth. We suggest that these processes require a lot of ATP and the cell must boost the chemiosmotic machinery. Small changes of available Put result in different energy output under the same light energy input (phases α and γ in **Figure 4**). The normal Put concentration inside cells is in the fractional millimolar range, so we suggest that a small increase or decrease of available Put can fine tune ATP production. *In vivo*, lower Put content characterizes the aged cells (Paschalidis and Roubelakis-Angelakis, 2005). "This is nicely correlated with the low metabolic rates that are common during senescence. Optimal ATP rates are about 1 mM Put that might be near the value of endogenous Put of actively growing cells (β phase). In the γ phase the rates of ATP synthesis gradually decline. This curve might explain why the same amount of exogenously supplied Put can give opposite effects" (Ioannidis et al., 2006). This is a puzzling phenomenon reported often by PA researchers.

CONSERVED FEATURES OF THE NEW CHEMIOSMOSIS AND FUTURE PERSPECTIVES

New ("polyaminylated") chemiosmosis as presented and explained in this contribution has at least four conserved features: (i) a coupling membrane, (ii) a proton-driven ATPase, (iii) a ΔpH, and iv) a pool of free PAs (e.g., Put). These four features are parts of the *pmf* machinery in microbes, animals, and plants. Chloroplast bioenergetics was the first field to investigate this concept and we urge colleagues from other fields to check whether such phenomena occur in their systems. Former studies in mitochondria have shown that PAs can play a stimulatory role (Toninello et al., 1984; Schuber, 1989). From a biochemical point of view there is no reason for inhibition of ion trapping phenomena in mitochondria. Chloroplast bioenergetics due to recent advances can test *in vivo* such hypotheses. If PAs act similarly in other non-photosynthetic systems as permeable buffers in chemiosmotic units then many up-to-date enigmatic processes may be explained. For example the molecular role of PAs in cancer cells is not well understood although their implication in the emergence of tumors is well documented (Jänne et al., 1978). Recently, the importance of mitochondrial $\Delta\psi$/pmf in cancer emergence and cancer cures was discovered (Dromparis and Michelakis, 2013).We suggest that a scientific question worth testing is whether $\Delta\psi$ in human cancer cells is regulated by PAs. If PAs modulate $\Delta\psi$ in thylakoids and in cancer cell mitochondria in a similar manner (i.e., through *pmf* modulation) then a longstanding question may finally be answered.

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