RESEARCH PAPER

Thermospermine catabolism increases Arabidopsis thaliana resistance to Pseudomonas viridiflava

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Abstract

This work investigated the roles of the tetraamine thermospermine (TSpm) by analysing its contribution to Arabidopsis basal defence against the biotrophic bacterium Pseudomonas viridiflava. The participation of polyamine oxidases (PAOs) in TSpm homeostasis and TSpm-mediated defence was also investigated. Exogenous supply of TSpm, as well as ectopic expression of the TSpm biosynthetic gene ACL5, increased Arabidopsis Col-0 resistance to P. viridiflava, while null acl5 mutants were less resistant than Col-0 plants. The above-mentioned increase in resistance was blocked by the PAO inhibitor SL-11061, thus demonstrating the participation of TSpm oxidation. Analysis of PAO genes expression in transgenic 35S::ACL5 and Col-0 plants supplied with TSpm suggests that PAO 1, 3, and 5 are the main PAOs involved in TSpm catabolism. In summary, TSpm exhibited the potential to perform defensive functions previously reported for its structural isomer Spm, and the relevance of these findings is discussed in the context of ACL5 expression and TSpm concentration in planta. Moreover, this work demonstrates that manipulation of TSpm metabolism modifies plant resistance to pathogens.

Key words: ACAULIS5, Arabidopsis thaliana, defence, polyamines, polyamine oxidase, Pseudomonas viridiflava, thermospermine.

Introduction

Polyamines (PAs) are natural aliphatic polycations ubiquitous in prokaryotic and eukaryotic cells and are essential for cell growth, proliferation, and differentiation (Cohen, 1998). Although the mechanism of PA action is not completely clear, they are known to modulate DNA–protein (Shah et al., 1999) and protein–protein interactions (Thomas et al., 1999), and RNA structure (Igarashi and Kashiwagi, 2000). Spermidine (Spd) and spermine (Spm) are well-studied PAs, together with the diamine putrescine (Put) (Cohen, 1998). In Arabidopsis thaliana, Spd is synthesized by Spd synthase (SPDS), an enzyme encoded by two genes, SPDS1 and SPDS2 (Hanzawa et al., 2002). Initially, it was reported that Spm synthase (SPMS), the enzyme involved in Spm biosynthesis, was also encoded by two genes, SPMS (Panicot et al., 2002) and ACL5 (Hanzawa et al., 2000). However, it was later demonstrated that ACL5 actually displays thermospermine (TSpm) synthase activity (Knott et al., 2007; Kakahi et al., 2008). The tetraamine TSpm synthesized by ACL5 is a structural isomer of Spm, first identified from the thermophilic bacterium Thermus thermophilus (Oshima, 1979). Recently, it was shown that TSpm is likely present throughout the whole plant kingdom, while Spm is found only in angiosperms (Minguet et al., 2008). In Arabidopsis, ACL5 is specifically expressed in xylem vessel elements and TSpm plays an active role in regulating vascular development (Muñiz et al., 2008) by preventing premature death of these elements. This safeguard action of
TSpm impacts on xylem cell morphology, cell wall patterning, and cell death, as well as overall plant growth (Vera Sirera et al., 2010). Thus, Arabidopsis loss-of-function acl5 mutants show a severe dwarf phenotype (Hanzawa et al., 2000). On the contrary, spnus mutants exhibit a normal phenotype (Imai et al., 2004). In spite of the information about the role of TSpm in the above-mentioned processes, the participation of this tetraamine in other physiological processes has not been investigated in depth so far. Thus, the possibility that, because of their structural similarity, TSpm and Spm play redundant roles and the mechanisms by which TSpm levels are regulated are worthy of being explored.

Several reports demonstrated the relevance of Spm in plant defence against pathogens (Yamakawa et al., 1998; Takahashi et al., 2003, 2004a,b; Urano et al., 2003; Uehara et al., 2005; Mitsuya et al., 2007, 2009). In this way, Spm was reported to induce pathogenesis-related gene expression in tobacco plants (Yamakawa et al., 1998) and to protect Arabidopsis against cucumber mosaic virus (CMV) by inducing the expression of a number of genes in common to CMV infection (Mitsuya et al., 2009). Apoplastic accumulation and further oxidation of Spm was shown to protect tobacco plants against Pseudomonas viridiflava (Marina et al., 2008) and it was recently demonstrated that genetic modifications of SPM5 expression strongly affect Arabidopsis resistance to this bacterium (Gonzalez et al., 2011). These and other works have shown that the protective effect of Spm against pathogens depends, to a large extent, on the activity of amine oxidases (Angelini et al., 1993; Cona et al., 2006). Moreover, oxidation of other PAs such as Spd and Put also contributes to tobacco and Arabidopsis resistance to Pseudomonas cichorii and Pseudomonas syringae, respectively (Yoda et al., 2009). Therefore, it could be speculated that PA catabolism itself is an important component of defence responses, regardless of the PA being oxidized by the plant catabolic machinery. In this regard, the role of TSpm oxidation in plant defence against pathogens has not been investigated so far. Exogenously supplied TSpm reduced cauliflower mosaic virus multiplication in A. thaliana and induced defence gene expression (Sagor et al., 2012), but it is still unknown whether this effect depends on TSpm oxidation. Oxidative catabolism of PAs is mediated by diamine and polyamine oxidases (DAOs and PAOs, respectively) that play an important role in PA homeostasis (Tiburcio et al., 1997). Arabidopsis has two putative DAO (ATAO1 and DA02) and five PAO (PAO1–5) genes. The action of DAO on Put yields pyrroline, hydrogen peroxide, and ammonia, while PAO action on Spd and Spm yields pyrroline and 1-(3-aminopropyl)pyrrolinium, respectively, as well as 1,3-diaminopropane and hydrogen peroxide (Cohen, 1998). PAO-mediated oxidation of TSpm has not been studied in detail, but is expected to yield the same products as Spm oxidation, in addition to other products such as Put.

On the basis of the information summarized above, it is evident that, notwithstanding the widespread distribution of TSpm in the plant kingdom, current knowledge about its biological functions is still limited. Therefore, this work aimed to understand the functional specificity of TSpms. For this purpose, A. thaliana acl5-5 and acl5-1 mutants previously characterized by Muñiz et al. (2008) and Imai et al. (2006), respectively, and transgenic ACL5 overexpressors were used. The ability of TSpm to increase A. thaliana resistance to P. viridiflava, as well as the consequences of TSpm depletion, were evaluated. The mechanism of TSpm oxidation in planta by PAOs and DAOs, and its contribution to pathogen defence was also evaluated. As far as is known, this is the first report about TSpm catabolism in planta and its relevance in defence responses.

Materials and methods

Plant material, transformation, and growth conditions

Arabidopsis thaliana L. ecotype Columbia (Col-0) was used as the wild type (WT). Two Arabidopsis ACL5 mutants were used. One of them, the null mutant acl5-5 (SALK_028736), harbours a T-DNA insertion in exon 6 of ACL5 and was obtained from a collection of mutants generated in a Col-0 background by Alonso et al. (2003) in the Salk Institute (San Diego, CA, USA) and was previously studied by Muñiz et al. (2008). The other, acl5-1, has a punctual mutation in exon 4 and was generated by Hanzawa et al. (2000) and studied by Imai et al. (2006). This mutant line exhibits a dwarf phenotype but less marked than acl5 mutants generated in a Landsberg erecta background (Hanzawa et al., 2000). Another Arabidopsis null mutant (pa01-1) obtained from the Salk Institute collection (SALK_013026), which harbours a T-DNA insertion in intron 6 of PAO1 and shows undetectable levels of PAO1 expression (Supplementary Fig. S1, available at JXB online) was also used.

Transgenic A. thaliana lines that ectopically expressed ACL5 were obtained as follows. A. thaliana ACL5 cDNA (GenBank AF184094) was excised from pGEX by digestion with EcoRI and SalI and then subcloned into pGEM-T Easy vector (Invitrogen). The pGEM::ACL5 vector thus obtained was digested with EcoRI and SacI and the excised fragment was subcloned into the pGPT-VKan binary vector downstream of the CaMV 35S promoter. The construction pGPTV Kan-35S::ACL5 was used to transform Agrobacterium tumefaciens GV3101. A. thaliana Col-0 plants were transformed by the floral dip method according to Clough and Bent (1998). Seeds of infiltrated plants were grown in MS agar (Murashige and Skoog, 1962) supplied with 50 μg ml−1 kanamycin. Homozygous lines were identified in plants grown from F2 seeds according to standard methodologies.

Bacterial strain, plant inoculation, and disease analysis

P. viridiflava (Burkholder) Dowson strain Pvh8 (Alippi et al., 2003) was cultivated at 28 °C in King's B medium (King et al., 1954). Xanthomonas campestris pv. campestris was cultivated at 28 °C in medium containing (w/v) 1% dextrose, 0.5% peptone, 0.3% yeast extract, and 0.3% malt extract. For plant inoculation, bacterial cells were harvested by centrifugation, washed and resuspended in 10 mM MgCl2, pH 7.0 to a final concentration of 5 × 10⁸ CFU ml⁻¹.

Leaves of 12-day-old plants were inoculated with 5 μl bacterial suspension (a single aliquot per leaf). Control plants were treated with 5 μl of 10 mM MgCl2 (pH 7.0). Plants were incubated in the growth chamber and sampled at different times after inoculation.
Prior to evaluating in planta bacterial growth, inoculated plants were surface washed several times with MgCl₂. Whole plants were then homogenized in 10 mM MgCl₂ and serial dilutions of the extracts thus obtained were plated on the above-mentioned media. The number of CFU was determined after 24 h (P. viridiflava) or 48 h (X. campestris) incubation at 28 °C. Disease severity was evaluated by estimating the percentage of diseased tissue in the inoculated leaves that showed symptoms. The percentage of diseased tissue was quantified after image acquisition with a SMZ binocular microscope (Nikon Instruments, Melville, NY, USA) coupled to a digital camera and further analysis with Image-Pro Plus version 4.1 (Media Cybernetics, MD, USA). On the basis of the percentage of the total leaf surface that exhibited symptoms, a severity rank between 1 and 5 was assigned to each diseased plant, as follows: 1, less than 10%; 2, 11–25%; 3, 26–50%; 4, 51–75%; 5, 76% to completely necrotic leaf and lesions spreading to the rest of the plant.

Chemicals and pharmacological treatments

Standard chemicals of the highest purity available were purchased from Sigma Chemical (St. Louis, MO), unless otherwise stated. TSpm was obtained from Chemiclones (Canada). The PAO inhibitor 1,19-bis(ethylamino)-5,10,15-triazanonadecane (SL-11061) was kindly gifted by Dr Benjamin Friedman (SLIL Biomedical, Madison, WI, USA). The DAO inhibitor N,N’-Diaminoguanidine monohydrochloride was purchased from ICN Biomedicals (Irvine, CA, USA). Inhibitors and polyamines were dissolved in distilled water and stored at −20 °C until use and were added to MS medium (final concentration 5 or 50 μM).

Polyamine analysis

The starting material for the extraction of polyamines was 100 mg of 12-day-old plants. Derivatization and quantitation of TSpm, Put, Spd, and Spm by GC-MS were performed as described by Rambla et al. (2010).

RNA extraction, cDNA synthesis, and quantitative reverse-transcription PCR

Total RNA was extracted from frozen tissue of 12-day-old plants using the E.Z.M.A Plant RNA Mini Kit (Omega Bio-tek). cDNA was synthesized with Superscript III reverse transcriptase (Invitrogen). cDNA template (1 μl) was used to perform the reactions with each pair of specific primers and FastStart Universal SYBR Green Master (Roche, Mannheim, Germany) to amplify the target genes. PCR reactions were performed using an Eppendorf Mastercycler Nexus SSF thermocycler. Amplification conditions were as follows: 1 cycle at 95 °C for 3 min, followed by 40 cycles at 95 °C for 30 s and 60 °C for 1 min. All reactions were checked for their dissociation curves. Relative expression levels were calculated using the ΔΔCT method (Applied Biosystems). Primer sequences are shown in Supplementary Table S1.

Statistics

Each experiment was independently conducted at least two times with similar results. Results from representative experiments are shown as means ± SD. Within each experiment, treatments consisted of 3–5 replicates, each of them consisting on pools of two plants for the analysis of bacterial growth in planta and pools of 10 plants for polyamine determination and quantitative reverse-transcription (qRT) PCR. Data were analysed by Student’s t-test or ANOVA followed by post-hoc comparisons by Tukey or Dunnet’s test. qRT-PCR results were analysed with REST version 2.0.7 (Pfaffl et al., 2002). Frequency distribution of disease severity data was analysed with GraphPad Prism.

Results

TSpm accumulation reduces P. viridiflava propagation in A. thaliana plants

As a first step to investigate the role of TSpm in A. thaliana defence, WT plants were grown in the presence of 5 μM TSpm prior to inoculation with P. viridiflava. This TSpm concentration was selected because it increased plant TSpm levels (Fig. 1A) without affecting growth. For comparative purposes, a group of plants was supplied with a similar concentration of Spm. Twelve days later, PA levels and resistance to P. viridiflava were evaluated. Exogenous supply of TSpm increased 4.9-fold the levels of this tetraamine in planta, while other PAs remained unaffected (Fig. 1A). In turn, plants supplemented with Spm exhibited no changes in the levels of any PA (Fig. 1A).

Propagation of P. viridiflava in TSpm-amended plants did not differ from WT plants with no amendments 24 hours after inoculation (HAI). However, bacterial propagation was reduced by TSpm supplementation at both 48 and 72 HAI, similarly to Spm-amended plants (Fig. 1B). In vitro bacterial growth was not affected by TSpm concentrations ranging from 2–50 μM (Supplementary Fig. S2), thus suggesting that increased resistance to P. viridiflava in plants supplied with TSpm is not due to toxic effects of this tetraamine towards the pathogen.

Perturbations of ACL5 expression affect A. thaliana resistance to P. viridiflava

In order to evaluate if endogenously produced TSpm is as effective as supplied TSpm in enhancing A. thaliana resistance to P. viridiflava, WT Col-0 plants were transformed with ACL5 under the control of the cauliflower mosaic virus (CaMV) 35S constitutive promoter. As a result, 10 lines that overexpressed ACL5 were obtained. Lines 35S::ACL5 10E and 20G, which showed ACL5 transcript levels 35- and 43-fold higher than WT plants (Fig. 2A), were used for further experiments. These lines exhibited a normal phenotype, with no obvious morphological or phenological alterations.

In spite of overexpressing ACL5, transgenic 35S::ACL5 plants contained TSpm levels similar to WT plants. Moreover, Put, Spd, and Spm levels were also similar in 35S::ACL5 and WT plants (data not shown). The similar TSpm levels in 35S::ACL5 and WT plants could indicate thatACL5 overexpression fails to increase TSpm biosynthesis in planta. Alternatively, the lack of TSpm accumulation in the 35S::ACL5 lines could be due to enhanced PA catabolism. This possibility is supported by the enhancement of transcript levels of some of the amine oxidases involved in PA catabolism in the 35S::ACL5 lines as compared to WT plants (Fig. 2B). In this sense, PAO1 mRNA levels in 35S::ACL5 lines 10E and 20G were, respectively, 3- and 9-fold higher than WT plants (Fig. 2B). In addition, the 35S::ACL5 20G line exhibited PAO3 mRNA levels 5-fold higher than WT plants, while the 35S::ACL5 10E line contained higher levels of PAO5 mRNA than WT plants (Fig. 2B). It should also be noted that PAO-mediated oxidation of TSpm produces...
Put, and enhanced TSpm oxidation could therefore result in Put accumulation. Even though Put was not altered in the 35S::ACL5 lines, these plants exhibited a 4–5-fold increase in the expression of ATAO1, a gene coding for an extracellular Cu²⁺-amine oxidase (Fig. 2C) that oxidizes Put (Møller and McPherson, 1998), but no changes in DAO2 were evident (Fig. 2C). Therefore, the most straightforward interpretation is that ACL5 overexpression enhances TSpm synthesis and catabolism, with a net result of TSpm levels similar to WT plants. Other points should also be considered regarding the difference in TSpm levels between the 35S::ACL5 lines and the WT plants amended with exogenous TSpm. TSpm derived from ACL5 overexpression is produced in the cytoplasm, while exogenously supplied TSpm enters the plant through the roots and gets to the leaf through the vascular system. Thus, taking into account that the analysis of PA levels is performed on extracts that contain PAs from different subcellular compartments and the apoplastic space as well, the method does not provide information about the subcellular location of TSpm. Therefore, the higher TSpm content in extracts obtained from exogenously supplied plants, as compared to ACL5 overexpressors, could be due, for instance, to the accumulation of this tetraamine in the apoplast or other subcellular compartment. Moreover, taking into account that PAOs involved in PA catabolism have several different subcellular locations and affinities for TSpm, multiple factors could be responsible for the difference in TSpm levels detected between exogenously supplied plants and ACL5 overexpressors.

Previously it has been demonstrated that increased resistance of Arabidopsis to P. viridiflava conferred by SPMS overexpression and Spm accumulation is due in part to Spm oxidation by amine oxidases (Gonzalez et al., 2011). Thus, if the lack of TSpm accumulation exhibited in this work by ACL5 overexpressors is due to enhanced TSpm oxidation by PAOs, these lines could still exhibit increased resistance to P. viridiflava. Therefore, resistance to P. viridiflava was evaluated in 35S::ACL5 lines 10E and 20G, which showed higher resistance than WT plants, as demonstrated by the 56–62% and 52–72% reduction of bacterial growth in planta detected 48 and 72 HAI, respectively (Fig. 3A). Moreover, these transgenic lines also showed increased resistance to the bacterial pathogen X. campestris pv. campestris (Supplementary Fig. S3).

Interestingly, and providing additional support to the view that ACL5 expression is related to P. viridiflava resistance, bacterial propagation in an acl5 mutant (acl5-5), which was unable to accumulate TSpm, was higher than in the WT...
Thermospermine catabolism in plant defence | 1397

Moreover, exogenous addition of TSpm raised bacterial resistance of acl5-5 to a level similar to WT plants (Fig. 3C). Another acl5 mutant (acl5-1) also exhibited higher P. viridiflava titres than WT plants (Supplementary Fig. S4), thus confirming that the lack of ACL5 expression reduces Arabidopsis resistance to P. viridiflava. These results, in conjunction with a
previous report about the xylem vessel-specific expression of ACL5 (Muñiz et al., 2008), suggest that the localized pattern of expression of this gene contributes to Arabidopsis defence against P. viridiflava.

Other indication that the 35S::ACL5 lines are more resistant to P. viridiflava than WT plants was obtained by inspection of disease symptoms in infected plants. Symptoms consisted on water-soaked translucent spots, which developed into chlorotic and subsequently necrotic lesions, as depicted by Jakob et al. (2002). Disease severity was higher in WT plants than in both transgenic lines 120 HAI. Thus, the WT was the only line for which plants showed the highest severity index 4 or 5 and only two of 12 plants ranked at intermediate index 3. On the contrary, 35S::ACL5 10E and 20G plants mainly fell within low disease severity ranks 1 and 2 and only one (line 10E) or two (line 20G) of eight plants ranked at intermediate index 3 (Fig. 3B). The statistical significance in frequency distributions between different lines was analysed and the transgenic 35S::ACL5 lines were different (P ≤ 0.05) from the WT plants. No differences in frequency distribution between the 10E and 20G transgenic lines were detected at this time after inoculation.

Both PAO-mediated oxidation of thermospermine and DAO-mediated oxidation of putrescine derived from thermospermine degradation enhance Arabidopsis resistance to P. viridiflava

The results presented thus far have demonstrated that TSpm accumulation increases resistance of WT A. thaliana to P. viridiflava. Moreover, resistance was also increased by ACL5 overexpression in transgenic 35S::ACL5 plants that showed no accumulation of TSpm. Increased expression of several PAO genes (Fig. 2B) suggested that the lack of TSpm accumulation in transgenic lines was due to enhanced TSpm oxidation. Moreover, the expression of PAO1, 3, and 5 was increased by the addition of exogenous TSpm to WT plants, similarly to exogenous Spm (Fig. 4). In addition, TSpm levels in the pao1-1 mutant were higher than in WT plants, while other PAs were unaffected (Supplementary Fig. S5), which provides evidence that PAO1 oxidizes TSpm in vivo. Thus, several lines of evidence suggest that the increase in resistance of the 35S::ACL5 lines could be due to enhanced TSpm oxidation.

Thus, the PAO inhibitor SL-11061 (Maiale et al., 2008; Marina et al., 2008) was used to evaluate the role of TSpm oxidation mediated by PAOs in Arabidopsis resistance to P. viridiflava. First, the effect of PAO inhibition on resistance of WT plants grown in the presence of TSpm was evaluated. As described already, TSpm addition to WT plants reduced bacterial growth 48 HAI, as compared to control plants without amendments (Figs. 1B and 5A). On the contrary, when TSpm was added in conjunction with SL-11061, bacterial propagation reached values similar to control plants (Fig. 5A). This result indicates that the increased resistance to P. viridiflava provided by exogenous TSpm depends on the oxidation of this tetraamine by PAOs. In the absence of exogenously supplied TSpm, SL-11061 addition exerted no effect on bacterial growth (Fig. 5A). This treatment has been previously demonstrated to decrease Arabidopsis resistance to P. viridiflava mediated by the oxidation of endogenous Spm (Gonzalez et al., 2011), but it should be noted that this effect becomes evident only at longer times after inoculation (72 HAI). Interestingly, 50 μM SL-11061 also prevented the increase in resistance associated with ACL5 overexpression in 35S::ACL5 10E (Fig. 5B), thus confirming the role of TSpm oxidation in disease resistance.

TSpm oxidation by PAOs produces Put (Takahashi et al., 2010), which in turn can be oxidized by DAOs. Therefore, DAO-mediated oxidation of Put derived from TSpm catabolism could also contribute to boost A. thaliana resistance to P. viridiflava. The role of DAO in resistance to P. viridiflava conferred by TSpm accumulation was confirmed by the fact that the DAO inhibitor N,N'-diaminoguanidine prevented the protective effect of TSpm against bacterial propagation in WT plants (Fig. 5A).

Discussion

In addition to the extensively studied PAs Put, Spd, and Spm, other PAs are also present in plants and microorganisms (Ober et al., 2003; Oshima et al., 2007). The model plant A. thaliana contains the tetraamine TSpm, a structural isomer of Spm that shows a widespread distribution in the plant kingdom. TSpm was demonstrated to regulate vascular development (Vera Sirera et al., 2010; Takano et al., 2012) and the TSpm synthase gene (ACL5) is mainly localized in xylem vessel elements (Muñiz et al., 2008). Thus, TSpm plays a specific developmental role in Arabidopsis, but the possibility that this tetraamine plays other functions, such as those of Spm, was not explored so far. Therefore, the present work investigated the role of TSpm in plant defence against pathogens. Reduction of P. viridiflava propagation in plants supplied with TSpm demonstrated that this tetraamine can contribute to Arabidopsis basal resistance to this bacterium. This hypothesis is also supported by the increase of resistance to P. viridiflava exhibited by transgenic plants that ectopically expressed ACL5. Moreover, it is interesting noting that ectopic expression of ACL5 also increased resistance to another bacterial pathogen such as X. campesstris pv. campesstris, thus demonstrating that the defensive role of TSpm is not restricted to P. viridiflava. It should be noted that gene expression controlled by the CaMV 35S promoter leads to increased mRNA levels of the ectopically expressed gene throughout the whole plant. On the contrary, under the control of its own promoter, ACL5 shows variable levels of expression in different organs and tissues, being highly expressed in xylem vessel elements (Muñiz et al., 2008). Thus, the results hereby presented demonstrate that genetic modification of the normal pattern of ACL5 expression can increase A. thaliana resistance to pathogenic bacteria. This is probably related to the
ability of plant PAOs to oxidize TSpm in a wide range of tissues and organs, as occurs when other PAs such as Spm are accumulated throughout the plant.

It should also be noted that two acl5 mutants evaluated in this work (acl5-1 and acl5-5) exhibited a decrease in resistance to *P. viridiflava*, as compared to WT plants. Taking into account the high expression of *ACL5* in vascular tissue of WT plants, it would be interesting to elucidate how this tissue-specific pattern of expression contributes to resistance. A possible explanation is that bacterial growth in vascular tissues of WT plants is reduced by PAO-mediated oxidation of the TSpm synthesized therein by the *ACL5* gene product, thus reducing systemic bacterial spreading. However, the decrease in resistance of acl5 mutants could also be due to the structural and anatomical alterations exhibited by these plants, which show a dwarf phenotype and a number of modifications in their vascular system and secondary growth, mainly in hypocotyls (Muñiz *et al*., 2008).

1,19-bis(ethylamino)-5,10,15-triazanodecane (SL-11061). Plants grown in MS without amendments were used as controls, and plants grown in the presence of inhibitors but without exogenously supplied TSpm served to analyse the contribution of the oxidation of endogenous PAs to resistance. (B) The effect of PAO inhibition on *P. viridiflava* propagation in transgenic 35S::ACL5 10E was evaluated in plants grown for 12 d in MS or in MS amended with 5 or 50 μM SL-11061. Plants were inoculated with a bacterial suspension and the number of CFU per plant was evaluated at 48 HAI. Results are mean ± SD of five (A) and three (B) replicates. Different letters indicate statistically significant differences in bacterial titres according to one-way ANOVA and Tukey’s test (*P* ≤ 0.05 in A; *P* ≤ 0.01 in B); in B, comparisons are only valid within a given line.
In this regard, it is worth remembering that exogenous TSpm raised acl5-5 resistance to bacterial infection to a level similar to WT plants. It has been reported that exogenous TSpm partly reverts the anatomical alterations of acl5 mutants. Thus, the exogenous TSpm-mediated increase in resistance of acl5-5 plants to bacterial infection detected in the current experiments confirms that the decreased resistance of this mutant is actually due to the lack of TSpm, but it remains to be established whether the anatomical changes of acl5-5 plants or the lack of TSpm used as a substrate by PAOs in specific organs and tissues is the main cause of the decreased resistance exhibited by acl5-5. Regardless of the mechanism involved in TSpm-mediated protection against P. viridiflava, the decrease in resistance exhibited by acl5-5 indicates that normal ACL5 expression is required by the defence machinery of Arabidopsis against this bacterium.

In the present work, ectopic expression of ACL5 did not increase TSpm levels, despite ACL5 expression was considerably higher than in WT plants. This finding prompted an analysis of how ACL5 overexpression and TSpm accumulation affect the expression of genes involved in PA catabolism. The enhanced expression of some of the five PAOs and ATAO1 exhibited by 35S::ACL5 plants suggests that the lack of TSpm accumulation in these lines is due to increased PA oxidation, PAO1, 3, and 5 probably being involved in maintaining TSpm levels. The view that PAO1, 3, and 5 contribute to TSpm homeostasis in Arabidopsis is also supported by the increase in transcript levels of these three PAO isoforms detected in WT plants supplied with TSpm. However, this conclusion is based only on the analysis of transcriptional changes of PAO isoforms. Therefore, it cannot be ruled out that translational or post-translational mechanisms regulate the expression of PAO isoforms in response to modifications of TSpm levels.

Kamada-Nobusada et al. (2008) demonstrated that AtPAO2, 3, and 4 are located in peroxisomes, while AtPAO1 and AtPAO5 were proposed to be cytoplasmic. Therefore, it is interesting noting that, in the present work, the expression of both cytoplasmic (PAO1 and 5) and peroxisomal (PAO3) AtPAOs were induced by increased TSpm levels or ACL5 expression. No information about the subcellular location of TSpm is available at the present, a subject that deserves further research to understand why both peroxisomal and cytoplasmic PAOs catabolize TSpm. In this regard, it should also be noted that recombinant ArPAO1, 2, 3, and 4 are all able to use TSpm as a substrate. Moreover, Fincato et al. (2011) found that ArPAO1 oxidizes TSpm with a \( k_{\text{cat}} \) value and a \( k_{\text{cat}}/K_{\text{m}} \) ratio 2- and 10-fold, respectively, higher than those for Spm, which suggests that TSpm is a better substrate than Spm for ArPAO1 and could thus be a physiological substrate of this enzyme in vivo. This view is consistent with the increased expression of PAO1 detected in the present work in WT A. thaliana plants supplied with TSpm and in the 35S::ACL5 lines. Additional support to this view is provided by the accumulation of TSpm and no other PA in the pao1 mutant. As a whole, the analysis of the expression of genes involved in PA catabolism not only contributed to understand the reasons why ectopic expression of ACL5 did not enhance TSpm levels, but also provided information about the mechanisms of TSpm oxidation in planta, an issue not addressed so far.

The participation of amine oxidases in PA-mediated defence against pathogens has been demonstrated in different pathosystems (Rea et al., 2002; Cona et al., 2006; Yoda et al., 2009). Yoda et al. (2003) demonstrated that tobacco infection by the tobacco mosaic virus augments PAO activity, which in turn enhances apoplastic H2O2 levels through PA oxidation. Similarly, PA oxidation contributes to the hypersensitive response of tobacco against tobacco mosaic virus (Marini et al., 2001). In addition, tobacco infection by P. syringae pv. tabaci provokes the accumulation of Spm and H2O2 in the leaf apoplast (Moschou et al., 2009). Previous reports by the current study group have also given evidence of the participation of PAOs in plant defence against pathogens (Marina et al., 2008). In particular, the enhancement of Arabidopsis resistance to P. viridiflava mediated by Spm has been demonstrated to depend, at least in part, on PAO activity (Gonzalez et al., 2011). In the present work, the use of the PAO inhibitor SL-11061 demonstrated that PAO activity is crucial for increased resistance against P. viridiflava exhibited by A. thaliana 35S::ACL5 lines, as well as by WT plants supplied with exogenous TSpm. Therefore, dependence on PAO activity is an additional feature shared by TSpm- and Spm-mediated protection against P. viridiflava in Arabidopsis. This suggests that increased resistance provided by PA oxidation does not depend on the PA being oxidized by PAOs, at least when two closely related tetramines such TSpm and Spm are considered.

Finally, it is worth mentioning that genetically modified A. thaliana plants that ectopically express SPMS constitutively express a variety of defence-related genes in absence of pathogens, which could contribute to the increased resistance to P. viridiflava exhibited by these plants (Gonzalez et al., 2011). The number of similarities found in the present work between 35S::ACL5 and 35S::SPMS plants in terms of resistance to P. viridiflava, as well as the decrease in resistance exhibited by acl5-5 mutants, raise the possibility that TSpm levels modulate the expression of defence-related genes. In this regard, a preliminary search for genes differentially expressed in 35S::ACL5 plants revealed that RPS2, which encodes a plasma membrane protein that confers resistance to P. syringae, is overexpressed in these plants as compared to WT plants (data not shown). Moreover, TSpm-mediated modulation of gene expression was recently analysed by Sagor et al. (2012). Interestingly, these authors demonstrated that TSpm can induce the expression of a number of genes also induced by Spm and participates in A. thaliana defence against CMV (Mitsuya et al., 2009). As a whole, the results obtained in the present work and those by others demonstrate that TSpm and TSpm oxidation contribute to Arabidopsis resistance to P. viridiflava.

Supplementary material

Supplementary data are available at JXB online.

Supplementary Table S1. Primers used in quantitative real-time PCR.
Supplementary Fig. S1. Isolation and characterization of the pao1-1 T-DNA insertional mutant.

Supplementary Fig. S2. Effect of thermospermine and spermine on growth of *P. viridiflava* in vitro.

Supplementary Fig. S3. Perturbation of *ACL5* expression affects *A. thaliana* resistance to *X. campestris pv. campestris*. 

Supplementary Fig. S4. Perturbation of *ACL5* expression affects *A. thaliana* resistance to *P. viridiflava*.

Supplementary Fig. S5. Polyamine concentration in the *A. thaliana* pao1-1 mutant.

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