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; Kakehi *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, *spms* 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 *SPMS* 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 *DAO2*) 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)pyrrollinium, 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 TSpm. 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 (*pao1-1*) 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 *Eco*RI and *Sal*I and then subcloned into pGEM-T Easy vector (*Invitrogen*). The pGEM::*ACL5* vector thus obtained was digested with *Eco*RI and *Sac*I and the excised fragment was subcloned into the pGPT-VKan binary vector downstream of the CaMV 35S promoter. The construction pGPTVKan-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.

Seeds of all the plant materials were disinfected with 75% (v/v) ethanol and 0.05% (v/v) Triton X-100 for 3 min and 95% (v/v) ethanol for 1 min. Disinfected seeds were dispensed in Petri dishes containing MS agar and incubated in a plant growth chamber. Plants were grown for 12–14 days under a 16/8 h light/dark cycle at 24/21 ± 2 °C and 55/75 ± 5% relative humidity and a photon flux density of 200 µmol m⁻² s⁻¹ provided by cool-white fluorescent and incandescent lamps.

Bacterial strain, plant inoculation, and disease analysis

P. viridiflava (Burkholder) Dowson strain Pvalb8 (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 5×10^8 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 MgCl$_2$. Whole plants were then homogenized in 10 mM MgCl$_2$ 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 Frydman (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 with ROX (Roche, USA). The reference gene *UBQ10* (At4g05320) was used as internal standard to normalize differences in template quantity. Quantitative PCR was carried out using a Stratagene Mx3005P Real Time qPCR System (LaJolla, CA, USA), with PCR cycling conditions as follows: 10 min at 95 °C, 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 $\Delta\Delta$threshold cycle (C_t) 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 that *ACL5* 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
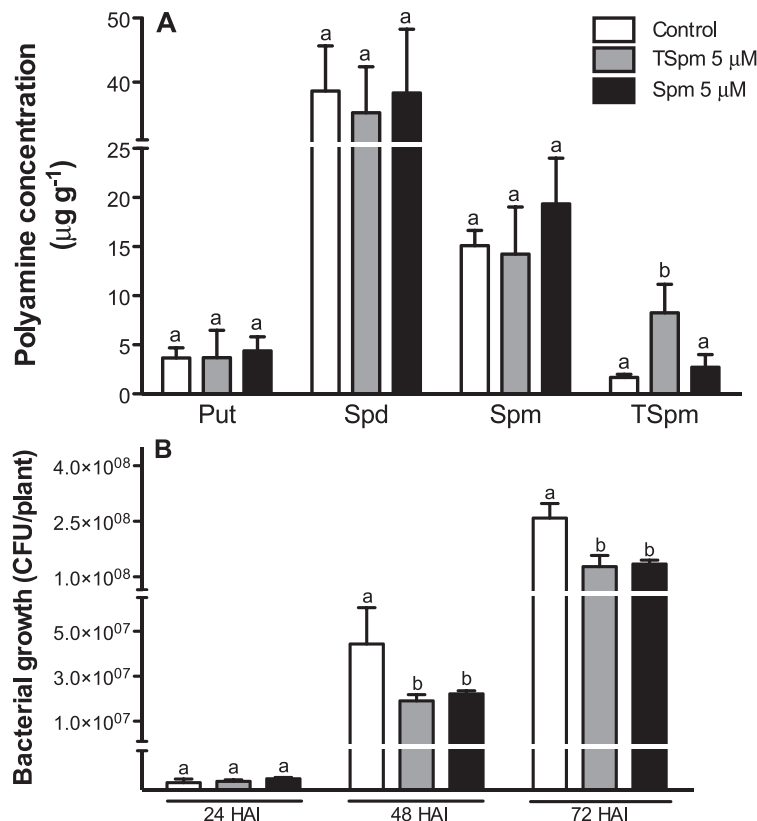
Fig. 1. Exogenously supplied TSpm increases endogenous *A. thaliana* Col-0 levels of TSpm and reduces *P. viridiflava* propagation. (A) Plants were grown for 12 d in MS medium (control) or MS supplemented with 5 μM TSpm or Spm and free polyamine levels (Put, Spd, Spm, and TSpm) were analysed by GC-MS. (B) A set of plants cultivated as described above was used to evaluate propagation of *P. viridiflava*. Plants were inoculated with a bacterial suspension and the number of CFU per plant was evaluated 24, 48, and 72 HAI. Results are mean ± SD of three (A) or five (B) replicates. Different letters indicate statistically significant differences between polyamine-amended and control plants according to one-way ANOVA and Tukey's test ($P \leq 0.05$).

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 Cu^{2+}-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
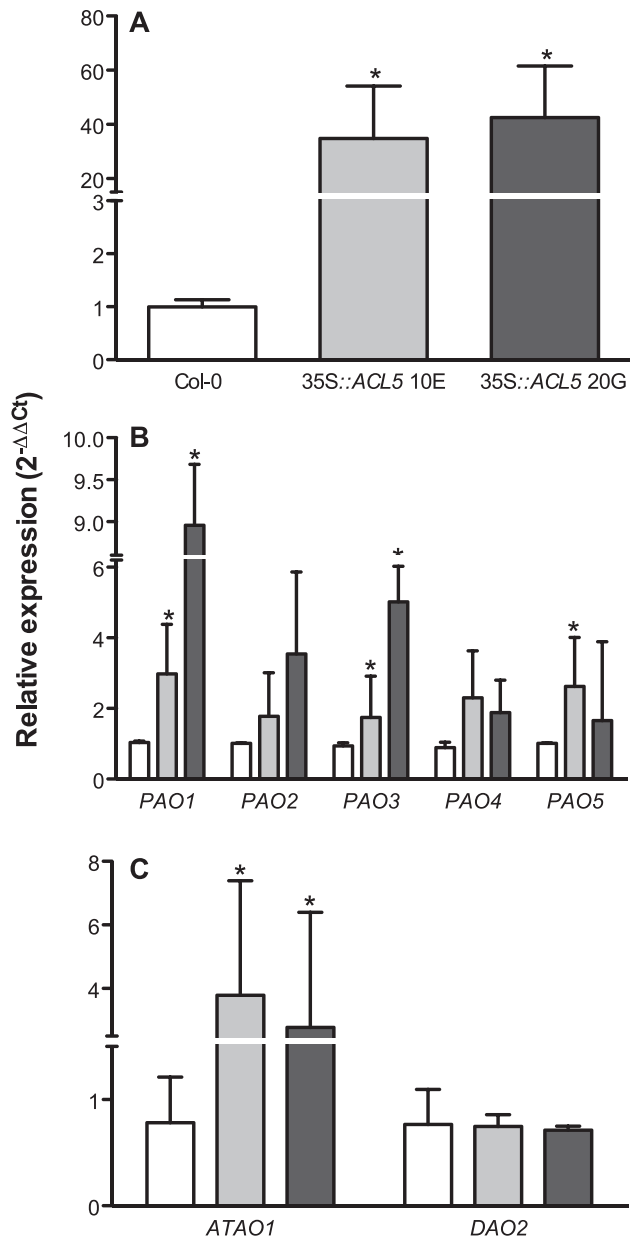
Fig. 2. *ACL5, diamine oxidase*, and *polyamine oxidase* expression in transgenic *Arabidopsis* lines that ectopically express *ACL5*. qRT-PCR was used to analyse abundance of mRNA from *ACL5* (A), five polyamine oxidase (*PAO1–PAO5*, B), and two diamine oxidase (*ATAO1* and *DAO2*, C) in 35S::*ACL5* line 10E and 20G. Col-0 plants were used as controls and *UBQ10* (At4g05320) was used as a reference gene. Results are mean ± SD of three replicates. Asterisks indicate statistically significant differences in gene expression between each line and Col-0 plants as analysed with the REST software ($P \leq 0.05$).

Fig. 3. (A) Perturbation of *ACL5* expression affects *Arabidopsis* resistance to *P. viridiflava*. WT Col-0 and 35S::*ACL5* 10E and 20G plants were inoculated with a suspension of *P. viridiflava* cells. The number of CFU per plant was evaluated 48 and 72 HAI. Results are mean ± SD of five replicates. Different letters indicate statistically significant differences at each time between the different plant lines according to one-way ANOVA and Tukey's test ($P \leq 0.05$). (B) Disease severity was evaluated at 120 HAI by assigning to each diseased plant a rank between 1 (lowest severity) and 5 (highest severity; as illustrated) and analysed by the number of plants in each rank. Statistically significant differences between distribution frequencies of each genotype were analysed with GraphPad Prism. (C) WT Col-0 and the *acl5-5* mutant (with and without exogenously added 10 μM TSpm) were inoculated with *P. viridiflava* and the number of CFU per plant was evaluated at 48 HAI as described in A. Differences in bacterial titres between different plants lines or

(Fig. 3C), in contrast to *ACL5* overexpressors (Fig. 3A). 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
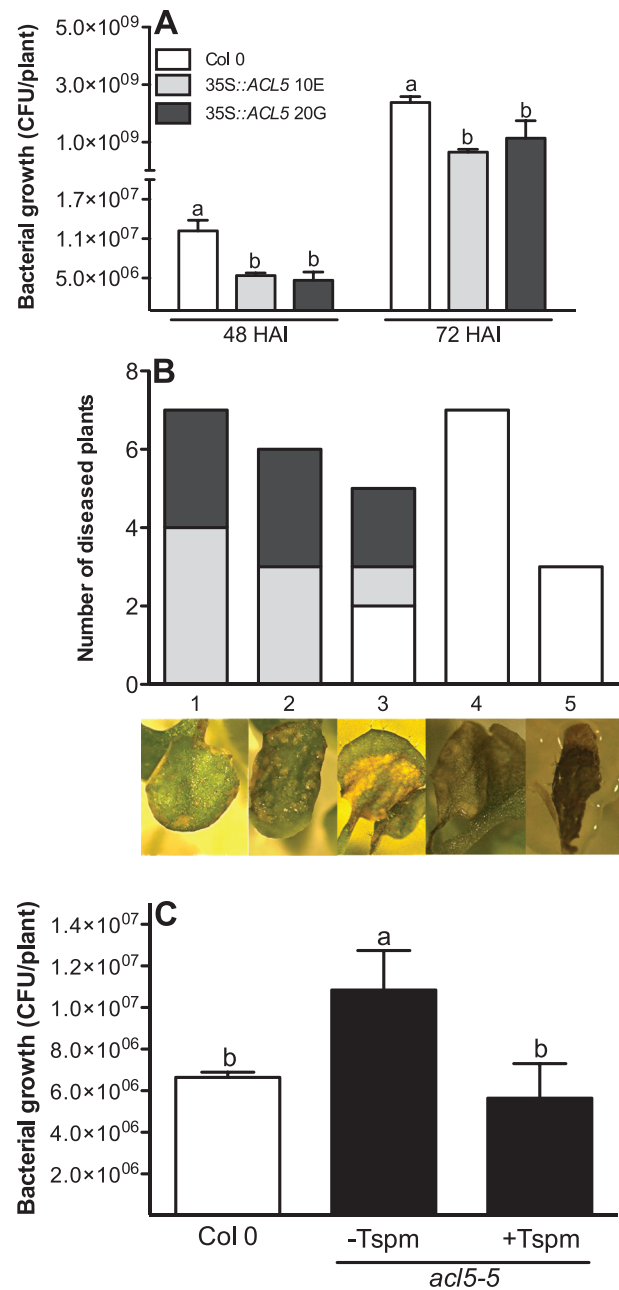
previous report about the xylem vessel-specific expression of *ACL5* (Muñiz *et al.*, 2008), suggest that the localized pattern of expression of this gene contributes to *Arabidopsis* defence against *P. viridiflava*.

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 ($P \leq 0.05$) 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

treatments according to one-way ANOVA and Tukey's test ($P \leq$ 0.05) (this figure is available in colour at *JXB* online).

(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. campestris* pv. *campestris*, 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
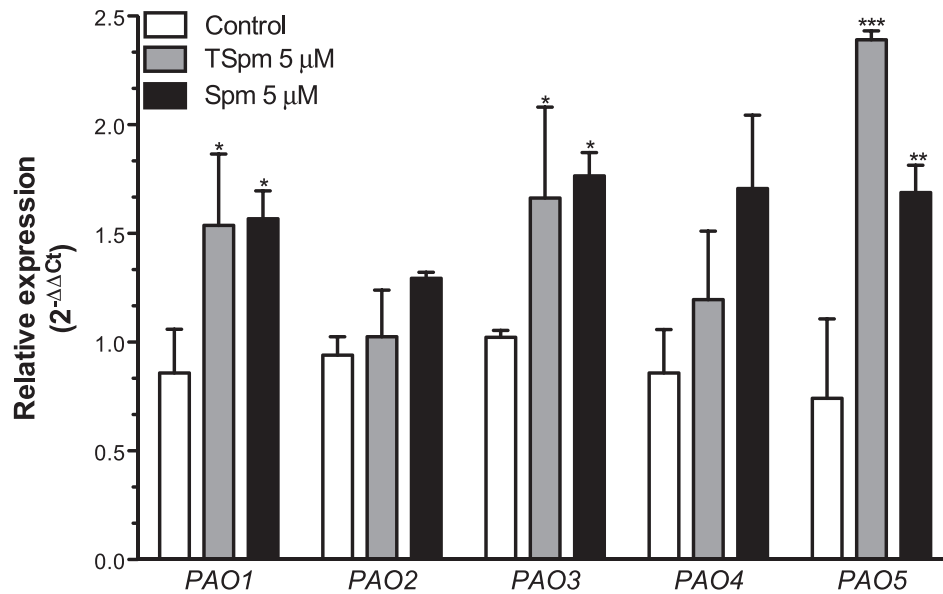
Fig. 4. Expression of *Arabidopsis PAO* genes in response to exogenously added TSpm. qRT-PCR was used to analyse the abundance of mRNAs of all known *PAO* genes (*PAO1–PAO5*). Results are mean ± SD of three replicates. Asterisks indicate statistically significant differences in the expression of each gene between polyamine-amended plants and controls as analysed with the REST software (*, $P \leq$ 0.05; **, $P \leq$ 0.01; ***, $P \leq$ 0.001).
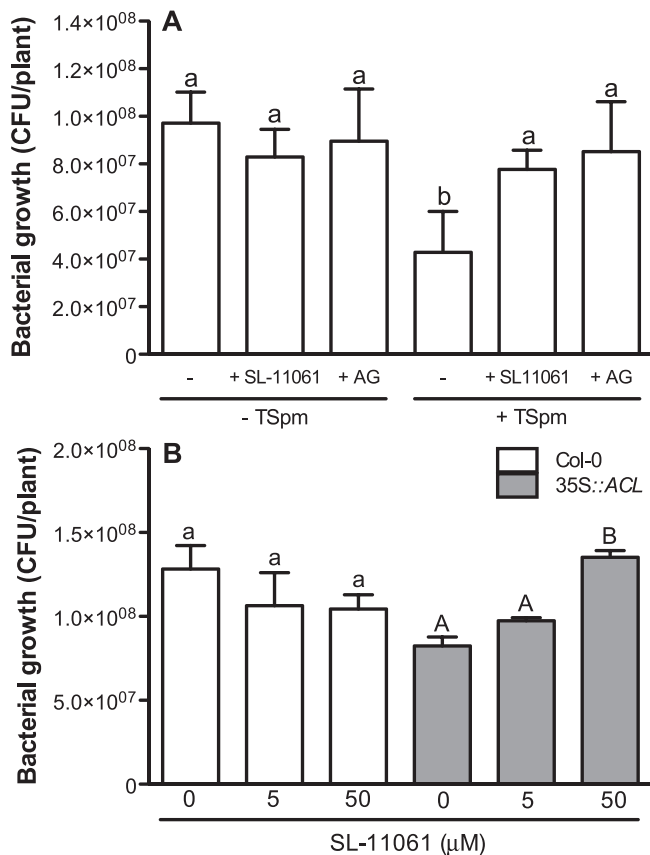

Fig. 5. Effect of PAO and DAO inhibitors on *P. viridiflava* propagation in *A. thaliana* plants that exhibit alterations in TSpm metabolism. (A) The effect of amine oxidase inhibitors on plant resistance conferred by exogenous TSpm was evaluated by growing Col-0 plants for 12 d in MS with 5 µM TSpm in combination with 5 µM *N,N'*-aminoguanidine (AG) or 5 µM

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-triazanonadecane (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 ($P \leq$ 0.05 in A; $P \leq$ 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* 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 *PAO*s 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 *At*PAO2, 3, and 4 are located in peroxisomes, while *At*PAO1 and *At*PAO5 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) At*PAO*s 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 *At*PAO1, 2, 3, and 4 are all able to use TSpm as a substrate. Moreover, Fincato *et al.* (2011) found that *At*PAO1 oxidizes TSpm with a k_{cat} value and a k_{cat}/K_m ratio 2- and 10-fold, respectively, higher than those for Spm, which suggests that TSpm is a better substrate than Spm for *At*PAO1 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 H_2O_2 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 H_2O_2 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.

Acknowledgements

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