

Perturbation of *spermine synthase* Gene Expression and Transcript Profiling Provide New Insights on the Role of the Tetraamine Spermine in *Arabidopsis* Defense against *Pseudomonas viridiflava*^{1[C][W]}

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The role of the tetraamine spermine in plant defense against pathogens was investigated by using the *Arabidopsis thaliana*-*Pseudomonas viridiflava* pathosystem. The effects of perturbations of plant spermine levels on susceptibility to bacterial infection were evaluated in transgenic plants (35S::*spermine synthase* [SPMS]) that overexpressed the SPMS gene and accumulated spermine, as well as in *spms* mutants with low spermine levels. The former exhibited higher resistance to *P. viridiflava* than wild-type plants, while the latter were more susceptible. Exogenous supply of spermine to wild-type plants also increased disease resistance. Increased resistance provided by spermine was partly counteracted by the polyamine oxidase inhibitor SL-11061, demonstrating that the protective effect of spermine partly depends on its oxidation. In addition, global changes in gene expression resulting from perturbations of spermine levels were analyzed by transcript profiling 35S::*SPMS-9* and *spms-2* plants. Overexpression of 602 genes was detected in 35S::*SPMS-9* plants, while 312 genes were down-regulated, as compared to the wild type. In the *spms-2* line, 211 and 158 genes were up- and down-regulated, respectively. Analysis of gene ontology term enrichment demonstrated that many genes overexpressed only in 35S::*SPMS-9* participate in pathogen perception and defense responses. Notably, several families of disease resistance genes, transcription factors, kinases, and nucleotide- and DNA/RNA-binding proteins were overexpressed in this line. Thus, a number of spermine-responsive genes potentially involved in resistance to *P. viridiflava* were identified. The obtained results support the idea that spermine contributes to plant resistance to *P. viridiflava*.

Plants are exposed to attack by a variety of bacteria, fungi, and insects. As a consequence, they have evolved defense mechanisms that involve complex

biochemical changes, regulated by an intricate signaling network (López et al., 2008). Plant infection usually leads to the accumulation of reactive oxygen species (ROS), an essential component of the hypersensitive response (HR) developed during many incompatible interactions (Apel and Hirt, 2004). In plants, several sources of ROS are known, among which polyamines (PAs) contribute to hydrogen peroxide (H₂O₂) formation in response to infection by several pathogens (Takahashi et al., 2003; Yoda et al., 2003, 2006; Marina et al., 2008; Moschou et al., 2009).

PAs are natural aliphatic polycations ubiquitous in prokaryotic and eukaryotic cells and are essential for cell growth, proliferation, and differentiation. PAs exist in free and conjugated forms, the latter being covalently bound to small molecules and proteins. The most common and abundant PAs are the diamine putrescine, the triamine spermidine, and the tetraamine spermine, synthesized from Orn and Arg by two alternative pathways. One of these pathways is shared by almost all organisms and involves Orn decarboxylation by the enzyme Orn decarboxylase

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(EC 4.1.1.17) to form putrescine. An alternative pathway occurs in plants and bacteria, where Arg is decarboxylated to agmatine by Arg decarboxylase (EC 4.1.1.19), and agmatine is then transformed into putrescine via *N*-carbamoyl-putrescine. Putrescine is then successively aminopropylated to produce spermidine and spermine by the spermidine (EC 2.5.1.16) and spermine (EC 2.5.1.22) synthase enzymes, respectively. The aminopropyl groups are donated by decarboxylated *S*-adenosyl-Met, a compound synthesized in a reaction catalyzed by *S*-adenosyl-Met decarboxylase (EC 4.1.1.50; Cohen, 1998).

PA catabolism is mediated by diamine oxidases and PA oxidases (PAOs). Diamine oxidases (EC 1.4.3.6) oxidize the diamines putrescine and cadaverine at the primary amino group, while PAOs (EC 1.5.3.11) catalyze the oxidation of spermidine, spermine, and/or their acetylated derivatives at the secondary amino groups (Angelini et al., 2010). Traditionally, plant PAOs were known to oxidize spermidine and spermine to 1,3-diaminopropane, H₂O₂, and the corresponding aldehyde (Cona et al., 2006). However, a PA back-conversion pathway similar to that of mammalian cells also operates in Arabidopsis (*Arabidopsis thaliana*), a species that harbors several PAO isoforms (Moschou et al., 2008).

A number of studies have provided evidence that pathogen attack induces modifications of PA metabolism in their hosts (for review, see Walters, 2003a, 2003b), but the physiological role of PAs in plant defense has not always been clear. For instance, both PA biosynthesis and catabolism are up-regulated during the HR induced by the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* and the Tobacco mosaic virus in barley (*Hordeum vulgare*; Cowley and Walters, 2002) and tobacco (*Nicotiana tabacum*; Marini et al., 2001), respectively. Some insights have been gained on the role of PAs in compatible interactions. In this sense, apoplastic spermine accumulation and further oxidation mediated by PAO led to H₂O₂ production in tobacco plants infected by the biotrophic bacterium *Pseudomonas syringae* pv *tabaci* (Moschou et al., 2009). Moreover, tobacco tolerance to *P. syringae* and the hemibiotrophic oomycete *Phytophthora parasitica* var *nicotianae* was enhanced by PAO overexpression (Moschou et al., 2009). Similarly, PA accumulation and further oxidation was also detected in the leaf apoplast of tobacco plants infected by the biotrophic bacterium *Pseudomonas viridiflava* and the necrotrophic fungus *Sclerotinia sclerotiorum*. This response exhibited contrasting effects on the ability of both pathogens to colonize host tissues, by restricting growth of *P. viridiflava* in planta, but enhancing tissue necrosis provoked by *S. sclerotiorum* (Marina et al., 2008).

In addition to ROS generation after oxidation by catabolic enzymes, other roles have been assigned to pathogen-induced PA accumulation. Spermine was proposed to act as a salicylate-independent inducer of acidic pathogenesis-related protein expression in to-

bacco mosaic virus-infected tobacco plants (Yamakawa et al., 1998). Later, Takahashi et al. (2003) demonstrated that spermine causes mitochondrial dysfunction via a signaling pathway that stimulates mitogen-activated protein kinases, which in turn activate a subset of HR-specific genes (Takahashi et al., 2004). Thus, evidences indicate that among the usual PAs, spermine stands out by its participation in plant defense against pathogens. In this regard, Mitsuya et al. (2009) investigated the effect of exogenously added spermine on the response of Arabidopsis to the *Cucumber mosaic virus* (CMV). By employing the super serial analysis of gene expression, the authors found that exogenously added spermine induced the expression of a number of Arabidopsis genes in common to CMV infection and proposed that spermine plays a role in signaling defense responses of Arabidopsis against this pathogen (Mitsuya et al., 2009; Sagor et al., 2009). Even though such approaches based on the addition of exogenous spermine allowed investigating the consequences of enhanced spermine levels on plant defense, the effects of spermine depletion were not explored so far and require a different approach, such as the use of mutants unable to accumulate this tetraamine. In addition, enhancement of spermine levels by overexpression of spermine biosynthetic genes has not been used as a tool to analyze the role of this tetraamine in plant defense up to date.

The aim of this work was to gather molecular and biochemical information to evaluate in vivo the role of spermine in plant defense against pathogens. For this purpose, Arabidopsis susceptibility to infection by its natural bacterial pathogen *P. viridiflava* (Jakob et al., 2002) was examined in plants altered in the endogenous content of spermine. To identify potential mechanisms for spermine action in this context, global gene expression analyses were performed in spermine overproducing lines, as well as in mutants with decreased spermine levels.

RESULTS

Infection of Arabidopsis Plants by *P. viridiflava* Leads to Spermine Accumulation

To evaluate the involvement of spermine in defense responses of Arabidopsis against *P. viridiflava*, the effect of infection on the levels of this tetraamine was analyzed. A transient increase of spermine levels was evident 24 h after inoculation (HAI) in infected plants (Fig. 1). Spermidine and putrescine levels were also increased 24 and 48 HAI, respectively (Supplemental Fig. S1). Spermine levels of infected plants nearly doubled those of controls 24 HAI. The effect of bacterial infection on spermine levels, along with previous evidences about the participation of this tetraamine in plant defense against *P. viridiflava* (Marina et al., 2008), prompted us to investigate pathogen response in plants altered in endogenous spermine levels.

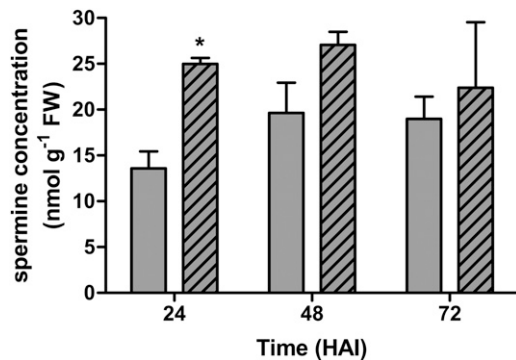


Figure 1. Time-course evolution of free spermine levels in Col-0 Arabidopsis plants infected by *P. viridiflava*. Infected plants (hatched bars) are compared to noninfected controls (gray bars). Statistically significant differences between both groups according to one-way ANOVA and Dunnett's test are shown as *, $P \leq 0.05$. Results are the mean of three to four replicates \pm SD.

Modification of Spermine Levels in *spermine synthase* Mutants and Transgenic Plants That Overexpress the *spermine synthase* Gene

Spermine synthase is encoded in Arabidopsis by a single copy of the *spermine synthase* (*SPMS*) gene (Knott et al., 2007). Thus, to manipulate the levels of endogenous spermine we generated lines expressing *SPMS* under the control of the constitutive 35S cauliflower mosaic virus (CaMV) promoter, and checked *SPMS* expression level in the available *spms* knockout mutants and in four of the *SPMS* overexpressing lines (35S::*SPMS*-7, 35S::*SPMS*-8, 35S::*SPMS*-9, and 35S::*SPMS*-15).

As expected, no *SPMS* gene expression was detectable in the two *spms* mutants analyzed (Fig. 2). On the contrary, steady-state levels of *SPMS* mRNA were 7- to 104-fold higher in transgenic 35S::*SPMS* lines than in wild-type plants. Moreover, changes in *SPMS* expression had an important impact on spermine synthesis, as demonstrated by the observation that all four *SPMS* overexpressing lines contained between 13 and 17 nmol g⁻¹ fresh weight (FW) of spermine, which represented between 2- and 3-fold higher spermine levels compared to wild type, while *spms* mutants contained lower spermine levels than wild-type plants (Fig. 3). Null *spms* mutants are expected to completely lack spermine, so the presence of this tetraamine in the extracts may be the consequence of joint quantification of other tetraamines (namely thermospermine) with our HPLC method. However, it should be kept in mind that gas chromatography coupled to mass spectrometry, an analytical technique with a higher resolution than the HPLC method hereby employed, demonstrated that the *spms* mutant employed in this work contains spermine traces, presumably as a contamination from the culture medium (Rambla et al., 2010). Moreover, Rambla et al. (2010) found that the *spms* mutant used in this work contains thermospermine levels similar to wild-type plants, thus demon-

strating that blocking spermine biosynthesis does not lead to enhanced thermospermine levels. Absolute spermine values were variable in different experiments, but relative spermine levels between the different genetically modified (GM) lines and wild-type plants were highly reproducible. The levels of the other two most abundant PAs (putrescine and spermidine) were also evaluated in the GM Arabidopsis lines and were found to be similar to those of wild-type plants (Supplemental Fig. S2).

In summary, it was verified that overexpression of the *SPMS* gene in Arabidopsis leads to spermine accumulation, and mutation of this gene results in decreased levels of this tetraamine.

Spermine Accumulation and Oxidation Modulate the Colonization of Arabidopsis Plants by the Biotrophic Bacterium *P. viridiflava*

The effect of perturbations of endogenous free spermine levels on Arabidopsis resistance to *P. viridiflava* was evaluated using the previously depicted *spms* mutants and transgenic lines, as well as wild-type plants for comparison. Plants were inoculated with a bacterial suspension and the number of colony forming units (CFU) per g plant FW was evaluated 72 HAI. As compared to wild-type plants, *P. viridiflava* propagation was 1.5- and 1.6-fold higher in the *spms*-2 and *spms*-1 lines, respectively (Fig. 4). On the contrary, bacterial propagation was strongly decreased in transgenic plants overexpressing the *SPMS* gene (Fig. 4). Similar evaluations were performed 24 and 48 HAI. At these times, a similar trend was observed (data not

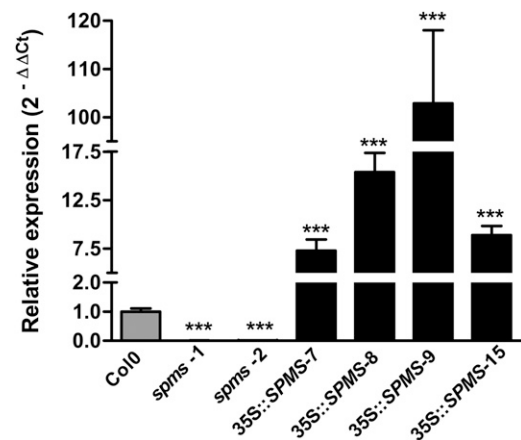


Figure 2. *SPMS* gene expression in GM Arabidopsis plants. qRT-PCR was used to analyze the abundance of *SPMS* transcripts in two *spms* mutants (white bars) and four transgenic lines that overexpress the *SPMS* gene under the control of the 35S CaMV promoter (black bars). Transcript levels were normalized to the average signal intensity of wild-type plants. Results are the mean of three replicates \pm SE and statistically significant differences in gene expression between GM lines and wild type (gray bar), as analyzed with the REST[®] software, are shown as ***, $P \leq 0.001$.

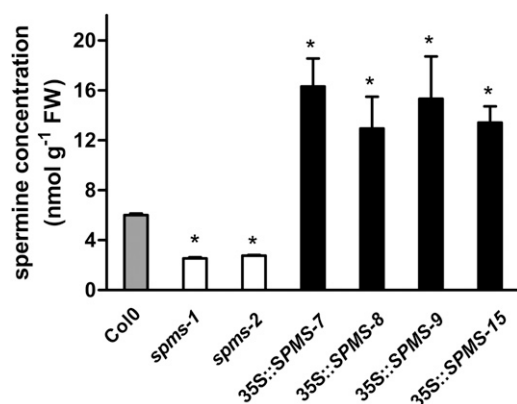


Figure 3. Free spermine levels in Arabidopsis plants GM in spermine biosynthesis. Spermine was quantified by HPLC in wild-type Arabidopsis Col-0 (gray bar), *spms* mutants (white bars), and transgenic lines that overexpress the *SPMS* gene under the control of the 35S CaMV promoter (black bars). Results are the mean of two to three replicates \pm SD and statistically significant differences in spermine levels between GM lines and wild type according to one-way ANOVA and Dunnett's test are shown as *, $P \leq 0.05$.

shown), but differences between GM and wild-type plants were not statistically significant.

Further indications for a protective role of spermine were obtained by inspection of the symptoms caused after infection. Symptoms consisted on water-soaked translucent spots, which developed into chlorotic and subsequently necrotic lesions 72 HAI, as depicted by Jakob et al. (2002). Disease incidence was 50% in wild-type plants 72 HAI. At this time point, a higher percentage (77%) of diseased plants was evident for the *spms-2* line. On the contrary, a lower percentage (28%) of diseased plants was found in the 35S::*SPMS-9* line (Fig. 5A). When evaluated later (144 HAI), disease incidence increased in all the plant lines, as compared to 72 HAI. At this time point, disease incidence was higher in wild type (81%) and the *spms-2* (91%) than in the 35S::*SPMS-9* line (52%; Fig. 5B).

Disease severity was higher in the *spms-2* line than in wild-type plants and the 35S::*SPMS-9* line 72 HAI. In this way, the *spms-2* mutant was the only line for which plants showing the highest severity index (5) were detected at this time point (Fig. 5C). Moreover, *spms-2* and wild-type plants that ranked at intermediate severity values (3) were also found at this time, but on the contrary, 35S::*SPMS-9* plants only fell within low disease severity ranks (1 and 2; Fig. 5C). When evaluated 144 HAI, disease severity was again higher in the *spms-2* than in the other lines, as denoted by the high number of mutant plants that showed the highest severity index (5; Fig. 5D). Thus, when frequency distribution of diseased plants in different severity ranks was analyzed 72 HAI, the transgenic 35S::*SPMS-9* line was found to be different ($P \leq 0.05$) from the *spms-2*. No significant differences in frequency distribution between wild-type plants and the other two plant lines were detected at this time after inoculation.

When analyzed 144 HAI, no significant differences between any plant line were detected, in spite of the high number of *spms-2* plants comprised in rank 5. A higher number of plants should probably be screened to verify if disease severity is significantly higher in the *spms-2* line than in the other plant lines at this time after inoculation.

As a whole, our results demonstrate that Arabidopsis infection by *P. viridiflava* strongly depends on the ability of the host to synthesize the tetraamine spermine. Thus, high levels of free spermine resulting from *SPMS* overexpression strongly decrease in planta propagation of *P. viridiflava* and disease symptoms, while the opposite is true for plants containing low spermine levels.

Given that PA oxidation, rather than PAs themselves, has been attributed a role in stress responses as a source for ROS (Marini et al., 2001; Marina et al., 2008; Moschou et al., 2009), it is possible that the increased resistance to *P. viridiflava* infection in plants overproducing spermine was due to an increase in spermine oxidation, rather than the accumulation of spermine. To distinguish between these two possibilities, we examined the susceptibility to *P. viridiflava* of Arabidopsis plants with high endogenous spermine content in the presence of the PAO inhibitor 1,19-Bis(ethylamino)-5,10,15-triazanonadecane (SL-11061; Bacchi et al., 2002). In addition to the 35S::*SPMS-9* line, which accumulates spermine, *spms-2* and wild-type plants with increased endogenous spermine levels as a consequence of supplementation with exogenous spermine were also employed in this experiment. Exogenous spermine concentrations were carefully selected to induce changes in plant spermine content that fell within the physiological range of concentrations and mimicked those of the 35S::*SPMS-9* line (Supplemental Fig. S3). Thus, wild-type and *spms-2*

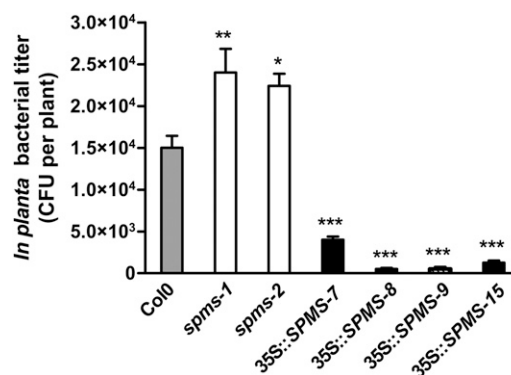


Figure 4. Propagation of *P. viridiflava* in Arabidopsis plants GM in spermine biosynthesis. Wild type (gray bar), *spms* mutants (white bars), and transgenic lines that overexpress the *SPMS* gene (black bars) were inoculated with *P. viridiflava*. The number of CFU per plant was evaluated 72 HAI. Results are the mean of three to six replicates \pm SD and statistically significant differences between GM lines and wild-type plants according to one-way ANOVA and Dunnett's test are shown as *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

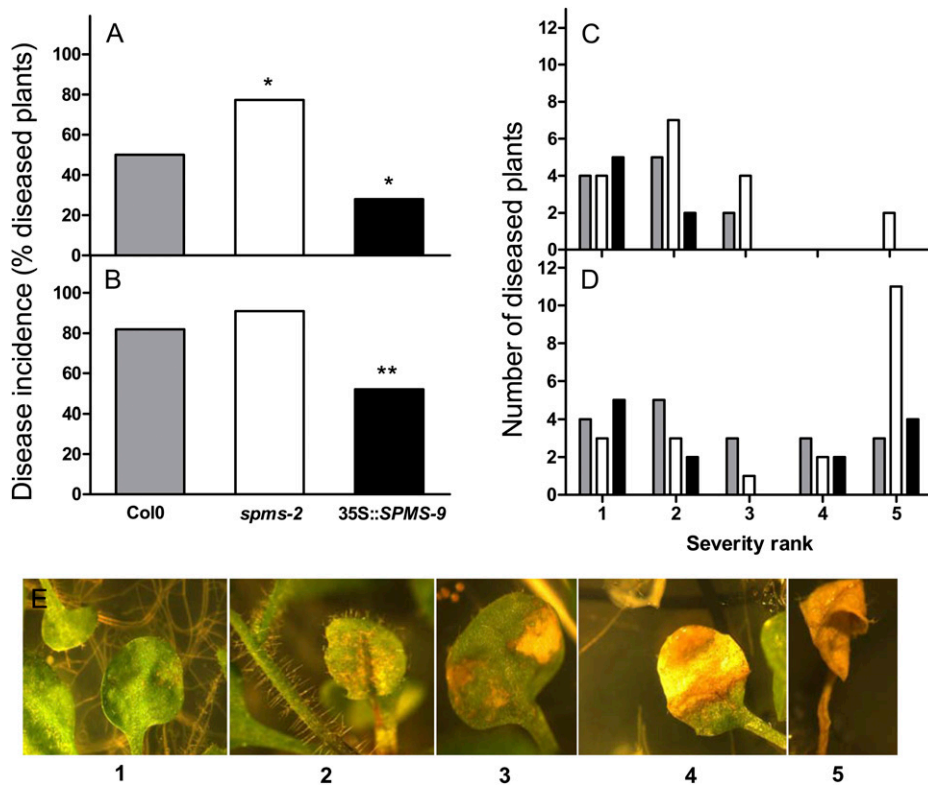


Figure 5. Disease incidence and severity caused by *P. viridiflava* infection of Arabidopsis plants GM in spermine biosynthesis. Wild type (gray bars), *spms* mutant (white bars), and a transgenic line that overexpress the *SPMS* gene (black bars) were inoculated with a suspension of *P. viridiflava* cells. Disease incidence was evaluated 72 (A) and 144 HAI (B) as the percentage of diseased plants over a total of 22 to 25 inoculated plants. Statistically significant differences between GM lines and wild type according to a nonparametric binomial test are shown as *, $P \leq 0.05$; **, $P \leq 0.01$. Disease severity was evaluated by estimating the percentage of diseased tissue in the inoculated leaves that showed symptoms, after image acquisition with a binocular microscope coupled to a digital camera, and further image analysis with the Image-ProPlus V 4.1 software. Disease severity was assessed 72 (C) and 144 HAI (D) by assigning a rank between 1 (lowest severity) and 5 (highest severity) to each diseased plant, and further analyzing the number of plants comprised in each rank. Statistically significant differences between distribution frequencies of each genotype were analyzed by the nonparametric Kruskal-Wallis test followed by Dunns' posthoc comparisons. Frequency distribution of the 35S::*SPMS-9* line was found to be different from the *spms-2* mutant ($P \leq 0.05$). E, Level of leaf damage corresponding to each severity rank. [See online article for color version of this figure.]

plants, respectively, supplemented with 30 and 50 μM spermine exhibited endogenous levels of this tetraamine similar to the transgenic line 35S::*SPMS-9* (Supplemental Fig. S3) and were used for the experiment described below.

As shown in Figure 3, *P. viridiflava* propagation was reduced in 35S::*SPMS-9*, as compared to wild-type plants, while the opposite picture was found in *spms-2* (Fig. 6). Supplementation of wild-type and *spms-2* plants with spermine strongly decreased in planta bacterial growth, thus reaching similar or even lower values than those of 35S::*SPMS-9* (Fig. 6). In this way, the increase of endogenous spermine levels by the exogenous supply of this tetraamine restricted bacterial infection in a similar way to *SPMS* overexpression. Supplementation with SL-11061 strongly increased bacterial multiplication in 35S::*SPMS-9*, which thus resembled *spms-2* with no amendments (Fig. 6). In addition, SL-11061 further increased the susceptibility

of wild-type plants to *P. viridiflava*. Moreover, the reduction of bacterial growth mediated by spermine addition to wild-type plants was completely prevented by SL-11061 (Fig. 6).

Accumulation of ROS

Generally, plant PAs can be oxidized by PAOs in two different ways. Many plant PAOs oxidize PAs through the so-called terminal catabolic pathway (Angelini et al., 2010). Arabidopsis contains five PAO isoforms, at least three of which (AtPAO1, AtPAO3, and AtPAO4) are known to operate through a different pathway that back converts PAs in a similar way to mammalian PAOs. Both types of PAO-mediated PA catabolism lead to H_2O_2 production. Thus, reduced bacterial propagation in 35S::*SPMS-9* plants observed in this work could be due to H_2O_2 accumulation, which could either be toxic to bacterial cells or activate plant defenses. Moreover,

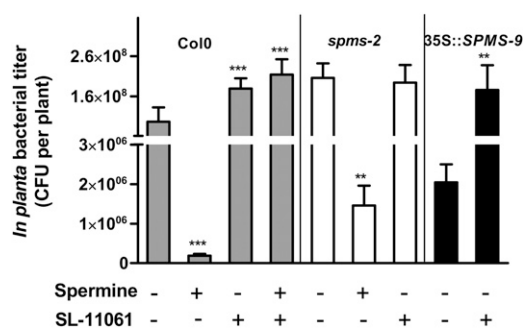


Figure 6. Oxidation is crucial for spermine-mediated restriction of *P. viridiflava* multiplication in Arabidopsis. Wild type (gray bars) and the *spms-2* line (white bars) were grown in culture medium amended with 30 and 50 μM spermine, respectively. When necessary, the PAO inhibitor SL-11061 (50 μM) was included in the culture medium. The absence and presence of each compound in the culture medium is indicated with – and + symbols below the horizontal axis. Plants grown without both compounds were used as controls. Black bars represent bacterial growth in plants of the *SPMS*-overexpressing line 35S::*SPMS-9* without exogenous supply of spermine. Plants were inoculated with *P. viridiflava* and the number of CFU per plant was evaluated 72 HAI. Results are the mean of three to six replicates \pm SD and statistically significant differences in bacterial growth between treatments and controls according to one-way ANOVA and Dunnett's test (wild-type Col-0 and *spms-2*) or Student's *t* test (35S::*SPMS-9*) are shown as *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. Comparisons are only valid within each plant line.

H_2O_2 could be converted to hydroxyl radical by the nonenzymatic reactions of Fenton and Haber-Weiss (Fenton, 1894; Haber and Weiss, 1934).

No ROS accumulation was detected by dichlorofluorescein diacetate staining in any of the three lines analyzed in this work and thus no evidence that 35S::*SPMS-9* plants constitutively accumulate higher ROS levels than wild-type plants was obtained. ROS levels were also analyzed in plants infected by *P. viridiflava*. Although infection elicited ROS accumulation, both 35S::*SPMS-9* and *spms-2* plants contained ROS levels similar to wild-type plants 48 and 72 HAI (data not shown). ROS levels were also analyzed in wild-type plants supplemented with different spermine concentrations. In this case, ROS accumulation was evident only after treatment with 500 μM or higher spermine concentrations (Supplemental Fig. S4), which resulted in spermine accumulation to levels far higher than those of 35S::*SPMS-9*. Thus, spermine accumulation enhanced ROS production only when the levels of this tetraamine were increased beyond its physiological levels.

Spermine Accumulation by Means of *SPMS* Overexpression Leads to Transcriptional Changes of a Wide Set of Genes

Although enhanced tolerance of *SPMS* overexpressors to *P. viridiflava* could be due, at least in part, to a direct effect of spermine oxidation on pathogen's ability to colonize host tissues, it is also possible that spermine levels affect the response of Arabidopsis

plants to *P. viridiflava* by other mechanisms. To test this hypothesis, an overview of transcriptional changes induced by perturbations of spermine levels in GM plants was obtained by a transcriptomic approach. Transcript profiles of the 35S::*SPMS-9* and *spms-2* plants were compared to wild-type Arabidopsis. In 35S::*SPMS-9*, 602 genes were overexpressed, while 312 were repressed. On the other hand, 211 genes were overexpressed and 158 genes were repressed in *spms-2*. Among the 602 overexpressed genes in the 35S::*SPMS-9* line, 148 were also overexpressed in *spms-2* (Supplemental Table S1). In this way, 454 (602 – 148) and 63 (211 – 148) genes were exclusively overexpressed in the 35S::*SPMS-9* (Supplemental Table S2) and *spms-2* lines (Supplemental Table S4), respectively. Similarly, among the 312 repressed genes in the 35S::*SPMS-9* line, 107 were also repressed in *spms-2* (Supplemental Table S1). Therefore, 205 (312 – 107) and 51 (158 – 107) genes were exclusively repressed in the 35S::*SPMS-9* (Supplemental Table S3) and *spms-2* lines (Supplemental Table S5), respectively. None of the genes overexpressed in the 35S::*SPMS-9* line was repressed in *spms-2*. Similarly, none of the genes repressed in the 35S::*SPMS-9* line was overexpressed in *spms-2*. Thus, those genes that are induced by spermine overproduction seem not to be down-regulated under low spermine conditions. Similarly, genes that are induced by spermine deprivation are not down-regulated when spermine is accumulated. In this regard, changes in the expression (either up- or down-regulation) of these groups of genes probably occur once spermine levels either surpass or fall below a threshold. This would explain why the expression of some genes is affected only in transgenic plants that accumulate spermine, while mutants unable to accumulate spermine show levels of gene expression similar to wild-type plants. Similarly, it would explain why some genes are up-regulated only in the *spms-2* mutant, while they are not down-regulated in 35S::*SPMS-9* plants.

Results of microarray analysis were validated by quantitative reverse transcription (qRT)-PCR analysis of eight genes declared changed in the 35S::*SPMS-9* line and 12 genes involved in PA biosynthetic and catabolic pathways. Among the 20 genes analyzed, nine were declared overexpressed by Significance Analysis of Microarrays (SAM3.0), which were also confirmed by qRT-PCR (Supplemental Table S6). Three genes were declared repressed by SAM3.0, two of which were confirmed by qRT-PCR, but one of them (*At5g15950*) was not (Supplemental Table S6). Regarding the remaining eight genes, declared unchanged by SAM3.0, six of them were confirmed by qRT-PCR, while the other two were repressed (Supplemental Table S6). As a whole, the validation detected a single false positive among those genes declared changed by SAM3.0. The fact that qRT-PCR detected changes in gene expression that were not revealed by SAM3.0 is in agreement with the higher sensitivity of the former technique, and demonstrates that the conditions employed for SAM were stringent.

Functional properties of the genes differentially expressed in 35S::SPMS-9 and *spms-2* lines as compared to wild-type plants, were analyzed by evaluating gene ontology (GO) term overrepresentation. GO terms involved in responses to carbohydrate, jasmonic acid, organic substances, biotic, endogenous, hormone, chemical, and other stimulus were overrepresented in the set of 454 genes only overexpressed in 35S::SPMS-9 ($P < 0.01$; Table I). In addition, GO terms involved in responses to other organisms, bacterium, and chitin were also overrepresented in this set of genes, as well as terms related with programmed cell death, wounding, stress, and notably, defense and immune responses ($P < 0.01$; Table I). Interestingly, the response to bacterium (GO:0009617), defense response to bacterium (GO:00042742), defense response to bacterium-incompatible interaction (GO:0009816), defense response-incompatible interaction (GO:0009814), plant-type HR (GO:0009626), and host programmed cell death induced by symbiont (GO:0034050) terms were also overrepresented in this set of genes ($P < 0.05$ for all of them; Table I). The set of 205 genes only down-regulated in the 35S::SPMS-9 line contained 15 overrepresented GO terms (Table II). The bulk of these terms were involved in cell wall-related processes and catalytic activities such as phosphatase and phosphoric ester hydrolase. GO terms associated to responses to organic substances, cold, and endogenous stimulus were also overrepresented in this set of genes (Table II).

The number of GO annotations overrepresented in the set of genes only up-regulated in *spms-2* was much lower than in 35S::SPMS-9. Only four GO terms, related to reductase activities, were overrepresented in this set of genes (Table III). No GO term was overrepresented in the set of genes only down-regulated in *spms-2*.

Finally, it is interesting to point out that 21 members of several disease resistance protein families were constitutively overexpressed in 35S::SPMS-9 (Supplemental Table S2). Moreover, the transmembrane receptors *RPS2* (*At4g26090*) and *RPS6* (*At5g46470*) involved in Arabidopsis responses to *P. syringae*, and a gene (*At1g35710*) that confers resistance to *P. syringae* strains harboring the *avrB* avirulence gene, were also overexpressed in 35S::SPMS-9 (Supplemental Table S2). Thirty transcription factors of the myb, bZIP, GATA, *scarecrow*, WRKY, ERF, and other families, as well as 41 kinases, 32 DNA/RNA, and 49 nucleotide-binding proteins involved in transduction of signals derived from different stimuli were also up-regulated in this line (Supplemental Table S2).

DISCUSSION

Spermine Accumulation, Oxidation, and Arabidopsis Resistance to *P. viridiflava*

It has been previously reported that spermine accumulation and further oxidation by PAOs affect plant

resistance to different pathogens (Marina et al., 2008; Mitsuya et al., 2009; Moschou et al., 2009; Sagor et al., 2009). An approach based on the exogenous supply of spermine and the super serial analysis of gene expression technique was used by Mitsuya et al. (2009) to gain insight on the role of this tetraamine as an inducer of defense gene expression during the response of Arabidopsis to CMV. However, the manipulation of plant spermine levels through the modification of *SPMS* gene expression has not been used as a tool to investigate how spermine affects plant defense responses and resistance to pathogens. Thus, in this work, GM Arabidopsis plants exhibiting modifications of spermine levels were used to gain insight on the role of spermine in plant defense, by using the biotrophic bacterium *P. viridiflava* as a pathogen.

Transgenic Arabidopsis plants that accumulated higher spermine levels than the wild type as a consequence of overexpression of the *SPMS* gene under the control of the 35S CaMV promoter were used, along with *spms* mutants with decreased spermine levels. Results obtained after analyzing the response of the above-mentioned plants to *P. viridiflava* infection demonstrate that spermine levels significantly affect the resistance of Arabidopsis to this bacterium. In this sense, the lack of *SPMS* expression and the consequent reduction of spermine levels rendered Arabidopsis plants more susceptible to *P. viridiflava*. Conversely, spermine accumulation as a consequence of *SPMS* overexpression drastically increased Arabidopsis resistance to *P. viridiflava*, both in terms of bacterial propagation in planta and disease development. Additional evidences of the protective role of spermine against *P. viridiflava* were obtained by the exogenous supply of this tetraamine to the growth medium of *spms* mutants and wild-type plants. It is worth pointing out that exogenous spermine concentrations were carefully selected to induce changes in plant spermine content that fell within the physiological range of concentrations and mimicked those of the 35S::SPMS-9 line, which are compatible with normal growth and development. The increase of Arabidopsis resistance to *P. viridiflava* provided by spermine accumulation could be due to different mechanisms. PA oxidation by different amine oxidases can lead to H₂O₂ production (Moschou et al., 2008; Angelini et al., 2010), which in turn could exert a direct toxic effect on the pathogen or activate defense mechanisms (Marini et al., 2001; Marina et al., 2008; Mitsuya et al., 2009; Moschou et al., 2009). Thus, the relevance of spermine oxidation on the increase of Arabidopsis resistance to *P. viridiflava* conferred by spermine was evaluated using the PA oxidase inhibitor SL-11061, a PA analog previously found to inhibit tobacco (Marina et al., 2008), maize, and oat (*Avena sativa*) PA oxidases (Maiale et al., 2008), which was also used to evaluate the role of PA oxidation in tobacco responses against pathogen infection (Marina et al., 2008). Results obtained after analyzing the consequences of inhibition of PA oxidation in wild-type plants supplemented with spermine and 35S::

Table 1. Overrepresented GO terms in the set of up-regulated genes in the SPMS overexpressing line 35S::SPMS-9

Genes called up-regulated by the significance analysis of microarrays and associated to GO terms were used to perform a GO overrepresentation analysis using Ontologizer software with settings Term-For-Term/Westfall-Young-Single-Step. A total of 22,766 genes were in the population set, 443 of which were in the study set. Overrepresented GO categories (adjusted P value ≤ 0.05) are listed.

GO ID ^a	Name ^b	NSP ^c	Study Count ^d	Population Count ^e	Adjusted P Value ^f
GO:0050896	Response to stimulus	B	133	2758	$<1.0 \times 10^{-300}$
GO:0006952	Defense response	B	51	586	$<1.0 \times 10^{-300}$
GO:0006950	Response to stress	B	84	1,571	$<1.0 \times 10^{-300}$
GO:0010200	Response to chitin	B	23	116	$<1.0 \times 10^{-300}$
GO:0010033	Response to organic substance	B	62	966	$<1.0 \times 10^{-300}$
GO:0042221	Response to chemical stimulus	B	74	1,377	$<1.0 \times 10^{-300}$
GO:0009743	Response to carbohydrate stimulus	B	24	180	$<1.0 \times 10^{-300}$
GO:0042742	Defense response to bacterium	B	18	132	$<1.0 \times 10^{-300}$
GO:0009719	Response to endogenous stimulus	B	43	768	$<1.0 \times 10^{-300}$
GO:0009617	Response to bacterium	B	20	187	$<1.0 \times 10^{-300}$
GO:0002376	Immune system process	B	19	173	$<1.0 \times 10^{-300}$
GO:0006955	Immune response	B	19	173	$<1.0 \times 10^{-300}$
GO:0051707	Response to other organism	B	31	461	$<1.0 \times 10^{-300}$
GO:0009753	Response to jasmonic acid stimulus	B	17	142	$<1.0 \times 10^{-300}$
GO:0009607	Response to biotic stimulus	B	31	478	$<1.0 \times 10^{-300}$
GO:0045087	Innate immune response	B	16	157	$<1.0 \times 10^{-300}$
GO:0051704	Multioorganism process	B	31	570	$<1.0 \times 10^{-300}$
GO:0016740	Transferase activity	F	79	2,390	$<1.0 \times 10^{-300}$
GO:0051716	Cellular response to stimulus	B	29	579	$<1.0 \times 10^{-300}$
GO:0009605	Response to external stimulus	B	19	294	$<1.0 \times 10^{-300}$
GO:0009816	Defense response to bacterium, incompatible interaction	B	6	26	$<1.0 \times 10^{-300}$
GO:0009725	Response to hormone stimulus	B	32	703	$<1.0 \times 10^{-300}$
GO:0008219	Cell death	B	11	109	0.002
GO:0016265	Death	B	11	109	0.002
GO:0009814	Defense response, incompatible interaction	B	10	92	0.004
GO:0009611	Response to wounding	B	12	136	0.004
GO:0048583	Regulation of response to stimulus	B	12	140	0.008
GO:0009626	Plant-type HR	B	6	33	0.036
GO:0034050	Host programmed cell death induced by symbiont	B	6	33	0.036
GO:0016301	Kinase activity	F	45	1,234	0.038

^aAccession number of the GO term. ^bGO term name. ^cNamespace or subontology (B, biological process; C, cellular component; F, molecular function). ^dThe number of genes in the study set annotated to the GO term in question. ^eThe number of genes in the population set annotated to the GO term in question. ^fAdjusted P value.

SPMS-9 plants, demonstrated that the increase in resistance of Arabidopsis to *P. viridiflava* mediated by spermine accumulation depends, at least in part, on the ability of the plant to oxidize this tetraamine. In agreement with this observation, spermine oxidation was found to play a role in tobacco resistance to *P. viridiflava* (Marina et al., 2008), *P. syringae* pv *tabaci*, and *P. parasitica* var *nicotianae* (Moschou et al., 2009), as well as in Arabidopsis resistance to CMV (Mitsuya et al., 2009). Moreover, Yoda et al. (2003, 2009) demonstrated that PA oxidase activity strongly contributes to the onset of both the host and nonhost HRs triggered in tobacco plants by different pathogens, thus suggesting that either spermidine or spermine oxidation are involved in this defense response.

On the basis of the above-mentioned results, it could be speculated that spermine-mediated protection of Arabidopsis plants against *P. viridiflava* is due to increased H₂O₂ levels in planta, as a result of spermine

oxidation. In this sense, postinfection H₂O₂ production by PA oxidation was found to be an essential component of defense responses (Yoda et al., 2003, 2006, 2009; Moschou et al., 2009). In this work, both pre- and postinfection levels of ROS in 35S::SPMS-9 plants were similar to those of wild-type plants, as demonstrated by staining plant tissues with a ROS-specific probe. Moreover, exogenous spermine was also ineffective in inducing ROS accumulation, except when tissues were infiltrated with high concentrations ($\geq 500 \mu\text{M}$), which results in the accumulation of nonphysiological levels of this tetraamine that negatively affect plant development. In addition, the *spms-2* line was found to contain ROS levels similar to those of wild-type plants. Therefore, the decreased resistance of this mutant to *P. viridiflava* is probably not related to lower ROS levels in planta. In summary, although plant resistance to *P. viridiflava* was directly related to preinfection levels of spermine and the ability to oxidize this tetraamine, a

Table II. Overrepresented GO terms in the set of down-regulated genes in the *SPMS* overexpressing line 35S::SPMS-9

Genes called down-regulated by the significance analysis of microarrays and associated to GO terms were used to perform a GO overrepresentation analysis using Ontologizer software with settings Term-For-Term/Westfall-Young-Single-Step. A total of 22,766 genes were in the population set, 201 of which were in the study set. Overrepresented GO categories (adjusted P value ≤ 0.05) are listed.

GO ID ^a	Name ^b	NSP ^c	Study Count ^d	Population Count ^e	Adjusted P Value ^f
GO:0042578	Phosphoric ester hydrolase activity	F	15	305	$<1.0 \times 10^{-300}$
GO:0009664	Plant-type cell wall organization	B	8	70	$<1.0 \times 10^{-300}$
GO:0007047	Cell wall organization	B	9	101	$<1.0 \times 10^{-300}$
GO:0045229	External encapsulating structure organization	B	9	118	$<1.0 \times 10^{-300}$
GO:0003993	Acid phosphatase activity	F	6	39	$<1.0 \times 10^{-300}$
GO:0016791	Phosphatase activity	F	12	252	0.002
GO:0005199	Structural constituent of cell wall	F	5	29	0.006
GO:0070882	Cell wall organization or biogenesis	B	9	160	0.010
GO:0010033	Response to organic substance	B	23	966	0.014
GO:0009719	Response to endogenous stimulus	B	20	768	0.016
GO:0042545	Cell wall modification	B	6	71	0.028
GO:0009827	Plant-type cell wall modification	B	5	44	0.032
GO:0009409	Response to cold	B	10	234	0.034

^aAccession number of the GO term. ^bGO term name. ^cNamespace or subontology (B, biological process; C, cellular component; F, molecular function). ^dThe number of genes in the study set annotated to the GO term in question. ^eThe number of genes in the population set annotated to the GO term in question. ^fAdjusted P value.

significant change in H₂O₂ homeostasis derived from PA oxidation does not seem to be the main cause of increased resistance. However, it cannot be fully discarded that subtle changes in ROS levels or localization actually occur in the above-mentioned GM Arabidopsis lines, either pre- or postinfection, which could be somehow related to differential resistance to *P. viridiflava*.

Spermine Levels and Transcriptional Changes Related to Defense Responses

In addition to the direct effects of spermine accumulation and oxidation on the ability of *P. viridiflava* to colonize and cause disease on host tissues, this tetraamine could also affect disease resistance at different levels, such as regulating the expression of genes involved in defense mechanisms. Other authors dem-

onstrated that exogenous spermine modulates the expression of Arabidopsis genes involved in cellular processes such as redox homeostasis and defense, as well as several transcription factors. Moreover, many genes induced by spermine are also up-regulated during Arabidopsis infection by CMV, thus suggesting the involvement of spermine in a defense signaling pathway (Mitsuya et al., 2009; Sagor et al., 2009).

In this work, it was demonstrated that constitutive enhancement of spermine levels by *SPMS* overexpression leads to changes in the transcriptome of Arabidopsis, as compared to wild-type plants. On the contrary, transcript profiles of an *spms* mutant showed less marked changes. Anyway, it should be kept in mind that, although in much lower levels than in wild-type plants, trace amounts of spermine are present in the *spms* mutants employed in this work. Therefore, it cannot be discarded that the transcriptome of plants

Table III. Overrepresented GO terms in the set of up-regulated genes in the *spms-2* mutant

Genes called up-regulated by the significance analysis of microarrays and associated to GO terms were used to perform a GO overrepresentation analysis using Ontologizer software with settings Term-For-Term/Westfall-Young-Single-Step. A total of 22,766 genes were in the population set, 63 of which were in the study set. Overrepresented GO categories (adjusted P value ≤ 0.05) are listed.

GO ID ^a	Name ^b	NSP ^c	Study Count ^d	Population Count ^e	Adjusted P Value ^f
GO:0008794	Arsenate reductase (glutaredoxin) activity	F	4	13	$<1.0 \times 10^{-300}$
GO:0030613	Oxidoreductase activity, acting on phosphorus or arsenic in donors	F	4	13	$<1.0 \times 10^{-300}$
GO:0030614	Oxidoreductase activity, acting on phosphorus or arsenic in donors, with disulfide as acceptor	F	4	13	$<1.0 \times 10^{-300}$
GO:0030611	Arsenate reductase activity	F	4	14	$<1.0 \times 10^{-300}$
GO:0016491	Oxidoreductase activity	F	11	988	0.048

^aAccession number of the GO term. ^bGO term name. ^cNamespace or subontology (B, biological process; C, cellular component; F, molecular function). ^dThe number of genes in the study set annotated to the GO term in question. ^eThe number of genes in the population set annotated to the GO term in question. ^fAdjusted P value.

that completely lack this tetraamine undergo even more profound changes than those hereby detected.

To understand how spermine increases Arabidopsis tolerance to *P. viridiflava*, a functional analysis of the genes over- and underexpressed in plants with enhanced levels of this tetraamine (35S::SPMS-9) was performed. Noteworthy, all the GO terms overrepresented in the set of genes up-regulated in 35S::SPMS-9 were related to perception and responses to different kind of stimuli associated with both biotic and abiotic stresses, thus supporting the idea that spermine plays a key role in stress responses. Moreover, the overrepresentation of GO terms related with responses to bacterium, biotic stimulus, as well as defense and immune responses, among others, could be related to increased resistance to *P. viridiflava* exhibited by the 35S::SPMS-9 line. In addition, disease resistance genes found in this work to be overexpressed in the 35S::SPMS-9 line could also be responsible for increased resistance. Similarly, some of the 47 transcription factors detected in 35S::SPMS-9 plants, 45 kinases, 32 DNA/RNA-binding proteins, and 49 nucleotide-binding proteins (Supplemental Table S1) could also be related to the activation of defense responses that increase Arabidopsis resistance to *P. viridiflava*.

Spermine-mediated enhancement of Arabidopsis resistance to *P. viridiflava* could also be due to the down-regulation of genes whose products somehow increase the susceptibility to this pathogen. In this regard, a link between some of the GO terms (phosphate and phosphoric acid-related enzyme activities) that were overrepresented in the down-regulated genes and the increase in resistance to *P. viridiflava* exhibited by the 35S::SPMS-9 line, cannot be easily envisaged. Taking into account the overrepresentation of GO terms related to cell wall biogenesis, modification, and organization among the genes down-regulated in 35S::SPMS-9, it would be interesting to evaluate if this line exhibits alterations of cell wall structure related to increased resistance to *P. viridiflava*. In this way, major differences are evident in the functional properties of the sets of genes up- and down-regulated in the spermine-accumulating transgenic line. Regarding genes involved in signaling and transcriptional regulation, several transcription factors and kinases different to the up-regulated ones were found to be down-regulated in the 35S::SPMS-9 line (Supplemental Tables S1 and S2). Therefore, further examination of the functional properties of the components of the signaling cascades that operate downstream of the transcription factors and other regulatory proteins hereby found to be up- and down-regulated by spermine could provide additional information about defense signaling mediated by this tetraamine.

Taking into account that SPMS overexpressors and mutants were respectively more resistant and susceptible to *P. viridiflava* than wild-type plants, it could be speculated that some genes related to defense responses exhibit contrasting patterns of expression in these two lines. However, no genes up-regulated in

35S::SPMS-9 and at the same time down-regulated in *spms-2* were detected in this study. Anyway, this finding does not rule out the possibility that transcriptional changes associated to modifications of spermine levels exert an effect on Arabidopsis resistance to *P. viridiflava*. In this way, spermine accumulation and deprivation could affect the expression of different sets of genes, both of which could differentially impact on Arabidopsis resistance to *P. viridiflava*.

Previously, it has been demonstrated that transcriptional changes induced by spermine in Arabidopsis are mainly related to signaling pathways triggered by H₂O₂ derived from spermine oxidation (Mitsuya et al., 2009; Sagor et al., 2009). In this work major changes in redox homeostasis were not evident, in that GO terms associated to ROS detoxification or related processes were not overrepresented in the 35S::SPMS-9 line. Moreover, transcription of many of the genes overexpressed in the 35S::SPMS-9 line was not reported to be regulated by ROS. These differences between the results of the above-mentioned reports and this one could be related to the different experimental approaches employed for enhancing plant spermine levels (exogenous supply versus genetic modifications of SPMS expression, respectively). Thus, only four out of 454 overexpressed genes in the 35S::SPMS-9 line used in this work were previously found to be up-regulated in Arabidopsis plants with exogenously supplied spermine (Mitsuya et al., 2009). Noteworthy, one of them was MPK3 (*At3g45640*), a kinase whose tobacco ortholog (WIPK) also participates in signaling cascades triggered by spermine (Takahashi et al., 2003). In this way, global changes in gene expression resulting from the accumulation of spermine caused by SPMS overexpression are not associated to major changes in redox homeostasis.

CONCLUSION

In summary, results obtained in this work show a direct link between spermine accumulation and oxidation and increased resistance to the biotrophic bacterium *P. viridiflava*, and demonstrate that increasing plant spermine levels by overexpression of the SPMS gene can lead to increased resistance. The mechanisms underlying spermine-mediated plant protection involve a variety of transcriptional changes. The identification of relevant spermine targets within this intricate signaling network represents a challenge for future research in this area.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The Columbia (Col-0) ecotype of Arabidopsis (*Arabidopsis thaliana*) was used as the wild type. Two Arabidopsis T-DNA insertion mutants of the SPMS gene were employed: the *spms-1* and *spms-2*. These mutants were obtained from the Kazusa DNA Research Institute and the Salk Institute (SALK_018902), respectively. The *spms-1* and *spms-2* mutants contain a T-DNA

insertion in the first intron, 180 and 553 bp upstream of the translational start codon, respectively (Imai et al., 2004). Four Arabidopsis transgenic lines (35S::SPMS-7, 35S::SPMS-8, 35S::SPMS-9, and 35S::SPMS-15) that overexpress the SPMS gene under the control of the CaMV 35S constitutive promoter (Minguet, 2008) were used. Both 35S::SPMS lines and *spms* mutants exhibit normal phenotypes in terms of growth and development, being similar to wild-type plants.

Seeds were surface disinfected before plating on Murashige and Skoog medium supplemented with 3% Suc w/v and stratified at 4°C for 2 d in the dark. Plates were incubated for 2 weeks in a growth chamber with a 16-h light/8-h dark photoperiod at 23°C/25°C, 55%/65% relative humidity (day/night), and a photon flux density of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool-white and GroLux fluorescent lamps.

Bacterial Strain, Plant Inoculation, and Disease Evaluation

Pseudomonas viridiflava strain Pvalb8 (Alippi et al., 2003) was kindly supplied by Dr. Adriana Alippi (Facultad de Agronomía, Universidad Nacional de La Plata, Argentina). This bacterium was cultivated at 28°C in King's B medium (King et al., 1954). For plant inoculation, bacterial cells were harvested by centrifugation, washed, and suspended in 10 mM MgCl₂ pH 7.0 to a final concentration of 4×10^8 CFU mL⁻¹.

Leaves of 15-d-old plants were inoculated with 5 μL of bacterial suspension. Five-microliter inoculums of 10 mM MgCl₂ pH 7.0 were used as controls. 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 disinfected with 70% (v/v) ethanol for 2 min and washed several times with sterile water. Plants were then homogenized in 10 mM MgCl₂ pH 7.0 and serial dilutions of the extracts thus obtained were plated on King's B agar medium. The number of CFU was determined after 24 h incubation at 28°C.

Disease incidence, defined as the percentage of diseased plants, was evaluated at different times after inoculation by determining the number of diseased plants among a total of 22 to 25 inoculated ones. Disease severity, defined as the level of damage in diseased plants, 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 Nikon SMZ binocular microscope (Nikon Instruments) coupled to a digital camera and further image analysis with the Image-ProPlus V 4.1 software (Media Cybernetics LP). 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. Ranks were as follows: 1, less than 10%; 2, 11% to 25%; 3, 26% to 50%; 4, 51% to 75%; and 5, 76% to completely necrotic leaf and lesions spreading to the rest of the plant.

Pharmacological Treatments and Chemicals

Putrescine, spermidine, and spermine were purchased from Sigma Chemical Co. (<http://www.sigmaaldrich.com>) and 1,7-heptanediamine from MP Biomedicals (formerly ICN Biomedicals, <http://www.mpbiomed.com>). 1,19-Bis(ethylamino)-5,10,15-triazanonadecane (SL-11061) was kindly gifted by Dr. Benjamin Frydman (SLIL Biomedical Corp.). All other chemicals were from commercial sources and of the highest purity available. Plant treatments with spermine and SL-11061 were performed by including these chemicals in the culture medium at the concentrations indicated elsewhere.

PA Analysis

PAs were extracted from 15-d-old plants by grinding samples (300 mg FW) in 1 mL of 5% (v/v) perchloric acid with a pestle and incubating at 4°C for 16 h. Extracts were dansylated as described previously (Marina et al., 2008). PA standards were treated in the same way as plant samples. Dansylated PAs were dissolved in 100 μL acetonitrile and analyzed by reversed-phase HPLC (Marcé et al., 1995).

Quantitative Real-Time PCR

Total RNA was extracted using TRI reagent (Sigma Chemical Co.) according to the manufacturer's instructions. First-strand cDNAs were synthesized using Moloney murine leukemia virus reverse transcriptase (Promega, <http://www.promega.com>). Quantitative real-time PCR reactions were per-

formed with specific primers using a FastStart universal SYBR green master with ROX (Roche, <http://www.roche.com>) on a Stratagene Mx3005P real-time qPCR system (Stratagene, http://www.gelifsciences.com/Apatrix/upp01077.nsf/content/argentina_hot_news_stratagene), according to the manufacturer's instructions. Two quantitative PCR replicates from two to four independent biological replications were performed on each cDNA sample. The relative expression was calculated as $2^{-(\Delta\text{Ct gene of interest}) - (\Delta\text{Ct reference gene})}$. Primers used for quantitative real-time PCR are listed in Supplemental Table S7.

Microarray Hybridization and Analyses

Total RNA from three biological replicates for each line (wild type, *spms-2*, and 35S::SPMS-9) under study (obtained as described previously), was treated as indicated by the GeneChip expression analysis technical manual (Affymetrix) to hybridize the ATH1 Arabidopsis GeneChip (Affymetrix) in the Unidad Central de Investigación de Medicina, Universidad de Valencia (Valencia, Spain; <http://scsie.uv.es/scsie-serv/ucim/equips.htm>). Raw data were processed with the RMAExpress software (Irizarry et al., 2003) for background adjustment and quantile normalization. The statistical technique of SAM3.0 was used to find differentially expressed genes in the set of microarray experiments (Tusher et al., 2001), by using a criteria for selection of fold change ≥ 1.5 and false discovery rate $\leq 5\%$. The Ontologizer 2.0 program (Shah and Fedoroff, 2004) was used to identify overrepresented GO terms in the sets of genes declared changed for each GM Arabidopsis line. Raw microarray data are presented in Supplemental Table S8.

ROS Analysis

ROS production was evaluated with the redox-sensitive dye 2',7'-dichlorofluorescein diacetate. Plants were incubated in 15 μM 2',7'-dichlorofluorescein diacetate for 4 h. Epifluorescence was observed with a Nikon Eclipse E600 microscope (Nikon, www.nikon.com) equipped with a 450 to 490 nm excitation filter and a 520 to 560 nm emission filter. Images were acquired with a Nikon DS-Qi1Mc video camera.

Statistical Analysis

Treatments consisted of three to five replicates, and each experiment was conducted at least twice with similar results. Results from representative experiments are shown as means \pm SD or SE, as indicated. Data were analyzed by ANOVA followed by posthoc comparisons by Dunnett's or Tukey's *t* test. qRT-PCR results were analyzed with REST© software V 2.0.7 (Pfaffl et al., 2002). Disease incidence data were analyzed by a nonparametric binomial test and frequency distribution of disease severity data were analyzed by the Kruskal-Wallis test followed by Dunnett's posthoc comparisons.

Sequence data from this article can be found in The Arabidopsis Information Resource or GenBank/EMBL data libraries under accession number At5g53120 (SPMS).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Free putrescine and spermidine levels in Arabidopsis wild-type plants infected by *P. viridiflava*.

Supplemental Figure S2. Free putrescine and spermidine levels in transgenic Arabidopsis 35S::SPMS lines and *spms* mutants.

Supplemental Figure S3. Modification of Arabidopsis spermine levels by exogenous addition of this tetraamine.

Supplemental Figure S4. Accumulation of ROS in wild-type Arabidopsis Col-0 plants supplemented with exogenous spermine.

Supplemental Table S1. List of genes declared changed both in Arabidopsis 35S::SPMS-9 and *spms-2*.

Supplemental Table S2. List of up-regulated genes in Arabidopsis 35S::SPMS-9.

Supplemental Table S3. List of down-regulated genes in Arabidopsis 35S::SPMS-9.

- Supplemental Table S4.** List of up-regulated genes in Arabidopsis *spms-2*.
- Supplemental Table S5.** List of down-regulated genes in Arabidopsis *spms-2*.
- Supplemental Table S6.** qRT-PCR validation of microarray data analysis.
- Supplemental Table S7.** Primers used for qRT-PCR.
- Supplemental Table S8.** Raw microarray data.

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LITERATURE CITED

- Alippi AM, Dal Bo E, Ronco LB, López MV, López AC, Aguilar OM (2003) *Pseudomonas* populations causing pith necrosis of tomato and pepper in Argentina are highly diverse. *Plant Pathol* **52**: 287–302
- Angelini R, Cona A, Federico R, Fincato P, Tavladoraki P, Tisi A (2010) Plant amine oxidases “on the move”: an update. *Plant Physiol Biochem* **48**: 560–564
- Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol* **55**: 373–399
- Bacchi CJ, Weiss LM, Lane S, Frydman B, Valasinas A, Reddy V, Sun JS, Marton LJ, Khan IA, Moretto M, et al (2002) Novel synthetic polyamines are effective in the treatment of experimental microsporidiosis, an opportunistic AIDS-associated infection. *Antimicrob Agents Chemother* **46**: 55–61
- Cohen SS (1998) *A Guide to Polyamines*. Oxford University Press, New York
- Cona A, Rea G, Botta M, Corelli F, Federico R, Angelini R (2006) Flavin-containing polyamine oxidase is a hydrogen peroxide source in the oxidative response to the protein phosphatase inhibitor cantharidin in *Zea mays L.* *J Exp Bot* **57**: 2277–2289
- Cowley T, Walters DR (2002) Polyamine metabolism in barley reacting hypersensitively to the powdery mildew fungus *Blumeria graminis f. sp. hordei*. *Plant Cell Environ* **25**: 461–468
- Fenton H (1894) Oxidation of tartaric acid in presence of iron. *J Chem Soc* **65**: 899–910
- Haber F, Weiss J (1934) The catalytic decomposition of hydrogen peroxide by iron salts. *Proc R Soc Lond A Math Phys Sci* **147**: 332–351
- Imai A, Akiyama T, Kato T, Sato S, Tabata S, Yamamoto KT, Takahashi T (2004) Spermine is not essential for survival of *Arabidopsis*. *FEBS Lett* **556**: 148–152
- Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP (2003) Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res* **31**: e15
- Jakob K, Goss EM, Araki H, Van T, Kreitman M, Bergelson J (2002) *Pseudomonas viridiflava* and *P. syringae*—natural pathogens of *Arabidopsis thaliana*. *Mol Plant Microbe Interact* **15**: 1195–1203
- King EO, Ward MK, Raney DE (1954) Two simple media for the demonstration of pyocyanin and fluorescin. *J Lab Clin Med* **44**: 301–307
- Knott JM, Römer P, Sumper M (2007) Putative spermine synthases from *Thalassiosira pseudonana* and *Arabidopsis thaliana* synthesize thermospermine rather than spermine. *FEBS Lett* **581**: 3081–3086
- López MA, Bannenberg G, Castresana C (2008) Controlling hormone signaling is a plant and pathogen challenge for growth and survival. *Curr Opin Plant Biol* **11**: 420–427
- Maiale SJ, Marina M, Sánchez DH, Pieckenstain FL, Ruiz OA (2008) In vitro and in vivo inhibition of plant polyamine oxidase activity by polyamine analogues. *Phytochemistry* **69**: 2552–2558
- Marcé M, Brown DS, Capell T, Figueras X, Tiburcio AF (1995) Rapid high-performance liquid chromatographic method for the quantitation of polyamines as their dansyl derivatives: application to plant and animal tissues. *J Chromatogr B Biomed Appl* **666**: 329–335
- Marina M, Maiale SJ, Rossi FR, Romero MF, Rivas EI, Gárriz A, Ruiz OA, Pieckenstain FL (2008) Apoplastic polyamine oxidation plays different roles in local responses of tobacco to infection by the necrotrophic fungus *Sclerotinia sclerotiorum* and the biotrophic bacterium *Pseudomonas viridiflava*. *Plant Physiol* **147**: 2164–2178
- Marini F, Betti L, Scaramagli S, Biondi S, Torrighiani P (2001) Polyamine metabolism is upregulated in response to tobacco mosaic virus in hypersensitive, but not in susceptible, tobacco. *New Phytol* **149**: 301–309
- Minguet EG (2008) Origen y función de las espermidina aminopropil transferasas en *Arabidopsis thaliana*. PhD thesis. Universidad Politécnica de Valencia, Valencia, Spain
- Mitsuya Y, Takahashi Y, Berberich T, Miyazaki A, Matsumura H, Takahashi H, Terauchi R, Kusano T (2009) Spermine signaling plays a significant role in the defense response of *Arabidopsis thaliana* to cucumber mosaic virus. *J Plant Physiol* **166**: 626–643
- Moschou PN, Sanmartin M, Andriopoulou AH, Rojo E, Sanchez-Serrano JJ, Roubelakis-Angelakis KA (2008) Bridging the gap between plant and mammalian polyamine catabolism: a novel peroxisomal polyamine oxidase responsible for a full back-conversion pathway in Arabidopsis. *Plant Physiol* **147**: 1845–1857
- Moschou PN, Sarris PE, Skandalis N, Andriopoulou AH, Paschalidis KA, Panopoulos NJ, Roubelakis-Angelakis KA (2009) Engineered polyamine catabolism preinduces tolerance of tobacco to bacteria and oomycetes. *Plant Physiol* **149**: 1970–1981
- Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* **30**: e36
- Rambla JL, Vera-Sirera F, Blázquez MA, Carbonell J, Granell A (2010) Quantitation of biogenic tetraamines in *Arabidopsis thaliana*. *Anal Biochem* **397**: 208–211
- Sagor GH, Cong RZ, Berberich T, Takahashi H, Takahashi Y, Kusano T (2009) Spermine signaling in defense reaction against avirulent viral pathogen in *Arabidopsis thaliana*. *Plant Signal Behav* **4**: 316–318
- Shah NH, Fedoroff NV (2004) CLENCH: a program for calculating Cluster ENrichment using the Gene Ontology. *Bioinformatics* **20**: 1196–1197
- Takahashi Y, Berberich T, Miyazaki A, Seo S, Ohashi Y, Kusano T (2003) Spermine signalling in tobacco: activation of mitogen-activated protein kinases by spermine is mediated through mitochondrial dysfunction. *Plant J* **36**: 820–829
- Takahashi Y, Uehara Y, Berberich T, Ito A, Saitoh H, Miyazaki A, Terauchi R, Kusano T (2004) A subset of hypersensitive response marker genes, including HSR203J, is the downstream target of a spermine signal transduction pathway in tobacco. *Plant J* **40**: 586–595
- Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* **98**: 5116–5121
- Walters DR (2003a) Polyamines and plant disease. *Phytochemistry* **64**: 97–107
- Walters DR (2003b) Resistance to plant pathogens: possible roles for free polyamines and polyamine catabolism. *New Phytol* **159**: 109–115
- Yamakawa H, Kamada H, Satoh M, Ohashi Y (1998) Spermine is a salicylate-independent endogenous inducer for both tobacco acidic pathogenesis-related proteins and resistance against tobacco mosaic virus infection. *Plant Physiol* **118**: 1213–1222
- Yoda H, Hiroi Y, Sano H (2006) Polyamine oxidase is one of the key elements for oxidative burst to induce programmed cell death in tobacco cultured cells. *Plant Physiol* **142**: 193–206
- Yoda H, Yamaguchi Y, Sano H (2003) Induction of hypersensitive cell death by hydrogen peroxide produced through polyamine degradation in tobacco plants. *Plant Physiol* **132**: 1973–1981