Running title: Spermine in *A. thaliana* defense

Corresponding author: Fernando L Pieckenstain

Unidad de Biotecnología 3

IIB-IINTECH/UNSAM-CONICET

Camino Circunvalación Laguna, Km. 6 CC 164

(B7130IWA) Chascomús, Provincia de Buenos Aires

ARGENTINA

Tel: 54 2241 424049

Fax: 54 2241 424048

Email: pieckenstain@intech.gov.ar

Research category

Plants interacting with other organisms
Perturbation of spermine synthase gene expression and transcript profiling provide new insights on the role of the tetraamine spermine in Arabidopsis thaliana defense against Pseudomonas viridiflava.

María Elisa Gonzalez1, Francisco Marco2,†, Eugenio Gómez Minguet3,††, Pedro Carrasco-Sorli2, Miguel Angel Blázquez3, Juan Carbonell3, Oscar Adolfo Ruiz1, and Fernando Luis Pieckenstain1*

1 Instituto de Investigaciones Biotecnológicas- Instituto Tecnológico de Chascomús (IIB-INTECh, UNSAM-CONICET); Camino Circunvalación Laguna, Km. 6 CC 164 (B7130IWA) Chascomús, Argentina
2 Departamento de Bioquímica y Biología Molecular, Universidad de Valencia. Facultad de Ciencias Biológicas, Dr. Moliner 50, 46100 Burjassot, Valencia, Spain
3 Instituto de Biología Molecular y Celular de Plantas (Universidad Politénica de Valencia-CSIC), 46022 Valencia, Spain
This work was financed by Agencia Española de Cooperación Internacional (PCI A/01190/07, Spain), Universidad Nacional de General San Martín (Project SA08/001, Argentina), CONICET (PIP 112-200801-00734 and PIP 04-5740) and Agencia Nacional de Promoción Científica y Tecnológica (PICT 04-26517, Argentina).

† Present address: Departamento de Biología Vegetal. Universidad de Valencia. Facultad de Farmacia. Av Vicent Andres Estelles s/n, 46100 Burjassot, Valencia, Spain

†† Present address: Laboratoire Physiologie Cellulaire Végétale - CNRS–CEA, 17 rue des Martyrs, 38 054 Grenoble Cedex 09, France

*Author for correspondence: Fernando L Pieckenstain

E-mail: pieckenstain@intech.gov.ar
ABSTRACT

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::SPMS) that overexpressed the spermine synthase (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 (WT) plants, while the latter were more susceptible. Exogenous supply of spermine to WT 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 analysed 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 WT. 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, 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*. 
INTRODUCTION

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 signalling network (Lopez 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 formation in response to infection by several pathogens (Takahashi et al., 2003; Yoda et al., 2003; Yoda et al., 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 ornithine and arginine by two alternative pathways. One of these pathways is shared by almost all organisms and involves ornithine decarboxylation by the enzyme ornithine decarboxylase (ODC, EC 4.1.1.17) to form putrescine. An alternative pathway occurs in plants and bacteria, where arginine is decarboxylated to agmatine by arginine decarboxylase (ADC, 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-methionine, a compound synthesized in a reaction catalyzed by S-adenosyl-methionine decarboxylase (AdoMetDC, EC 4.1.1.50) (Cohen, 1998).

Polyamine catabolism is mediated by diamine and polyamine oxidases (DAOs and PAOs, respectively). DAOs (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 A. thaliana, a species that harbours 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 reviews see (Walters, 2003; Walters, 2003)], 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 (TMV) 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 (Burkholder) Dowson and the necrotrophic fungus Sclerotinia sclerotiorum (Lib.) De Bary. 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 TMV-infected tobacco plants (Yamakawa et al., 1998). Later, Takahashi et al. (2003) demonstrated that spermine causes mitochondrial dysfunction via a signalling 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 A. thaliana to the cucumber mosaic virus (CMV). By employing the serial analysis of gene expression, the authors found that exogenously added spermine induced the expression of a number of A. thaliana genes in common to CMV infection and proposed that spermine plays a role in signalling defense responses of A. thaliana 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 analyse 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, A. thaliana 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. In order to identify potential mechanisms for spermine action in this context, global gene expression analyses were performed in spermine over-producing lines, as well as in mutants with decreased spermine levels.
RESULTS

Infection of *A. thaliana* plants by *P. viridiflava* leads to spermine accumulation

In order to evaluate the involvement of spermine in defense responses of *A. thaliana* against *P. viridiflava*, the effect of infection on the levels of this tetraamine was analysed. 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 (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.

Modification of spermine levels in *spms* mutants and transgenic plants that overexpress the *SPMS* gene

Spermine synthase is encoded in *A. thaliana* by a single copy of the *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 CaMV promoter, and checked *SPMS* expression level in the available *spms* knock-out 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 analysed (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 (WT) 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\(^{-1}\) FW of spermine, which represented between 2- and 3-fold higher
spermine levels compared to WT, while spms mutants contained lower spermine levels than WT 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 the present 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 the present work contains thermospermine levels similar to WT plants, thus demonstrating 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 lines and WT plants were highly reproducible. The levels of the other two most abundant PAs (putrescine and spermidine) were also evaluated in the genetically modified (GM) A. thaliana lines and were found to be similar to those of WT plants (Fig. S2).

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

Spermine accumulation and oxidation modulates the colonization of A. thaliana plants by the biotrophic bacterium P. viridiflava.

The effect of perturbations of endogenous free spermine levels on A. thaliana resistance to P. viridiflava was evaluated using the previously depicted spms mutants and transgenic lines, as well as WT 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 WT 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
shown), but differences between genetically modified and WT 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
watersoaked translucent spots, which developed into chlorotic and
subsequently necrotic lesions 72 HAI, as depicted by Jakob et al. (2002).
Disease incidence was 50% in WT 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 WT (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 WT 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 WT 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
analysed 72 HAI, the transgenic 35S::*SPMS*-9 line was found to be different
(*P*≤0.05) from the *spms*-2. No significant differences in frequency distribution
between WT plants and the other two plant lines were detected at this time
after inoculation. When analysed 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
in order 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 *A. thaliana* 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 polyamine 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 over-producing 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 *A. thaliana* 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 WT 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 in order to induce changes in plant spermine content that fell within the physiological range of concentrations and mimicked those of the 35S::SPMS-9 line (Fig. S3). Thus, wild-type and *spms*-2 plants, respectively supplemented with 30 and 50 µM spermine exhibited endogenous levels of this tetraamine similar to the transgenic line 35S::SPMS-9 (Fig. S3) and were used for the experiment described below.

As shown in Fig. 3, *P. viridiflava* propagation was reduced in 35S::SPMS-9, as compared to WT plants, while the opposite picture was found in *spms*-2 (Fig. 6). Supplementation of WT 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 WT plants to *P. viridiflava*. Moreover, the reduction of
bacterial growth mediated by spermine addition to WT plants was completely
prevented by SL-11061 (Fig. 6).

Accumulation of reactive oxygen species

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). *A. thaliana* 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$_2$O$_2$
production. Thus, reduced bacterial propagation in 35S::*SPMS*-9 plants
observed in the present work could be due to H$_2$O$_2$ accumulation, which
could either be toxic to bacterial cells or activate plant defenses. Moreover,
H$_2$O$_2$ could be converted to hydroxyl radical by the non-enzymatic 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 analysed in this work and thus no evidence
that 35S::*SPMS*-9 plants constitutively accumulate higher ROS levels than
WT plants was obtained. ROS levels were also analysed in plants infected by
*P. viridiflava*. Although infection elicited ROS accumulation, both 35S::*SPMS*-9
and *spms*-2 plants contained ROS levels similar to WT plants 48 and 72
HAI (data not shown). ROS levels were also analysed in WT plants
supplemented with different spermine concentrations. In this case, ROS
accumulation was evident only after treatment with 500 µM or higher
spermine concentrations (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 spermine synthase 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 A. thaliana plants to P. viridiflava by other mechanisms. To test this hypothesis, an overview of transcriptional changes induced by perturbations of spermine levels in genetically modified plants was obtained by a transcriptomic approach. Transcript profiles of the 35S::SPMS-9 and spms-2 plants were compared to WT A. thaliana. 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 (Table S1). In this way, 454 (602-148) and 63 (211-148) genes were exclusively overexpressed in the 35S::SPMS-9 (Table S2) and spms-2 lines (Tables S4), respectively. Similarly, among the 312 repressed genes in the 35S::SPMS-9 line, 107 were also repressed in spms-2 (Table S1). Therefore, 205 (312-107) and 51 (158-107) genes were exclusively repressed in the 35S::SPMS-9 (Table S3) and spms-2 lines (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 over-production 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 WT 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 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 analysed, nine were declared overexpressed by SAM3.0, which were also confirmed by qRT-PCR (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 (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 (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 WT plants, were analysed by evaluating GO term over-representation. GO terms involved in responses to carbohydrate, jasmonic acid, organic substances, biotic, endogenous, hormone, chemical and other stimulus were over-represented 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 over-represented 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 over-represented 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 over-represented 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 over-represented in this set of genes (Table II).

The number of GO annotations over-represented 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 over-represented in this set of genes (Table III). No GO term was over-represented in the set of genes only down-regulated in spms-2.

Finally, it is interesting to point out that twenty-one members of several disease resistance protein families, were constitutively overexpressed in 35S::SPMS-9 (Table S2). Moreover, the transmembrane receptors RPS2 (At4g26090) and RPS6 (At5g46470) involved in A. thaliana responses to P. syringae, and a gene (At1g35710) that confers resistance to P. syringae strains harbouring the avrB avirulence gene, were also overexpressed in 35S::SPMS-9 (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 (Table S2).
DISCUSSION

Spermine accumulation, oxidation, and *A. thaliana* 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 SAGE 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 *A. thaliana* to CMV. However, the manipulation of plant spermine levels through the modification of *SPMS* gene expression has not been used as tool to investigate how spermine affects plant defense responses and resistance to pathogens. Thus, in the present work, genetically modified *A. thaliana* 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 *A. thaliana* plants that accumulated higher spermine levels than the WT as a consequence of overexpression of the *SPMS* gene under the control of the 35SCaMV promoter were used, along with *spms* mutants with decreased spermine levels. Results obtained after analysing the response of the above-mentioned plants to *P. viridiflava* infection demonstrate that spermine levels significantly affect the resistance of *A. thaliana* to this bacterium. In this sense, the lack of *SPMS* expression and the consequent reduction of spermine levels rendered *A. thaliana* plants more susceptible to *P. viridiflava*. Conversely, spermine accumulation as a consequence of *SPMS* overexpression drastically increased *A. thaliana* 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 WT plants. It is worth pointing out that exogenous spermine concentrations were carefully selected in order 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 *A. thaliana* resistance to *P. viridiflava* provided by spermine accumulation could be due to different mechanisms. PA oxidation by different amine oxidases can lead to H$_2$O$_2$ 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 *A. thaliana* resistance to *P. viridiflava* conferred by spermine was evaluated using the PA oxidase inhibitor SL-11061, a PA analogue previously found to inhibit tobacco (Marina et al., 2008), maize and oat 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 analysing the consequences of inhibition of PA oxidation in WT plants supplemented with spermine and 35S::SPMS-9 plants, demonstrated that the increase in resistance of *A. thaliana* to *P. viridiflava* mediated by spermine accumulation depends, at least in part, on the ability of the plant to oxidise 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 *A. thaliana* resistance to cucumber mosaic virus (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 non-host 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 *A. thaliana* plants against *P. viridiflava* is due to increased H$_2$O$_2$ levels *in planta*, as a result of spermine oxidation. In this sense, post-infection H$_2$O$_2$ 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 the present work, both pre- and post-infection levels of ROS in 35S::SPMS-9 plants were similar to those of WT 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 (≥ 500 µM), which results in the accumulation of non-physiological 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 WT 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 pre-infection levels of spermine and the ability to oxidise this tetraamine, a significant change in H$_2$O$_2$ 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 genetically modified *A. thaliana* lines, either pre- or post-infection, 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 colonise 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 demonstrated that exogenous spermine modulates the expression of *A. thaliana* 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 *A. thaliana* infection by CMV, thus suggesting the involvement of spermine in a defense signalling pathway (Mitsuya et al., 2009; Sagor et al., 2009).

In the present work, it was demonstrated that constitutive enhancement of spermine levels by *SPMS* overexpression leads to changes in the transcriptome of *A. thaliana*, as compared to WT 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 WT plants, trace amounts of spermine are present in the *spms* mutants...
employed in the present work. Therefore, it cannot be discarded that the transcriptome of plants that completely lack this tetraamine undergo even more profound changes than those hereby detected.

To understand how spermine increases *A. thaliana* 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 over-represented 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 over-representation 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 the present 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 (Table S1) could also be related to the activation of defense responses that increase *A. thaliana* resistance to *P. viridiflava*.

Spermine-mediated enhancement of *A. thaliana* 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 over-represented 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 over-representation 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 signalling 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 (Tables S1 and S2). Therefore,
further examination of the functional properties of the components of the
signalling 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 signalling
mediated by this tetraamine.

Taking into account that SPMS overexpressors and mutants were
respectively more resistant and susceptible to P. viridiflava than WT 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 the present study. Anyway, this finding does not rule out the
possibility that transcriptional changes associated to modifications of
spermine levels exert an effect on A. thaliana 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 A.
thaliana resistance to P. viridiflava.

Previously, it has been demonstrated that transcriptional changes
induced by spermine in A. thaliana are mainly related to signalling pathways
triggered by H₂O₂ derived from spermine oxidation (Mitsuya et al., 2009;
Sagor et al., 2009). In the present work major changes in redox homeostasis
were not evident, in that GO terms associated to ROS detoxification or
related processes were not over-represented 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 the
present one could be related to the different experimental approaches
employed for enhancing plant spermine levels (exogenous supply vs. genetic
modifications of SPMS expression, respectively). Thus, only four out of 454
overexpressed genes in the 35S::SPMS-9 line used in the present work were
previously found to be up-regulated in A. thaliana 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
signalling 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 the present 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 signalling network represents a challenge for future research in this area.
EXPERIMENTAL PROCEDURES

Plant material and growth conditions

The Columbia (Col-0) ecotype of *A. thaliana* was used as the WT. Two *A. thaliana* 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 *A. thaliana* 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 WT plants.

Seeds were surface disinfected before plating on MS medium supplemented with 3% sucrose w/v and stratified at 4ºC for two days in the dark. Plates were incubated for two weeks in a growth chamber with a 16-h light/8-h dark photoperiod at 23/25ºC, 55/65% relative humidity (day/night) and a photon flux density of 200 µmol m⁻² sec⁻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 Dra. Adriana Alippi *(Facultad de Agronomía, Universidad Nacional de La Plata, Argentina)*. This bacterium was cultivated at 28ºC 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 4x10⁸ CFU mL⁻¹.

Leaves of 15-day-old plants were inoculated with 5 µL of bacterial suspension. Five µL 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 Inc, Melville, NY, USA) coupled to a digital camera and further image analysis with the Image-Pro®Plus V 4.1 software (Media Cybernetics LP, 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. Ranks were as follows: 1, less than 10%; 2, 11 to 25%; 3, 26 to 50%; 4, 51 to 75%; 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 ICN Biomedicals (http://www.icnbiomed.com/). 1,19-Bis(ethylamino)-5,10,15-triazanonadecane (SL-11061) was kindly gifted by Dr. Benjamin Frydman (SLIL Biomedical Corporation). 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.
Polyamine analysis

PAs were extracted from 15-day-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). Polyamine standards were treated in the same way as plant samples. Dansylated PAs were dissolved in 100 µL acetonitrile and analysed 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 (MMLV-RT) (Promega, http://www.promega.com). Quantitative real-time PCR reactions were performed 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.gelifesciences.com/Aptrix/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 $2^{-\Delta \Delta Ct \text{ gene of interest}}$ – $\Delta \Delta Ct \text{ reference gene}$. Primers used for quantitative real-time PCR are listed in Table S7.

Microarray hybridization and analyses

Total RNA from three biological replicates for each line (WT, spms-2 and 35S::SPMS-9) under study (obtained as described previously), was treated as indicated by the GeneChip® Expression Analysis technical manual (Affymetrix) in order 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 Significance Analysis of Microarrays (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 ≤5%. The Ontologizer 2.0 program (Shah and Fedoroff, 2004) was used to identify over-represented GO terms in the sets of genes declared changed for each GM A. thaliana line. Raw microarray data are presented in table S8.

Reactive Oxygen Species analysis

Reactive oxygen species (ROS) production was evaluated with the redox-sensitive dye 2′,7′-dichlorofluorescein diacetate (DCFH-DA). Plants were incubated in 15 µM DCFH-DA for 4 h. Epifluorescence was observed with a Nikon Eclipse E600 microscope (Nikon, www.nikon.com) equipped with a 450–490 nm excitation filter and a 520–560 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 ± SD or SE, as indicated. Data were analysed by ANOVA followed by post-hoc comparisons by Dunnet’s or Tukey’s T test. QRT-PCR results were analysed with REST© software V2.0.7 (Pfaffl et al., 2002). Disease incidence data were analysed by a non-parametric binomial test and frequency distribution of disease severity data was analysed by the Kruskal-Wallis test followed by Dunns’ post-hoc comparisons.
ACKNOWLEDGEMENTS

MEG is a doctoral fellow of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). OAR and FLP are members of the Research Career of CONICET.

LITERATURE CITED


Fenton H (1894 ) Oxidation of tartaric acid in presence of iron. . Journal of Chemical Society 65 899–910


Marina M, Maiale SJ, Rossi FR, Romero MF, Rivas E1, Garriz 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 Physiology **147**: 2164-2178


Pfaffl M, Horgan G, Dempfle L (2002) Relative expression software tool (REST(C)) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Research **30**: e36


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 Signaling & Behavior **4**: 316-318


FIGURE LEGENDS

Figure 1. Time-course evolution of free spermine levels in Col0 A. thaliana plants infected by P. viridiflava. Infected plants (hatched bars) are compared to non-infected controls (gray bars). Statistically significant differences between both groups according to one-way ANOVA and Dunnett's test are shown as *, $P \leq 0.05$. HAI, hours after inoculation. Results are the mean of 3-4 replicates ± SD.

Figure 2. Spermine synthase gene expression in genetically modified A. thaliana plants. QRT-PCR was used to analyse the abundance of SPMS transcripts in two spermine synthase mutants (white bars) and four transgenic lines that overexpress the SPMS gene under the control of the 35SCaMV promoter (black bars). Transcript levels were normalized to the average signal intensity of WT plants. Results are the mean of 3 replicates ± SE and statistically significant differences in gene expression between GM lines and WT (gray bar), as analysed with the REST© software, are shown as ***, $P \leq 0.001$.

Figure 3. Free spermine levels in A. thaliana plants genetically modified in spermine biosynthesis. Spermine was quantified by HPLC in WT A. thaliana Col0 (gray bars), spermine synthase mutants (white bars) and transgenic lines that overexpress the spermine synthase gene under the control of the 35SCaMV promoter (black bars). Results are the mean of 2-3 replicates ± SD and statistically significant differences in spermine levels between GM lines and WT according to one-way ANOVA and Dunnett’s test are shown as *, $P \leq 0.05$.

Figure 4. Propagation of P. viridiflava in A. thaliana plants genetically modified in spermine biosynthesis. WT (gray bars), spermine synthase mutants (white bars) and transgenic lines that overexpress the spermine synthase gene (black bars) were inoculated with P. viridiflava. The number of colony forming units (CFU) per plant was evaluated 72 h after inoculation.
Results are the mean of 3-6 replicates ± SD and statistically significant differences between GM lines and WT plants according to one-way ANOVA and Dunnett’s test are shown as *, P≤0.05; **, P≤0.01; ***, P≤0.001.

**Figure 5.** Disease incidence and severity caused by *P. viridiflava* infection of *A. thaliana* plants genetically modified (GM) in spermine biosynthesis. WT (gray bars), *spermine synthase* mutant (white bars) and a transgenic line that overexpress the *spermine synthase* gene (black bars) were inoculated with a suspension of *P. viridiflava* cells. Disease incidence was evaluated 72 (A) and 144 h after inoculation (HAI) (B) as the percentage of diseased plants over a total of 22 to 25 inoculated plants. Statistically significant differences between GM lines and WT according to a non-parametric binomial test are shown as *, P≤0.05; **, P≤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-Pro®Plus 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 analysing the number of plants comprised in each rank. Statistically significant differences between distribution frequencies of each genotype were analysed by the non-parametric Kruskal-Wallis test followed by Dunns’ post-hoc comparisons. Frequency distribution of the 35S::*SPMS-9* line was found to be different from the *spms-2* mutant (P≤ 0.05). (E) Level of leaf damage corresponding to each severity rank.

**Figure 6.** Oxidation is crucial for spermine-mediated restriction of *P. viridiflava* multiplication in *A. thaliana*. WT (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 axe. Plants grown without both compounds were used as controls. Black bars represent bacterial growth in plants of the *spermine synthase*-overexpressing line 35S::*SPMS-9* without exogenous supply of...
spermine. Plants were inoculated with \textit{P. viridiflava} and the number of colony forming units (CFU) per plant was evaluated 72 h after inoculation. Results are the mean of 3-6 replicates \pm SD and statistically significant differences in bacterial growth between treatments and controls according to one-way ANOVA and Dunnett's test (WT Col0 and \textit{spms}-2) or Student's T test (\textit{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.
Table I. Over-represented GO terms in the set of up-regulated genes in the *spermine synthase* overexpressing line 35S::SPMS-9

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Genes called up-regulated by the significance analysis of microarrays and associated to GO terms were used to perform a gene ontology (GO) over-representation 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. Over-represented GO categories (Adj. P-value ≤ 0.05) are listed. 1 Accession number of the GO term, 2 GO term name, 3 namespace or subontology (B, biological process; C, cellular component; F, molecular function), 4 and 5 represent the number of genes in the study and population sets annotated to the GO term in question, 6 adjusted P-Value.
Table II.- Over-represented GO terms in the set of down-regulated genes in the spermine synthase overexpressing line 35S::SPMS-9

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<td>structural constituent of cell wall</td>
<td>F</td>
<td>5</td>
<td>29</td>
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<td>GO:0070882</td>
<td>cell wall organization or biogenesis</td>
<td>B</td>
<td>9</td>
<td>160</td>
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<tr>
<td>GO:0010033</td>
<td>response to organic substance</td>
<td>B</td>
<td>23</td>
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</tr>
<tr>
<td>GO:0009719</td>
<td>response to endogenous stimulus</td>
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<td>20</td>
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<td>GO:0042545</td>
<td>cell wall modification</td>
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<td>6</td>
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<td>GO:0009827</td>
<td>plant-type cell wall modification</td>
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<td>response to cold</td>
<td>B</td>
<td>10</td>
<td>234</td>
<td>0.034</td>
</tr>
</tbody>
</table>

Genes called down-regulated by the significance analysis of microarrays and associated to GO terms were used to perform a gene ontology (GO) over-representation 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. Over-represented GO categories (Adj. P-value ≤0.05) are listed. 1 Accession number of the GO term; 2 GO term name; 3 namespace or subontology (B, biological process; C, cellular component; F, molecular function); 4 and 5 represent the number of genes in the study and population sets annotated to the GO term in question; 6 adjusted P-Value.
Table III.- Over-represented GO terms in the set of up-regulated genes in the *spms*-2 mutant

<table>
<thead>
<tr>
<th>GO ID</th>
<th>Name</th>
<th>NSP</th>
<th>Study count</th>
<th>Pop. Count</th>
<th>Adj. P-value</th>
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<td>GO:0008794</td>
<td>arsenate reductase (glutaredoxin) activity</td>
<td>F</td>
<td>4</td>
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<td>&lt;1.0 x 10^-300</td>
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<tr>
<td>GO:0030613</td>
<td>oxidoreductase activity, acting on phosphorus or arsenic in donors</td>
<td>F</td>
<td>4</td>
<td>13</td>
<td>&lt;1.0 x 10^-300</td>
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<tr>
<td>GO:0030614</td>
<td>oxidoreductase activity, acting on phosphorus or arsenic in donors, with disulfide as acceptor</td>
<td>F</td>
<td>4</td>
<td>13</td>
<td>&lt;1.0 x 10^-300</td>
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<td>F</td>
<td>4</td>
<td>14</td>
<td>&lt;1.0 x 10^-300</td>
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<td>GO:0016491</td>
<td>oxidoreductase activity</td>
<td>F</td>
<td>11</td>
<td>988</td>
<td>0.048</td>
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</tbody>
</table>

Genes called up-regulated by the significance analysis of microarrays and associated to GO terms were used to perform a gene ontology (GO) over-representation 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. Over-represented GO categories (Adj. P-value ≤0.05) are listed. ¹Accession number of the GO term, ²GO term name, ³namespace or subontology (B, biological process; C, cellular component; F, molecular function), ⁴and ⁵represent the number of genes in the study and population sets annotated to the GO term in question, ⁶adjusted P-Value.
SUPPLEMENTAL DATA

Figure S1. Free putrescine and spermidine levels in A. thaliana WT plants infected by P. viridiflava.

Figure S2. Free putrescine and spermidine levels in transgenic A. thaliana 35S::SPMS lines and spms mutants.

Figure S3. Modification of A. thaliana spermine levels by exogenous addition of this tetraamine.

Figure S4. Accumulation of reactive oxygen species in WT A. thaliana Col0 plants supplemented with exogenous spermine.

Table S1. List of genes declared changed both in A. thaliana 35S::SPMS-9 and spms-2.

Table S2. List of up-regulated genes in A. thaliana 35S::SPMS-9.

Table S3. List of down-regulated genes in A. thaliana 35S::SPMS-9.

Table S4. List of up-regulated genes in A. thaliana spms-2.

Table S5. List of down-regulated genes in A. thaliana spms-2.

Table S6. QRT-PCR validation of microarray data analysis

Table S7: Primers used for qRT-PCR

Table S8: Raw microarray data
Figure 1. Time-course evolution of free spermine levels in Col0 Arabidopsis thaliana plants infected by P. viridiflava (hatched bars) as compared to non-infected plants (gray bars). Statistically significant differences between infected plants and controls according to one-way ANOVA and Dunnett's test are shown as *, P=0.05. HAI, hours after inoculation. Results are the mean of 3-4 replicates ± SD.
Figure 2: Gonzalez et al
Running Title: Spermine in A. thaliana defense

Figure 2. Spermine synthase gene expression in genetically modified A. thaliana plants. QRT-PCR was used to analyse the abundance of SPMS transcripts in two spermine synthase mutants (white bars) and four transgenic lines that overexpress the SPMS gene under the control of the 35SCaMV promoter (black bars). Transcript levels were normalized to the average signal intensity of WT plants. Results are the mean of 3 replicates ± SE and statistically significant differences in gene expression between GM lines and WT (gray bar), as analysed with the REST© software, are shown as ***, \( P=0.001 \).
Figure 3: Gonzalez et al  
Running Title: Spermine in A. thaliana defense

Figure 3. Free spermine levels in A. thaliana plants genetically modified in spermine biosynthesis. Spermine was quantified by HPLC in WT A. thaliana Col0 (gray bars), spermine synthase mutants (white bars) and transgenic lines that overexpress the spermine synthase gene under the control of the 35SCaMV promoter (black bars). Results are the mean of 2-3 replicates ± SD and statistically significant differences in spermine levels between GM lines and WT according to one-way ANOVA and Dunnett's test are shown as *, $P \leq 0.05$. 

Spm levels GM lines (Fig 3) Gonzalez et al.pzf:Spermine [Data Set-A] graph - Sun Oct 31 23:41:27 2010
**Figure 4.** Propagation of *P. viridiflava* in *A. thaliana* plants genetically modified in spermine biosynthesis. WT (gray bars), spermine synthase mutants (white bars) and transgenic lines that overexpress the spermine synthase gene (black bars) were inoculated with *P. viridiflava*. The number of colony forming units (CFU) per plant was evaluated 72 h after inoculation. Results are the mean of 3-6 replicates ± SD and statistically significant differences between GM lines and WT plants according to one-way ANOVA and Dunnett's test are shown as *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. 

**Running Title:** Spermine in *A. thaliana* defense
Figure 5. Disease incidence and severity caused by P. viridiflava infection of A. thaliana plants genetically modified (GM) in spermine biosynthesis. WT (gray bars), spermine synthase mutant (white bars) and a transgenic line that overexpress the spermine synthase gene (black bars) were inoculated with a suspension of P. viridiflava cells. Disease incidence was evaluated 72 (A) and 144 h after inoculation (HAI) (B) as the percentage of diseased plants over a total of 22 to 25 inoculated plants. Statistically significant differences between GM lines and WT according to a non-parametric binomial test are shown as *, P ≤ 0.05; **, P ≤ 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-Pro®Plus 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 analysing the number of plants comprised in each rank. Statistically significant differences between distribution frequencies of each genotype were analysed by the non-parametric Kruskal-Wallis test followed by Dunns’ post-hoc comparisons. Frequency distribution of the 35S::SPMS-9 line was found to be different from the spms-2 mutant (P ≤ 0.05). (E) Level of leaf damage corresponding to each severity rank.
Figure 6. Oxidation is crucial for spermine-mediated restriction of *P. viridiflava* multiplication in *A. thaliana*. WT (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 *spermine synthase*-overexpressing line 35S::SPMS-9 without exogenous supply of spermine. Plants were inoculated with *P. viridiflava* and the number of colony forming units (CFU) per plant was evaluated 72 h after inoculation. Results are the mean of 3-6 replicates SD and statistically significant differences in bacterial growth between treatments and controls according to one-way ANOVA and Dunnett’s test (WT Col0 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.
Figure S1. Gonzalez et al
Running Title: The tetraamine spermine in A. thaliana defense against P. viridiflava

Figure S1. Time-course evolution of free putrescine (A) and spermidine (B) levels in Col0 A. thaliana plants infected by P. viridiflava. Infected plants (hatched bars) are compared to non-infected controls (gray bars). Statistically significant differences between both groups according to one-way ANOVA and Dunnett's test are shown as *, $P \leq 0.05$. HAI, hours after inoculation. Results are the mean of 3-4 replicates ± SD.
**Figure S2** Gonzalez *et al*

**Running Title:** The tetraamine spermine in *A. thaliana* defense against *P. viridiflava*

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**Figure S2.** Free putrescine (A) and spermidine (B) levels in *A. thaliana* plants genetically modified in spermine biosynthesis. Polyamines were quantified by HPLC in WT *A. thaliana* Col0 (gray bars), *spermine synthase* mutants (white bars) and transgenic lines that overexpress the *spermine synthase* gene under the control of the 35SCaMV promoter (black bars). Results are the mean of 2-3 replicates ± SD and statistically significant differences in spermine levels between GM lines and WT according to one-way ANOVA and Dunnett's test are shown as *, *P*≤0.05.
**Figure S3** Gonzalez *et al*

**Running Title:** The tetraamine spermine in *A. thaliana* defense against *P. viridiflava*

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**Figure S3.** Modification of *A. thaliana* endogenous spermine levels by supplementation with exogenous spermine. Wild type Col0 (grey bars) and the spermine synthase mutant *spms-2* (white bars) were grown in culture medium amended with different spermine concentrations (up to 100 µM). Plant spermine concentration was analysed in 15-day-old plants by HPLC. Spermine concentration of the spermine synthase-overexpressing line 35S::SPMS-9 (black bar) is included for comparison. Results are the mean of 3-4 replicates ± SD.
Figure S4 Gonzalez et al
Running Title: The tetraamine spermine in *A. thaliana* defense against *P. viridiflava*

Accumulation of reactive oxygen species in wild type *A. thaliana* Col0 plants supplemented with exogenous spermine. Plants were grown for 15 days in MS medium amended with 30; 100; 500 and 1,000 μM spermine and then transferred to a 15 μM dichlorofluorescein diacetate solution. Epifluorescence microscopy images of leaf portions from plants grown with 30 (B) and 500 μM spermine (C) are shown. Plants grown without spermine served as controls (A). Scale bar represents 200 μm.