RNAi-mediated silencing of spermidine synthase gene results in reduced reproductive potential in tobacco

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Abstract Spermidine belongs to a class of polycationic compounds known as polyamines. Polyamines are known to be involved in a wide range of biological processes but the exact role and contribution of different polyamines to these processes are still not clear. In the present study, we have tried to understand the contribution of triamine spermidine to the growth and development of tobacco by downregulating spermidine synthase gene (*SPDS*) using RNA interference. Down-regulation of *SPDS* gene resulted in decreased spermidine levels and a slight increase in the levels of its precursor, the diamine putrescine and the molecule downstream of Spd, the tetraamine spermine. While the vegetative growth of the transgenics remained largely unaffected, *SPDS* down-regulation resulted in smaller size of flowers, decreased pollen viability and seed setting, and a reduced and delayed seed germination. When subjected to abiotic stress, the transgenics showed an increased tolerance to salinity and drought conditions owing to a steady intracellular pool of putrescine and spermine. The results not only highlight the importance of spermidine in determining reproductive potential in plants but have also help delineate its function from that of putrescine and spermine.

Keywords Polyamines · Spermidine synthase · RNAi · Reproductive potential · Abiotic stress tolerance · Tobacco

Abbreviations

PA Polyamine

RNAi	RNA interference
Put	Putrescine
Spd	Spermidine
Spm	Spermine
eIF5A	Eukaryotic translation initiation factor 5A
ODC	Ornithine decarboxylase
ADC	Arginine decarboxylase
SPDS	Spermidine synthase
SAM	S-Adenosylmethionine
dcSAM	Decarboxylated S-adenosylmethionine
SAMDC	S-Adenosylmethionine decarboxylase
SPM	Spermine synthase
PMT	Putrescine N-methyltransferase

Introduction

Polyamines (PAs) have been a quintessential part of prokaryotic and eukaryotic organisms since time immemorial. They are low molecular weight, aliphatic cations that are ubiquitously found in all living organisms, except for the two orders of Archaeobacteria, Methanobacteriales and Halobacteriales (Wallace et al. 2003). The three most common forms of PAs include diamine putrescine (Put), triamine spermidine (Spd) and tetraamine spermine (Spm). By the virtue of remaining protonated at physiological pH, they regulate a number of cellular functions ranging from cell division, differentiation and development to cell death (Kaur-Sawhney et al. 2003). They function through a variety of growth and developmental processes including organogenesis, root formation, floral induction, pollination, micro- and mega- sporogenesis, seed development, embryogenesis, fruit ripening and senescence, besides mediating biotic and abiotic stress responses (Baron and Stasolla 2008; Kusano et al. 2008;

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Igarashi and Kashiwagi 2010; Moschou et al. 2012; Tiburcio et al. 2014; Sequera-Mutiozabal et al. 2017). In eukaryotes, PAs affect translation by undertaking Spd-dependent hypusine modification of eukaryotic translation initiation factor 5A (eIF5A) at a specific lysine residue (Cooper et al. 1983). Lysine is modified to the unusual amino acid hypusine by the addition of the 4-aminobutyl moiety from Spd (Park et al. 2010). Hypusinated eIF5A is essential for the translation of polyproline motifs (Gutierrez et al. 2013).

The first PA to be synthesized is diamine Put, which in most organisms, is formed by decarboxylation of amino acid ornithine in a step catalyzed by the enzyme ornithine decarboxylase (ODC, EC 4.1.1.17). In addition to ODC pathway, plants and bacteria have an additional pathway for Put formation, which is mediated by arginine decarboxylase (ADC; EC 4.1.1.19). Put is further converted to higher PAs, Spd and Spm by sequential addition of an aminopropyl group derived from decarboxylated S-adenosylmethionine (dcSAM) in a reaction that is catalyzed by the enzyme Spd synthase (SPDS; EC 2.5.1.16) and Spm synthase (SPM; EC 2.5.1.22) respectively. The aminopropyl group donor, dcSAM, is produced from S-adenosylmethionine (SAM) by SAM decarboxylase (SAMDC; EC 2.5.1.16) (Slocum 1991). Another PA, garnering much attention these days, is a structural isomer of spermine called thermospermine that has been shown to have a specific role of repressing xylem differentiation in higher plants (Takano et al. 2012). Thermospermine synthase, the gene which has not been characterized in tobacco so far, uses Spd as a substrate to synthesize thermospermine. The diamine Put is also acted upon by Putrescine N-methyltransferase (PMT), which catalyzes S-adenosylmethionine (SAM)-dependent methylation of Put for alkaloid biosynthesis. Due to sequence similarity between *PMT* and *SPDS*, it is hypothesized that the gene for *PMT* may have been derived from *SPDS* (Biastoff et al. 2009).

In dicotyledons, there are two paralogous genes with conserved intron–exon structures that are known to code for *SPDS* (Carbonell and Blázquez 2009). *SPDS* has been shown to be essential in plants as double mutants for the genes encoding for *SPDS1* and *SPDS2*, are embryo lethal (Imai et al. 2004). PAs, including Spd, are also known to play a significant role in plant stress responses- protecting plants during severe stressful events such as drought and soil salinization (Sequera-Mutiozabal et al. 2017). Constitutive expression of *SPDS* gene from *Cucurbita ficifolia* in *Arabidopsis thaliana* resulted in a significant increase in *SPDS* activity and Spd content in leaves and showed enhanced tolerance to various abiotic stresses, including chilling, freezing, salinity, hyperosmosis, drought, and paraquat toxicity (Kasukabe et al. 2004). Apart from these few reports, most of the studies on Spd in plants have

focused on the manipulation of *SAMDC* (Waie and Rajam 2003; Chen et al. 2014) for altering Spd levels rather than manipulating *SPDS* expression.

Manipulation of intracellular PA titres is more effective when the expression levels of PA biosynthesis genes are lowered compared to the manipulation achieved by over-expression of PA biosynthesis genes. There are many studies to show an increased level of Put titres in response to over-expression of *ADC* or *ODC* gene without any changes in the levels of Spd (DeScenzo and Minocha 1993; Burtin and Michael 1997; Masgrau et al. 1997; Mayer and Michael 2003). Similarly, over-expression of *SAMDC* or *SPDS* results in the increase in Spd levels (Franceschetti et al. 2004). On the other hand, reduction in the endogenous levels of *ADC/ODC* transcripts leads to depletion of both Put and Spd pools (Trung-Nghia et al. 2003). There are number of inhibitor, mutant and down-regulation studies that have shown the decreased levels of *SAMDC* or *SPDS* transcripts reduce Spd and Spm titres along with an accumulation of Put, which makes the down-regulation of PA biosynthesis genes a more efficient approach for altering PA titres and studying their functions (Chen et al. 2014).

The aim of the present study was to study the morphological and physiological implications of *SPDS* knock-down in order to evaluate its importance and contribution to PA functions. Another objective was to see the effects of lowered *SPDS* transcripts and altered PA levels on the transcript abundance of other PA-pathway related genes to assess the extent of genetic contribution to PA homeostasis. In many dictos, two genes are known to code for *SPDS*, but only one gene is reported in tobacco. Since only the cDNA sequence of the gene was available for tobacco, we have deciphered the whole gene sequence for tobacco *SPDS* gene and multiple introns were detected in the sequence.

Materials and methods

Deciphering the complete gene sequence of *SPDS* gene

SPDS gene is known to contain introns in many other plant species, but the whole gene sequence was not available for *Nicotiana tabacum*. Overlapping primers were designed to obtain end-to-end sequence by amplifying the gene in three parts. The cDNA sequence (AF321139.1) was used as reference and the three segments were amplified from the tobacco genomic DNA using respective primers. Genomic DNA was isolated from young tobacco leaves following CTAB method as described by Doyle and Doyle (1990). For the first segment, forward primer (FP), 5'-

GGAGGAACAACAACAGGAAGAG-3', was taken from 5' UTR and the reverse primer (RP), 5'-CACCTTTCCA-TAAGTTGATGACTG-3', was picked ~ 200 bp downstream to it. For amplifying the second segment, forward primer (5'-CAGTCATCAACTTATGGAAAGGTG-3') was designed from the same DNA stretch as the reverse primer for the first segment while the reverse primer (5'-GGCAATTGGCAACGATTTCTTC-3') was picked ~ 500 bp downstream to it. Similarly, for the third part, FP, 5'-GAAGAAATCGTTGCCAATTGCC-3', was designed from inside of the second segment while the RP, 5'-GGTATGGCTGTCATCGTCGTC-3' was taken from 3' UTR. After an initial denaturation at 94 °C for 5 min, we carried out 30 amplification cycles: denaturation (94 °C, 30 s), annealing (58 °C, 30 s), and extension (72 °C, 45 s) with a final extension at 72 °C for 10 min. The products were visualized on agarose gel (1% Tris–borate/EDTA, TBE), eluted (Thermo Scientific | Life Technologies, USA), cloned into pGEM®-T Easy Vector (Promega Life Science, USA), sequenced and analyzed.

Preparation of *SPDS*-hpRNAi construct and tobacco transformation

SPDS-hpRNAi construct was prepared by cloning a 434 bp long partial gene sequence in sense (using *AscI* and *SwaI* sites) and in antisense orientation (using *BamHI* and *XbaI* sites) in plant binary vector, pMVRhp (prepared in the laboratory by cloning an entire hairpin forming cassette from pFGC5941 to pCAMBIA 2300). The sense fragment was amplified using a FP 5'-TAGGCGCGCC CTTCTGTTC-3' and a reverse primer, 5'-CGCATTTA AATTGCAACTCC-3'. The antisense fragment was amplified using the FP 5'-GTGGATCCCTCGTATCCT AC-3' and RP, 5'-TATCTAGACTTCTGTTCTTCC-3'. PCR amplification was carried out by an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min. PCR products were cloned into pGEM®-T Easy Vector (Promega Life Science, USA), confirmed by sequencing and eventually cloned into the RNAi vector using appropriate restriction sites. The construct was mobilized into *Agrobacterium tumefaciens* LBA4404 strain, which was then used to carry out tobacco transformations according to the protocol as described by Waie and Rajam (2003). Xanthi variety of *Nicotiana tabacum* was used in this study. Plant cultures were maintained under controlled growth conditions (26 ± 2 °C, 16 h photoperiod with irradiance of 40 IE mol/m²/s1).

PCR and Southern blot hybridization

The putative transformants were screened for the presence of transgene by PCR using *npt II* (marker gene) specific primers. The sequence of FP was 5'-AGGACACGCTGAAATCACCAG-3' and that of RP was 5'-GCCACAGTCGATGAATCCAG-3'. PCR was carried out as: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation (94 °C, 30 s), annealing (60 °C, 30 s), extension (72 °C, 45 s) and final extension of 10 min at 72 °C. Transgene integration for a selected few PCR-positive lines was further confirmed by Southern blot hybridization using *npt II* (marker gene) specific probe. For Southern analysis, 15 µg genomic DNA was restricted with *EcoRI* restriction enzyme, the fragments were resolved on 0.8% agarose gel and transferred onto positively charged nylon membrane (MDI, India) according to the standard protocol as described in Sambrook et al. 2001. The probe was prepared by labeling it with P³² using a random primer kit by following the manufacturer's guidelines (GE Healthcare Amersham Megaprime DNA Labeling Systems, Fisher Scientific). Pre-hybridization, hybridization and washing processes were all carried out as described in Sambrook et al. 2001.

RNA isolation and expression analysis

Total RNA was isolated from the leaves of age-matched PA-RNAi lines and wild-type using TRIzol kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. One microgram of DNase treated (Fermentas, Canada) RNA samples was used as template for cDNA synthesis using two-step RT-PCR kit (Thermo Scientific, Life Technologies) as per manufacturer's guidelines. Quantitative RT-PCR was performed in ABI7900HT Fast Real-Time PCR System (Applied Biosystems Inc., Foster City, CA) in a reaction volume of 10 µl using SYBR Green PCR Master Mix (Applied Biosystems | Life Technologies, USA) following manufacturer's instructions. Each reaction consisted of 200 ng of diluted cDNA in 1 × *Power* SYBR® Green PCR Master Mix and 5 µM each of gene-specific forward and reverse primers. Two biological replicates with two technical replicates each were kept for each line along with negative controls (without cDNA) for every primer set. Cycling conditions used: initial hold at 50 °C for 2 min, a 10 min denaturation step at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s and an annealing and extension step at 60 °C for 1 min. For melt curve analysis, an additional step was added consisting of 15 s at 95 °C, 15 s at 60 °C followed by another 15 s at 95 °C. Cycle threshold (CT) values for the genes under study were calculated using the ABI Software and exported to Excel spreadsheets for additional analysis. Expression

analysis was carried out for *SPDS*, *ADC* and *ODC*. *Ubiquitin (Ubiq)* was used as the reference gene for normalization of target gene expression. Primer sequences used: For *SPDS*, FP: 5'-CCAATTGCCGCCAAATCTTC-3', RP: 5'-AAGTAGAGCAGAGCATGAAACC-3'; for *ADC*, FP: 5'-CGTTGTTTCGGTTCCTGATA-3', RP: 5'-CACCTTGGTAGTGAGCTTCAT-3'; for *ODC*, FP: 5'-GATGCCGATTCAAACGCTTATC-3', RP: 5'-CCGACGTCTAGAACAGTCATTT-3'; for *Ubiq*, FP: 5'-ACCTTGCTGACTACAATATCC-3', RP: 5'-CGACA-CAACGACATCAACAAC-3'.

Polyamine analysis

PA estimation was done as per the protocol originally described by Flores and Galston (1982). Age matched leaf samples from three *SPDS*-RNAi lines and wild-type were taken for PA analysis. Tissue samples weighing ~ 100 mg each, were homogenized in 1 ml of cold 10% perchloric acid (PCA) and centrifuged at 18,000×g (14,000 rpm in a Sigma 92 Nr.12154-H rotor) for 20 min at 4 °C. The supernatant fraction forms the source of free and conjugated PAs whereas the pellet contains bound PAs. The aliquots of both the supernatant and pellet were subjected to acid hydrolysis to release conjugated and bound PA fractions, respectively. After dansylation, the samples were run on high-resolution silica gel TLC plates (Whatman LK6D) and quantified using spectrofluorometer, with excitation at 350 nm and emission at 495 nm.

Measurement of leaf pigments

A measurement of total chlorophyll and carotenoid content was carried out in the leaves of tobacco wild-type and RNAi lines using DMSO as solvent (Tait and Hik 2003). Leaf tissue (100 mg) from each line was placed in 5 ml of DMSO and the pigments were allowed to leach out into the solvent by incubating the tissue at 65 °C. Three biological replicates were taken for each line. Absorbances of the DMSO extracts were recorded in a spectrophotometer (BIORAD SmartSpec™ Plus) at the wavelengths of 480, 649 and 665 nm. Concentration of total chlorophyll content (a + b) and carotenoids was calculated according to Wellburn's equations (Wellburn 1994).

Pollen viability assay

Pollen from freshly dehiscent anthers of tobacco wild-type and *SPDS*-RNAi lines were collected and stained with 1% acetocarmine. Pollen grains were then checked for viability under a bright-field microscope. Spherical-shaped, darkly stained pollen grains were considered fertile whereas non-

stained and deformed pollen were scored as sterile (Prabhavathi and Rajam 2007).

Scanning electron microscopy

Anther tissue samples from untransformed control and RNAi lines were fixed in a mix of 2% paraformaldehyde and 2% glutaraldehyde, processed and viewed using Zeiss EVO40 at AIRF, JNU, New Delhi.

Seed viability and germination rate

Seeds were sterilized as per the protocol described by Leatherwood et al. (2007) and inoculated on MS basal medium for germination in a growth chamber at 28 °C with 16/8 (light/dark). At least 120 seeds per line were inoculated. The germinated seed numbers were recorded daily.

Abiotic stress tolerance assays

Floating leaf disc assay

The leaves of selected *SPDS*-RNAi lines and untransformed control were punched with 1.0 cm cork borer to obtain uniform sized leaf discs. The leaf discs were floated on sterile solution containing 0, 100, 200 and 400 mM NaCl. Plates were maintained under culture room conditions. Observations were made after 3 and 5 days from the start of the experiment.

Salt stress assay with test tube grown plantlets

A sub-lethal concentration of 150 mM NaCl was optimized after an initial standardization by challenging wild-type tobacco plants with different NaCl concentrations of 100, 150 and 200 mM. Test tube grown plants were suspended in a thermocol sheet that was then floated on 1/10th strength of MS medium containing 150 mM NaCl. The response was monitored for over a period of 7 days under controlled culture room conditions. Appropriate water controls were maintained and three clones for each line were tested. All the seven Southern positive RNAi lines were chosen for the experiment and the plants were incubated in different concentrations of NaCl solution, suspended through a sheet of thermocol.

Drought tolerance assay

Test tube grown plants were transferred to pots and grown for 1 month before they were deprived of water and kept under observation for over 2 weeks. All Southern positive RNAi lines were chosen for the experiment along with the wild-type control.

Data analysis

All results were obtained from at least three independent experiments and data presented are the average (mean) with standard deviation from all the experiments. Statistical analyses consisted of data being subjected to Student's *t* test at $P < 0.05$ for determining significant differences between the test samples and wild-type control.

Results

Complete gene sequence of SPDS gene

The three sets of primers were designed to cover the whole of 1272 bp long cDNA sequence. The primers were used on genomic DNA that gave amplicons of lengths much larger than the expected sizes. The first primer set gave an amplicon of ~ 1 kb instead of the expected 226 bp; the second set amplified ~ 1.2 kb fragment against an expected size of 514 bp and the third amplicon of 177 bp swelled to 750 bp. Each fragment was cloned, sequenced from both ends, analyzed and arranged to give a continuous stretch of sequence. Eight exons and seven introns were identified, and the 3536 bp long complete gene sequence was submitted to the NCBI database with an accession number of KM516788.1.

Generation of tobacco transgenics with *SPDS*-hpRNAi construct

Twenty-three kanamycin resistant primary transformants were raised by *Agrobacterium*-mediated tobacco transformation using *SPDS*-hpRNAi construct. Primary screening of the putative *SPDS*-RNAi tobacco transformants was done by PCR amplification of the *npt-II* marker gene. The transgene integration was further confirmed in seven of the twenty-three PCR positive lines by Southern analysis (Fig. 1a). Each line showed a unique integration pattern, indicating independent transformation events. During the vegetative growth phase, *SPDS*-RNAi lines appeared phenotypically similar to the untransformed tobacco plants (Fig. 1b). *SPDS* down-regulation, however, resulted in delayed flowering (by an average of over two weeks at least), reduced flower-size (Fig. 1c) and reduced male and female fertility in the RNAi lines. There was no apparent difference in the morphology of anther buds and ovaries between transgenics and the wild-type.

Fig. 1 Southern blot hybridization and morphological features of *SPDS*-RNAi lines **a** Southern blot analysis of *SPDS*-RNAi tobacco lines using *nptII* gene probe, **b** representative pictures of wild-type and *SPDS*-RNAi line under field conditions, **c** flower morphology of wild-type and a representative *SPDS*-RNAi line. Numbers denote the transgenic lines and Wt denote untransformed control (wild-type)

Reduced *SPDS* expression in RNAi lines without any change in the transcript levels of other pathway-related genes

Quantitative RT-PCR revealed a significant decrease in the transcript levels of *SPDS* in all the seven Southern-positive

RNAi lines, 2, 4, 11, 12, 15, 24 and 28 as compared to the wild-type (Fig. 2a). Relative gene expression of *ADC* and *ODC* was unchanged in the RNAi lines as compared to untransformed wild-type control (Fig. 2b, c). Target gene

expression was normalized with ubiquitin gene in all the samples.

Reduced spermidine and steady putrescine, spermine levels

Three *SPDS*-RNAi lines viz, 4, 12 and 15 were selected for PA analysis. Free, bound and conjugated PA fractions were quantified in the age matched leaf samples of untransformed wild-type control and compared with that in the three RNAi lines. Although small but significant increase was seen in the free and conjugated fractions of Put in RNAi lines, most distinctly in the line 4 (Fig. 3a). Significant changes were detected for bound and conjugated fractions of Spd in lines 4, 12 and 15 (1.3–1.5 fold), whereas the change was least in the free Spd fraction as compared to the other two (Fig. 3b). Interestingly, an increase in Spm levels was also seen in the RNAi lines, though it was statistically significant only in line 4 (Fig. 3c). Total PA content was reduced in all the three *SPDS*-RNAi lines (Fig. 3d).

Reduced chlorophyll content

Total chlorophyll levels (chlorophyll a and b) were decreased in all the *SPDS*-RNAi lines tested (Fig. 4a). There was a marginal change in the carotenoid levels, significant only for three *SPDS*-RNAi lines, 12, 24 and 28 (Fig. 4b). Overall, leaf pigments were only slightly altered by reduced Spd levels.

Delayed flowering, reduced pollen viability, altered pollen morphology, reduced seed setting and viability

Fertility was noticeably affected in all the RNAi lines as they showed reduced pollen viability and seed setting in comparison with the wild-type. Pollen viability was studied by acetocarmine staining, which stains the viable pollen red but do not stain the non-viable pollen. Although complete male sterility was not detected in any of the lines tested, it was reduced to as low as ~ 20% in line 4, and ranged between 40 and 60% in lines 11, 12 and 15 (Fig. 5a). Even among the viable mix of pollen, they were present in different sizes (Fig. 5b). Some looked round and normal in shape while others were smaller and underdeveloped. Therefore, there was an apparent lack of synteny in the development of pollen grains. Many of the smaller size pollen grains did not take up the stain properly. The primary staining results of pollen from PA-RNAi lines had shown the unstaining, sterile pollen to be shrunken and distorted. In order to further examine the pollen morphology, the pollen surface and integrity of pollen wall, pollen

Fig. 2 Relative gene expression (fold change) in wild-type and *SPDS*-RNAi lines as revealed by real time analysis. **a** *SPDS* gene expression, **b** *ADC* gene expression, **c** *ODC* gene expression; values were subjected to Student's *t* test. *Significant difference at $P < 0.05$

Fig. 3 The levels of free, conjugated and bound forms of Put (a), Spd (b) and Spm (c) as well as total PA content (d) in tobacco wild-type and *SPDS*-RNAi lines. Values were subjected to Student's *t* test.

*Significant difference at $P < 0.05$ for free fraction, †significant difference at $P < 0.05$ for conjugated fraction and *significant difference at $P < 0.05$ for bound fraction

grains were observed under scanning electron microscope (SEM, Fig. 5c). As seen with acetocarmine staining, pollen grains from wild-type tobacco plants were spherical in shape and had a smooth-walled exine. In contrast, pollen from RNAi lines 4 and 15 exhibited altered pollen morphology as they looked shrunken and distorted in shape, suggesting *SPDS* downregulation was detrimental to pollen development.

Seed setting was also affected in all the five *SPDS*-RNAi lines observed, 2, 4, 11, 12 and 15. In addition to these Southern positive lines, one PCR-positive line 39, which was tested only for its pollen viability (10%), did not show any seed setting. The capsules from RNAi lines were small and showed variation in size, which was corroborated with the reduction in seed number as compared to wild-type (Fig. 6a). Seed viability was severely affected in all the five RNAi lines tested, ranging from near zero in lines 2 and 11 to ~20% in line 4 and ~30% in lines 12 and 15 (Fig. 6b). In accordance with the degree of non-viability of seeds, germination time also differed in different lines. In the untransformed control, ~60% seeds germinated after the first week of incubation and the rest ~35% germinated by the second week. In case of transgenic lines, however, seed germination was delayed by as much as

2 weeks. In *SPDS* 15, germination time was delayed by a week and beyond whereas in other two lines, 4 and 12, seed germination was delayed by 2 weeks and beyond as compared to wild-type (Fig. 6c).

Improved abiotic stress tolerance

In floating leaf disc assay, salt stress causes a gradual loss (leaching) of chlorophyll in response to the increased salinity. The RNAi lines, which took longer to bleach out were considered as more stress tolerant. The leaf discs of *SPDS*-RNAi line 4 were floated in different concentrations of NaCl (100, 200 mM) along with the wild-type and incubated over a period of five days until the wild-type leaf discs were completely bleached out. It was found that the *SPDS*-RNAi line showed a better stress tolerance response as it only showed a partial bleaching, compared to wild-type, which had completely bleached out by the fifth day (Fig. 7a).

Also, in case of whole plant salt stress tolerance assay in hydroponics, *SPDS*-RNAi lines showed an increased stress tolerance as compared to wild-type (Fig. 7b). Taking cues from the salt stress assays, *SPDS*-RNAi lines, which had shown improved tolerance to salt stress as compared to

Fig. 4 Pigment levels in *SPDS*-RNAi lines **a** total chlorophyll content and **b** total carotenoid content in untransformed control and *SPDS*-RNAi lines. Values were subjected to Student's *t* test. *Significant difference at $P < 0.05$

wild type, were studied further for drought tolerance. Even after a weeklong incubation, no wilting symptoms were evident, except for the beginning of yellowing of lower leaves in wild-type and *SPDS* lines 2 and 22. Wilting symptoms began to appear in plants after 12 days. Plants were maintained without water until complete drooping was observed, which took 17 days after the commencement of the experiment (Fig. 7c). Before permanent wilting could set in, they were watered to initiate their recovery. Out of the eight *SPDS*-RNAi lines tested (including seven Southern positive lines), four lines, 4, 11, 15 and 28, showed an enhanced tolerance to drought stress as compared to wild-type. After watering the plants, all the plants could recover within 12–15 h, with recovery seen faster in drought tolerant *SPDS* lines as compared to the wild-type control.

Discussion

Reduced *SPDS* expression, unchanged expression profile of other pathway related genes and reduced PA levels

RNAi-mediated down-regulation of *SPDS* gene brought down its transcript levels along with a significant decrease in Spd levels, particularly of its bound and conjugated fractions. A cell maintains the intracellular PA levels within a definite range and follows strict homeostatic mechanisms to regulate their levels (Kusano et al. 2008). To know whether or not the mechanisms involve change in the expression of other PA pathway related genes, relative expression levels of *ADC* and *ODC* were studied in the RNAi lines, and no significant change in their expression was observed. This confirmed a preconceived hypothesis based on a few discreet reports, which suggest that PA homeostasis functions only at the biomolecular level and not at the genetic level. Trung-Nghia et al. (2003) had reported that down-regulation of *ADC* resulted in a decreased *ADC* activity with a concomitant decrease in intracellular PA levels but the expression of other PA biosynthesis pathway related genes, *ODC*, *SAMDC* and *SPDS*, was unaltered. Similar results were also obtained in tobacco transgenics with the down-regulation of *ODC* gene (Choubey and Rajam, 2016). In tobacco, *ADC* gene is present along with its two paralogues, *ADC1* and *ADC2*. *ADC* activity is low or near about absent in roots and flowers whereas the other two isoforms are expressed predominantly in the roots, flowers and seeds (Borrell et al. 1995). This is the reason why expression was checked only for *ADC* in the leaf samples.

Although PA levels were reduced in response to reduced *SPDS* transcripts, changes in PA titres were not as severe as seen in the cases where other PA pathway related genes are knocked out or downregulated (Chen et al. 2014; Sinha and Rajam 2013). The fact that the *SPDS* is not a rate-limiting enzyme of the pathway and is very stable in nature, the target gene knockdown was not absolute in *SPDS*-RNAi lines. This might explain why Spd levels could not be reduced in high proportions. Marginal increase in the levels of Put and Spd can be attributed to the fact that when *SPDS* is downregulated, the conversion of Put to Spd slows down, but is not completely stopped. This results in reduced Spd titres and an accumulation of Put, but Spm biosynthesis continues, further reducing the Spd levels. An occasional rise in Spm levels is seen due to the channeling of dcSAM from Spd synthesis to Spm synthesis (Yatin 2002). An indirect support to this assertion comes from the reports where *SAMDC* is targeted. In such cases, Spm levels go down along with Spd as lowering of *SAMDC* activity

Fig. 5 Study of pollen viability and morphology **a** percentage pollen viability in tobacco *SPDS*-RNAi lines as compared to wild-type, **b** acetocarmine staining to determine pollen viability in wild type and

SPDS-RNAi lines, **c** SEM pictures of pollen from untransformed control and tobacco RNAi lines 4 and 15

deprives the cells of the precursor molecule, dcSAM (Chen et al. 2014; Sinha and Rajam 2013).

Effect of reduced Spd levels on flower size, pollen development, seed setting and viability

Involvement of PAs in floral development is reported in a wide variety of crops (Liu et al. 2007). Our results suggest that Spd is directly involved in pollen development and pollen viability as the pollen viability was reduced up to 60% in different *SPDS*-RNAi lines. The importance of PAs, especially Spd, in pollen germination and pollen tube growth has been substantiated in many systems like *Lilium longiflorum* Thunb (Rajam 1989), *Helianthus annuus* (Cetin et al. 2000), tomato (Song et al. 2001), *Prunus mume* cv. 'Zaohua' (Wolukau, et al. 2004), *Actinidia*

deliciosa (Falasca et al. 2010), *Arabidopsis* (Wu et al. 2010) and tobacco (Ma et al. 2012). The reduction in SAMDC activity by its inhibitor has been shown to reduce pollen tube growth (Wu et al. 2010), while the down-regulation of *SAMDC* gene expression led to the abnormal pollen development (Chen et al. 2014; Sinha and Rajam 2013). Spd has been correlated with regulating pollen tube growth via Spd-derived H_2O_2 . This hydrogen peroxide triggers the activation of Ca^{2+} -permeable channels, leading to an increased cytosolic Ca^{2+} that accompanies pollen tube growth. *Arabidopsis* mutants that fail to induce opening of the Ca^{2+} -permeable channels in the presence of Spd, exhibit reduced pollen tube growth and seed number (Wu et al. 2010). Spd, thus, plays a critical role from microsporogenesis to pollen–pistil interaction during fertilization, affecting not only pollen viability but also the

Fig. 6 Seed capsules and seed viability **a** comparison of seed capsules from wild-type and *SPDS*-RNAi lines, **b** percentage seed germination and germination rate of transgenic seeds as compared to

control, **c** evaluating seed viability in *SPDS*-RNAi lines by germinating seeds on MS basal medium

seed number (Aloisi et al. 2016). In our study also, *SPDS*-RNAi lines have shown a significantly reduced seed number in addition to reduced pollen viability.

The inability of the seeds from *SPDS*-RNAi lines to germinate indicates a putative role of Spd in seed development and emergence of seedlings. Mutation in the *SPDS* genes in *Arabidopsis* was shown to result in abnormally shrunken seeds, giving an embryo lethal phenotype and this indicates the importance of Spd in embryo growth and seed development (Imai et al. 2004). There are not many reports where *SPDS* has been specifically targeted genetically and thus, most of the studies have relied upon the use of inhibitors or physiological estimation of Spd during different developmental stages. High Spd content was reported during the formation and development of globular embryo in *Picea abies* (Santanen and Simola 1992) and *Camellia* (Pedroso et al. 1997) indicating the importance of Spd during seed development. Specific targeting of the enzyme *SPDS* and reduced viability of transgenic seeds further

supports this supposition. PA-conjugates are also hypothesized to affect seed viability as they act as storehouse for supplying free PAs during seed germination (Bonneau et al. 1994). PAs bound to proteins have been located in flower petals, pollens and seeds (Del Duca and Serafini-Fracassini 2005). Our results have also shown a sharp decline in Spd conjugates and its bound fraction, which might have resulted in reduced seed viability and delayed germination rate. This was the main reason why the study was carried out only on T_0 transgenics. Due to non-viable transgenic seeds, they could not be taken to subsequent generations for analysis.

Effect of altered PA levels on abiotic stress tolerance

In a large number of plants, PAs have been shown to accumulate in response to environmental stress conditions (Bhattacharya and Rajam 2006; Cheng et al. 2009; Marco et al. 2015). Improved stress tolerance is always observed

Fig. 7 Qualitative abiotic stress assays **a** Representative pictures showing phenotypic differences between leaf discs of wild-type and *SPDS*-RNAi line 4 after treatment with 100 and 200 mM NaCl solution for 3 and 5 days, **b** Representative pictures showing

phenotypic differences between wild-type and *SPDS*-RNAi lines after treatment with 150 mM NaCl solution for 7 days, **c** in vivo drought stress assay for *SPDS*-RNAi lines in pots

with elevated levels of Put and/or Spd and Spm (Alcázar et al. 2011; Marco et al. 2011; Sequera-Mutiozabal et al. 2017). Spm has been shown to play a significant role in providing protection against high salt and drought stress in *Arabidopsis* (Cheng et al. 2009; Yamaguchi et al. 2006, 2007). In the present study, Put and Spm accumulation in *SPDS*-RNAi lines resulted in improved stress tolerance against saline and drought stress, underlining the protective role of Put and Spm against abiotic stresses.

Conclusions

PA analysis in *SPDS*-RNAi tobacco lines exhibited a reduction in only Spd content, suggesting that the effects on reproductive potential such as pollen viability, seed setting and viability (embryo development), could be a direct ramification of Spd and its conjugates. The results obtained through *SPDS* knockdown may also find applications in crop improvement. Firstly, *SPDS* can be targeted in agronomically important plant species to ascertain its utility in imparting abiotic stress tolerance. Since it also

leads to reproductive defects, RNAi construct can be prepared under a stress inducible promoter so that *SPDS* silencing occurs only in response to stress. Secondly, *SPDS* down-regulation clearly had an adverse effect on pollen development, as evident by the presence of abnormally shaped, non-viable pollen grains. This feature can be exploited for engineering male sterility in crop plants, a desirable trait for gene containment or hybrid breeding, by targeting *SPDS* under the control of tapetum-specific promoter.

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Compliance with ethical standards

Conflict of interest The authors declare that they do not have conflict of interests.

References

- Alcázar R, Cuevas JC, Planas J, Zarza X, Bortolotti C, Carrasco P, Salinas J, Tiburcio AF, Altabella T (2011) Integration of polyamines in the cold acclimation response. *Plant Sci* 180:31–38
- Aloisi I, Cai G, Serafini-Fracassini D, Del Duca S (2016) Polyamines in pollen: from microsporogenesis to fertilization. *Front Plant Sci* 7:155
- Baron K, Stasolla C (2008) The role of polyamines during in vivo and in vitro development. *In Vitro Cell Dev Biol Plant* 44:384–395
- Bhattacharya E, Rajam MV (2006) Polyamine biosynthesis as a novel target for engineering crop plants for abiotic stress tolerance. *J Plant Biol* 33:99–105
- Biaostoff S, Reinhardt N, Reva V, Brandt W, Dräger B (2009) Evolution of putrescine N-methyltransferase from spermidine synthase demanded alterations in substrate binding. *FEBS Lett* 583:3367–3374
- Bonneau L, Carré M, Martin-Tanguy J (1994) Polyamines and related enzymes in rice differing in germination potential. *Plant Growth Regul* 15:75–82
- Borrell A, Culiñez-Macia FA, Altabella T, Besford RT, Flores D, Tiburcio AF (1995) Arginine decarboxylase is localized in chloroplasts. *Plant Physiol* 109:771–776
- Burtin D, Michael T (1997) Over-expression of arginine decarboxylase in transgenic plants. *Biochem J* 325:331–337
- Carbonell J, Blázquez MA (2009) Regulatory mechanisms of polyamine biosynthesis in plants. *Genes Genom* 31:107–118
- Cetin E, Yildirim C, Palavan-Unsal N, Unal M (2000) Effect of spermine and cyclohexylamine on in vitro pollen germination and tube growth in *Helianthus annuus*. *Can J Plant Sci* 80:241–245
- Chen M, Chen J, Fang J, Guo Z, Lu S (2014) Down-regulation of S-adenosylmethionine decarboxylase genes results in reduced plant length, pollen viability, and abiotic stress tolerance. *Plant Cell, Tissue Organ Cult* 116:311–322
- Cheng L, Zou YJ, Ding SL, Zhang JJ, Yu XL, Cao JS, Lu G (2009) Polyamine accumulation in transgenic tomato enhances the tolerance to high temperature stress. *J Integr Plant Biol* 51:489–499
- Choubey A, Rajam MV (2016) Transcriptome response and developmental implications of RNAi-mediated *ODC* knockdown in tobacco. *Funct Integr Genomics*. <https://doi.org/10.1007/s10142-016-0539-3>
- Cooper HL, Park MH, Folk JE, Safer B, Braverman R (1983) Identification of the hypusine-containing protein Hy + as translation initiation factor eIF-4D. *Proc Natl Acad Sci USA* 80:1854–1857
- Del Duca S, Serafini-Fracassini D (2005) Transglutaminases of higher, lower plants and fungi. *Prog Exp Tumor Res* 38:223–247
- DeSzenzo RA, Minocha SC (1993) Modulation of cellular polyamines in tobacco by transfer and expression of mouse ornithine decarboxylase cDNA. *Plant Mol Biol* 22:113–127
- Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. *Focus* 12:13–15
- Falasca G, Franceschetti M, Bagni N, Altamura MM, Biasi R (2010) Polyamine biosynthesis and control of the development of functional pollen in kiwifruit. *Plant Physiol Biochem* 48:565–573
- Flores HE, Galston AW (1982) Analysis of polyamines in higher plants by high performance liquid chromatography. *Plant Physiol* 69:701–706
- Franceschetti M, Fornale S, Tassonia A, Zuccherelli K, Mayer MJ, Bagni N (2004) Effects of spermidine synthase overexpression on polyamine biosynthetic pathway in tobacco plants. *J Plant Physiol* 161:989–1001
- Gutierrez E, Shin B-S, Woolstenhulme CJ et al (2013) eIF5A promotes translation of polyproline motifs. *Mol Cell* 51:35–45
- Igarashi K, Kashiwagi K (2010) Modulation of cellular function by polyamines. *Int J Biochem Cell Biol* 42:39–51
- Imai A, Matsuyama T, Hanzawa Y, Akiyama T, Tamaoki M, Saji H, Shirano Y, Kato T, Hayashi H, Shibata D, Tabata S, Komeda Y, Takahashi T (2004) Spermidine synthase genes are essential for survival of *Arabidopsis*. *Plant Physiol* 135:1565–1573
- Kasukabe Y, He L, Nada K, Misawa S, Ihara I, Tachibana S (2004) Overexpression of spermidine synthase enhances tolerance to multiple environmental stresses and upregulates the expression of various stress-regulated genes in transgenic *A. thaliana*. *Plant Cell Physiol* 45:712–722
- Kaur-Sawhney R, Tiburcio AF, Altabella T, Galston A (2003) Polyamines in plants: an overview. *J Cell Mol Biol* 2:1–12
- Kusano T, Berberich T, Tateda C, Takahashi Y (2008) Polyamines: essential factors for growth and survival. *Planta* 228:367–381
- Leatherwood WR, Pharr DM, Dean LO, Williamson JD (2007) Carbohydrate content and root growth in seeds germinated under salt stress. *J Am Soc Hortic Sci* 132:876–882
- Liu JH, Kitashiba H, Wang J, Ban Y, Moriguchi T (2007) Polyamines and their ability to provide environmental tolerance in plants. *Plant Biotechnol* 24:117–126
- Ma W, Wang J, Hu J, Guan Y, Li Y, Zheng Y (2012) Relation between changes in polyamine, protective enzyme activity and pollen vigor of tobacco in different flowering stages. *Afr J Agric Res* 7:5491–5497
- Marco F, Alcázar R, Tiburcio AF, Carrasco P (2011) Interactions between polyamines and abiotic stress pathway responses unraveled by transcriptome analysis of polyamine overproducers. *OMICS* 15:775–781
- Marco F, Bitrián M, Carrasco P, Rajam MV, Alcázar R, Tiburcio AF (2015) Genetic engineering strategies for abiotic stress tolerance in plants. In: Bahadur B, Rajam MV, Sahijram L, Krishnamurthy KV (eds) *Plant biology and biotechnology*. Vol. II: Plant genomics and biotechnology. Springer, New Delhi, pp 579–609

- Masgrau C, Altabella T, Farrás R, Flores D, Thompson AJ, Besford RT, Tiburcio AF (1997) Inducible overexpression of oat arginine decarboxylase in transgenic tobacco plants. *Plant J* 11:465–473
- Mayer MJ, Michael MJ (2003) Polyamine homeostasis in transgenic plants overexpressing ornithine decarboxylase includes ornithine limitation. *J Biochem* 134:765–772
- Moschou PN, Wu J, Cona A, Tavladoraki P, Angelini R, Roubelakis-Angelakis KA (2012) The polyamines and their catabolic products are significant players in the turnover of nitrogenous molecules in plants. *J Exp Bot* 63:5003–5015
- Park MH, Nishimura K, Zanelli CF, Valentini SR (2010) Functional significance of eIF5A and its hypusine modification in eukaryotes. *Amino Acids* 41:2538–2545
- Pedroso MC, Primikiros N, Roubelakis-Angelakis KA, Pais MS (1997) Free and conjugated polyamines in embryogenic and non-embryogenic leaf regions of *Camellia* leaves before and during direct somatic embryogenesis. *Plant Physiol* 101:213–219
- Prabhavathi VR, Rajam MV (2007) Polyamine accumulation in transgenic eggplant enhances tolerance to multiple abiotic stresses and fungal resistance. *Plant Biotechnol* 24:273–282
- Rajam MV (1989) Restriction of pollen germination and tube growth in lily pollen by inhibitors of polyamine metabolism. *Plant Sci* 59:53–56
- Sambrook J, Fritsch ED, Maniatis T (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
- Santanen A, Simola LK (1992) Changes in polyamine metabolism during somatic embryogenesis in *Picea abies*. *J Plant Physiol* 104:475–480
- Sequera-Mutiozabal M, Antoniou C, Tiburcio AF, Alcázar R, Fotopoulos V (2017) Polyamines: emerging hubs promoting drought and salt stress tolerance in plants. *Curr Mol Biol Rep* 3:28–36
- Sinha R, Rajam MV (2013) RNAi silencing of three homologues of S-adenosylmethionine decarboxylase gene in tapetal tissue of tomato results in male sterility. *Plant Mol Biol* 82:169–180
- Slocum RD (1991) Polyamine biosynthesis in plants. In: Slocum RD, Flores HE (eds) The biochemistry and physiology of polyamines in plants. CRC Press, Boca Raton, pp 23–40
- Song J, Nada K, Tachibana S (2001) The early increase of S-adenosylmethionine decarboxylase activity is essential for the normal germination and tube growth in tomato (*Lycopersicon esculentum* Mill.) pollen. *Plant Sci* 161:507–515
- Tait MA, Hik DS (2003) Is dimethylsulfoxide a reliable solvent for extracting chlorophyll under field conditions? *Photosynth Res* 78:87–91
- Takano A, Kakehi JI, Takahashi T (2012) Thermospermine is not a minor polyamine in the plant kingdom. *Plant Cell Physiol* 53:606–616
- Tiburcio AF, Altabella T, Bitrián M, Alcázar R (2014) The roles of polyamines during the lifespan of plants: from development to stress. *Planta* 240:1–18
- Trung-Nghia P, Bassie L, Safwat G, Thu-Hang P, Lepri O, Rocha P, Christou P, Capell T (2003) Reduction in the endogenous arginine decarboxylase transcript levels in rice leads to depletion of the putrescine and spermidine pools with no concomitant changes in the expression of downstream genes in the polyamine biosynthetic pathway. *Planta* 218:125–134
- Waie B, Rajam MV (2003) Effect of increased polyamine biosynthesis on stress responses in transgenic tobacco by introduction of human S-adenosylmethionine gene. *Plant Sci* 164:727–734
- Wallace HM, Fraser AV, Hughes AA (2003) Perspective of polyamine metabolism. *Biochem J* 376:1–14
- Wellburn AR (1994) The spectral determination of chlorophyll a and chlorophyll b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *J Plant Physiol* 144:307–313
- Wolukau JN, Zhang SL, Xu GH, Chen D (2004) The effect of temperature, polyamines and polyamine synthesis inhibitor on in vitro pollen germination and pollen tube growth of *Prunus mume*. *Sci Hortic* 99:289–299
- Wu J, Shang Z, Wu J, Jiang X, Moschou PN, Sun W, Roubelakis-Angelakis KA, Zhang S (2010) Spermidine oxidase-derived H₂O₂ regulates pollen plasma membrane hyperpolarization-activated Ca²⁺ permeable channels and pollen tube growth. *Plant J* 63:1042–1053
- Yamaguchi K, Takahashi Y, Berberich T, Imai A, Miyazaki A, Takahashi T, Michael A, Kusano T (2006) The polyamine spermine protects against high salt stress in *Arabidopsis thaliana*. *FEBS Lett* 580:6783–6788
- Yamaguchi K, Takahashi Y, Berberich T, Imai A, Takahashi T, Michael AJ, Kusano T (2007) A protective role for the polyamine spermine against drought stress in *Arabidopsis*. *Biochem Biophys Res Commun* 352:486–490
- Yatin M (2002) Polyamines in living organisms. *J Cell Mol Biol* 1:57–67