Evaluation of arginine decarboxylase (*FvADC*) and spermidine synthase (*FvSPDS*) genes of woodland strawberry (*Fragaria vesca* L.) in *Nicotiana tabacum*

Ákos MENDEL[1*], Erzsébet KISS[2]

1 Institute of Horticultural Sciences, Hungarian University of Agriculture and Life Sciences, Budapest, Hungary

2 Institute of Genetics and Biotechnology, Hungarian University of Agriculture and Life Sciences, Gödöllő, Hungary

*Correspondance: mendel.akos@uni-mate.hu

ORCIDs:

Ákos MENDEL: https://orcid.org/0000-0002-8355-7255

Erzsébet KISS: https://orcid.org/0000-0002-8933-5311

Abstract: A comparative analysis was performed under *in vitro* conditions using *Nicotiana tabacum* lines overexpressing the genes of arginine decarboxylase (FvADC) and spermidine synthase (FvSPDS) enzymes. The transgenic and native lines were tested under controlled conditions and exposed to long-term treatment of arginine (150 mg/l), putrescine (10 mg/l) and spermidine (10 mg/l). Chlorophyll and lignin content of the samples were mesured spectrophotometrically, proline, putrescine, spermidine and spermine contents were determined by HPLC methods. The experimental results showed that the arginine decarboxylase enzyme has an effect on polyamine metabolism. As it is involved in several other biosynthetic pathways, this effect is significant but not

outstanding. Spermidine synthase is more abundant: it directly enhances the accumulation of higher polyamine forms (SPD, SPM) without a direct negative feedback. A complex regulatory mechanism plays an important role in the precise adjustment of the amount and proportion of polyamines, an equilibrium that cannot be disrupted by minor influences.

Key words: *Fragaria vesca*, polyamine, strawberry, transgenic, *Nicotiana tabacum*

1. Introduction

Our primary goal is to better understand the genes encoding the arginine decarboxylase and spermidine synthase enzymes and their mechanism of action. The function of genes involved in polyamine biosynthesis has been studied in several plant species, but their exact mechanism of action and interactions are not known. Knowledge of the function of the enzymes involved in the biosynthetic pathway is perhaps even more important than that of the genes encoding them. If we know how substrate-dependent the function of each enzyme is, we will also know the expected efficacy of possible interventions.

The strawberry (*Fragaria x ananassa* Duch.) plant is a good example of non-climacteric fruit ripening. This compact model plant is perennial, early fruiting, can be propagated generatively and vegetatively, and tolerates *in vitro* conditions well (Jiu et al., 2018). In strawberry, ethylene levels are highest in the green ripening phase, decline to the white phase, and then increase slightly again until the red ripening phase (Kovács et al., 2020), with no ethylene peak at strawberry fruit ripening. Thus, strawberry fruit ripening is not only regulated by ethylene, but by a combination of several plant hormones (Shen and Rose, 2014). During strawberry fruit development, an increase in abscisic acid (ABA) levels and a decrease in indoleacetic acid (IAA) levels suggest that the ratio of these

hormones serves as a signal for the next stage of fruit ripening (Perkins-Veazie, 1995). Indoleacetic acid alone induces elongation of the receptor cells, simultaneously inhibiting the progress of ripening (Given et al., 1988) In contrast, elevated ABA (abscisic acid) concentrations promote fruit ripening (Jia et al., 2011). Our previous experiments have shown that although ethylene and polyamines are involved in fruit ripening, other biosynthetic pathways may also involve genes active in polyamine metabolism (Mendel et al., 2018, Mendel et al., 2021). Stress is any external effect that prevents a biological system (in this case a plant) from functioning normally. Different types of abiotic stresses cause significant economic damage worldwide every year. The most common are cold, drought, heat, heavy metal and salt stresses (Mahajan and Tuteja, 2005). Ethylene and polyamines play an important role in the biotic and abiotic stress responses of plants (Romero et al., 2018).

Polyamines and proline are the most important nitrogen-containing plant osmolytes (Kavi Kishore et al., 1995; Bouchereau et al., 1999). Spermidine (Spd), spermine (Spm) and their direct precursor molecule, diamine putrescine (Put), are essential and vital polyamines present in all living organisms (Liu et al., 2016; Liu et al., 2017). Polyamines play an important role in the development of abiotic and biotic stress tolerance and stress response (Kasukabe et al., 2004; Alcázar et al., 2010). Polyamines improve membrane stability through the regulation of osmotic state, help to counteract the negative effects of free radicals, and also affect stomatal openness (Roy et al., 2005).

Our previous experiments have shown that although ethylene and polyamines are involved in fruit ripening, other biosynthetic pathways may also involve genes which are active in polyamine metabolism (Mendel et al., 2018; Mendel et al., 2021). Different types of abiotic stresses cause significant economic damage worldwide every year.

Ethylene and polyamines play an important role in the biotic and abiotic stress responses of plants (Peng et al., 2014), although many researchers agree that the metabolism of ethylene and polyamine is antagonistic (Li et al., 2004; Nambeesan et al., 2012; Yu et al., 2016).

Ethylene biosynthesis occurs through the SAM-ACC-ethylene pathway (Yang and Hoffmann, 1984). The expression of genes encoding the enzymes ACC synthase (ACS) and ACC oxidase (ACO), which are involved in ethylene production, increases under stress, thus increasing the ethylene content of tissues. Exogenous ACC and ethylene treatments also increase stress tolerance, while reducing ethylene concentrations makes the plants studied more susceptible (Peng et al., 2014; Chen et al., 2014).

In addition to the reactive oxygen radicals accumulated in cells, the amount of proline is also a good indicator of the stress state of plants. Proline accumulation has been shown in many plant species under environmental stresses, and is therefore considered as an amino acid indicative of stress status in addition to representing a large rapidly utilizable nitrogen reserve (Kovács et al., 2014; Borgo et al., 2015). Various forms of polyamines also play a role in the protection and stabilization of chloroplasts, mitochondria and plasma membranes (Jia et al., 2010). Spermine preserves plasma membrane integrity under stress and prevents superoxide generation by inhibiting NADPH oxidase activation (Shen et al., 2000). Higher order polyamines (spermidine and spermine) increase the activity of tonoplast ion transporters (H^+-ATPase and H^+-PPase), which thereby pump Na^+ from the cytosol to the vacuoles, thus establishing ion and pH balance in the cell (Roy et al., 2005). Polyamines also play a role in salt-induced stress signaling through spermidine-induced phosphorylation (Gupta et al., 2012).

Polyamine forms can modify protein activity and function during post-translational processes, but can also reduce the efficiency of transcription (Mustafavi et al., 2018). These interventions contribute to changes in protein content in plant tissues (Yuan et al., 2014; Du et al., 2017; Sequera-Mutiozabal et al., 2017). Rapid degradation of proteins by senescence is prevented by both spermidine and spermine (Wang and Shi, 2004; Serafini-Fracassini et al., 2010). Spermidine increases the activity of the enzyme nitrate reductase, thereby positively affecting nitrogen metabolism (Miura, 2013).

Divergent results have been obtained in studies of polyamine content in stressed plants, with endogenous polyamines decreasing in apple, maize and bean under salt stress, while increasing in grape, wheat and Chinese cabbage (Liu et al., 2008; Kim et al., 2010; Upreti and Murti, 2010; Legocka and Sobieszczuk-Nowicka, 2012). The increased tolerance has been attributed to the effect of increased levels of spermidine and spermine (Ahmad et al., 2009; Ben et al., 2009). The role of polyamines in the development of abiotic stress tolerance is increasingly understood, but their mechanism of action still raises many questions. What seems certain, however, is that increased polyamine contents help plants respond to stresses, thus maintaining their normal functioning.

The use of polyamines (especially putrescine) is also of great help in the *in vitro* cultivation of fruit species with poor rooting, but also increases tuber development, tuber size and yield in several species (Pedros et al., 1999; Ondo Ovono et al., 2010). Rhizome mass can also be increased *in vitro* by polyamines. Bulb yield of tulip cultivars has also been shown to benefit from both polyamines and arginine, with a clear outline of the positive effect of exogenous polyamine treatment (Podwyszyńska et al., 2015).

S-adenosyl-L-methionine (SAM) is the common precursor molecule for ethylene and polyamine metabolism (Minocha, 1988). The S-adenosyl-L-methionine synthase

synthesizes SAM from L-methionine and ATP, which is the second most widely used enzyme substrate after ATP (Cantoni, 1975). SAM is required for the methylation of DNA, RNA and proteins in both mitochondria and chloroplasts (Tabor and Tabor 1984; Boerjan et al., 1994; Block et al., 2002). Lignin is the major metabolic consumer of SAM, but only in certain cells and at certain developmental stages is lignin accumulation observed between the primary and secondary cell wall (Hanson et al., 1994) is used for the methylation of putrescine, nicotine, tropane and nortropane alkaloids (Biastoff et al., 2009). In the case when SAM decarboxylase (SAMDC) decarboxylates SAM and produces decarboxylated SAM (dcSAM), it cannot participate in ethylene biosynthesis. In this case, dcSAM provides the aminopropyl group for the synthesis of higher order polyamines (spermidine and spermine). As shown in Figure 1., the key enzymes for spermidine (Spd) and spermine (Spm) biosynthesis, in addition to SAMDC, are arginine decarboxylase (ADC) and spermidine synthase (SPDS) (Mehta et al., 2002; Khan and Singh, 2010). Competition for SAM between the ethylene and polyamine pathways does not occur because SAM is available in large excess: only 10% of SAM is used by ethylene and polyamine biosynthesis (Bregoli et al., 2002). The biosynthesis of polyamines in plants is mainly carried out by the enzymes ADC, SAMDC, SPDS and spermine synthase (SPMS) (Minocha, 1988; Lechowska et al., 2021). Enzymes involved in the biosynthesis of polyamines are ODC (ornithine decarboxylase), ADC (arginine decarboxylase), agmatine-iminohydrolase, N-carbomylputrescine amidohydrolase, SAMDC (SAM decarboxylase), SPDS (spermidine synthase), SPMS (spermidine synthase) and LDC (lysine decarboxylase). This biosynthetic pathway in plants is regulated by arginine decarboxylase (ADC), ornithine decarboxylase (ODC), SAM decarboxylase (SAMDC) and spermidine synthase (SPDS) (Hasegawa et al., 2000).

The number of ADC genes in plants varies, with some plants having 2 (*Arabidopsis*) or 3 (*Brassica juncea*) (Galloway et al., 1998; Mo and Pua, 2002). In *Arabidopsis thaliana*, the *AtADC1* gene is active in the petiole and basal part of the leaves, whereas the *AtADC2* gene is active in the petiole, true leaves, root vascular bundles and floral organs (Urano et al., 2003). This dichotomy holds true for both adult and seedling plants (Hummel et al., 2004). *AtADC1* gene expression is barely detectable at seed formation, but *AtADC2* expression is enhanced 12 days after flowering and remains high at all stages of embryo development. Both ADC genes show increased activity in the shoot apex and root apex growing cone. Under stress, only *AtADC2* is more active in transport tissues (Urano et al., 2003). Studies on the ADC genes of *Nicotiana tabacum*, *N. benthamiana*, *Oryza sativa*, have shown similar results (Wang et al., 2000; Bortolotti et al., 2004; Akiyama et al., 2007). Several plant species have only one ADC gene (Rastogi et al., 1993; Nam et al., 1997; Primikirios and Roubelakis-Angelakis, 1999), such as *Avea fatua* and *Fragaria vesca* (Mattoo et al., 2015). ADC paralogs show 80% sequence identity in the *Brassicaceae* family. The expression of the *arginine decarboxylase* (ADC) gene is increased by exogenous spermidine treatment (Lazzarato et al., 2009). The oat *ADC* gene encodes a 66 kDa protein from which an enzymatically active ADC enzyme of 24 kDa in size is derived . In tomato, the same enzyme is derived from a 55 kDa (502 amino acid) protein (Rastogi et al., 1993). In *N. tabacum*, 54 kDa of the 77 kDa polypeptide (721 amino acids) is functional and can be detected in the nucleus and chloroplast (Bortolotti et al., 2004). The main ORF of *FvADC* (NC_020497.1) encodes 708 amino acids, with no intron. At the genomic level, it is closest to genes encoding the arginine decarboxylase enzyme of the genus *Prunus* of the family *Rosaceae*: *Prunus persica* (XM_007200245.2) has 88% similarity, *Prunus avium* (XM_021950639.1) 81% similarity, *Prunus dulcis*

(XM_034370861.1) 81% similarity. There is also a high degree of similarity with the same genes in *Malus* and *Pyrus* species. There is 74% sequence similarity with the gene encoding ADC in *Nicotiana tabacum* (NM_001325190.1). At the amino acid level, the *Fragaria vesca* ADC enzyme shows an 85% identity with *Prunus persica* (XP_007200307.1), 84% with *Prunus avium* (XP_021806331.1), 84% with *Prunus armeniaca* (CAB4320369.1) and even 74% with *Nicotiana tabacum* (AAF42972.1).

The *Arabidopsis thaliana* spermidine synthase enzyme has a mass of 36 kDa. *AtSPDS1* and *AtSPDS2* are highly expressed in roots, while *AtSPDS3* is expressed in shoot internodes and flower buds (Hanzawa et al., 2002). The pea *PsSPDS1* gene is active in dividing plant tissues (shoot apex, root apex, developing fruit), while *PsSPDS2* is more highly expressed later in fruit development and during shoot elongation (Alabadí et al., 1999). Similar expression patterns have been described in *Nicotiana tabacum*, *Medicago falcata*, *Zea mays* (Rodríguez-Kessler et al., 2006; Zhuo et al., 2018). An *SPDS* gene is also present in *Olea europaea*, *Prunus pseudocerasus*, *Prunus avium*, *Prunus persica*, *Fragaria x ananassa*, and *Fragaria vesca* (Gomez-Jimenez et al., 2010; Kovács et al., 2020; Mendel et al., 2018; Wu et al., 2020).The main ORF of *FvSPDS* (XM_004297595.2) encodes 336 amino acids. In nucleotide sequence order, *Ipomopsis aggregate* (GT313702.1, 96%), *Prunus armeniaca* (CV046039.1, 88%) and *Quercus robur* (FP026861.1, 86%) show the highest sequence similarity to the sequence under study. The sequence of the *Nicotiana tabacum SPDS* gene is 80% identical to the *FvSPDS* gene. Also in terms of amino acid sequence, the genus *Prunus* shows the highest similarity: *Prunus armeniaca* (KAH0991643.1) 87%, *Prunus dulcis* (XP_034218472.1) 87%, *Prunus persica* (XP_007222503.1) 87%. The amino acid sequence of *Nicotiana tabacum* SPDS shows 84% similarity with the FvSPDS enzyme.

Importance of *S-adenosyl-L-methionine synthase* (*FvSAMS*), involved in ethylene metabolism, and the *S-adenosyl-L-methionine decarboxylase* (*FvSAMDC*) gene (responsible for encoding the common enzyme of ethylene and polyamine metabolism) against abiotic stresses is highlighted previously (Kovács *et al*., 2020). From these experiments we concluded, that enhanced expression of both genes affects positively the salt tolerance of the *Nicotiana benthamiana* plants. Previous studies have already investigated the effects of ADC and SPDS enzyme overexpression separately and demonstrated that both ADC and SPDS overexpression enhance plant tolerance to various abiotic stresses. However, these two enzymes have not yet been tested in one experimental system. In this experiment, wild-type tobacco plants were treated *in vitro* with arginine, putrescine, and spermidine. The *FvADC* transgenic lines were treated with arginine and putrescine, and the *FvSPDS* lines with putrescine and spermidine. The chlorophyll a and b, lignin and proline contents, the amount of polyamines and the ratio of polyamines to each other were determined.

2. Materials and methods

2.1. Plant material and genetic transformation

The sequence of the whole genome of *Fragaria vesca* L. is available in the database of National Center for Biotechnology Information (NCBI). Basic Local Alignment and Search Tool (BLAST) analysis was carried out to identify the main ORF of *FvADC* and *FvSPDS*. Total RNA of *F. vesca* cv. 'Rügen' was isolated according to the manufacturer's protocol (Total RNA Mini Kit Plant, Geneaid®, New Taipei City, Taiwan). cDNA was synthesized from total RNA using Oligo(dT)18 primer using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, USA). ORF of *FvADC* is 2856

bp (putative *FvADC* - XM_004306397.2), ORF of *FvSPDS* is 1378 bp (putative *FvSPDS* - XM_004297595.2). From the cDNA library, the *FvADC* and *FvSPDS* genes were amplified using the primer pairs Fv_ADC_RT_F- FvADC_RT_R and Fv_SPDS_RT_F-FvSPDS_RT_R, respectively (Table 1.). Fragments of the appropriate size were re-isolated from the agarose gel (Promega Wizard® SV Gel and PCR Clean-Up System Kit, Promega, Madison, USA). Gateway® was used to assemble the vector constructs. Following the manufacturer's protocol, *FvADC* and *FvSPDS* sequences were ligated into pENTRTM/SD/D-TOPO cloning vector. The forward primers also contained the required CAC sequence, while the reverse primer was used to eliminate the stop codon at the end of the coding regions. With these changes, we were able to C terminally fuse the cDNA ORFs to the sGFP reporter gene in the pGWB405 binary vector. (Figure 2.).

Agrobacterium tumefaciens strain GV3101 was transformed with the binary vector constructs pGWB405::FvADC and pGWB405::FvSPDS. Colony PCR was performed on colonies of transformed strains grown on selection LB medium containing 50 µg/ml spectinomycin. The transformed colonies were cultured on liquid YEP medium (50 µg/ml spectinomycin and 100 µg/ml rifampicin) and incubated for 12 h at 28ºC.

Leaves of *in vitro* grown sterile tobacco plants (*N. tabacum* L.) were used as explants. Rectangular pieces of 1 cm^2 surface area were cut and shaken in previously described liquid culture (YEP) for 60 min, for inoculation. The explants were placed on solid MS medium, and plant tissues were co-cultured for four days with *Agrobacterium tumefaciens* GV3101 containing pGWB405::FvADC or pGWB405::FvSPDS constructs in the dark at 28°C. At the end of co-cultivation, leaf sections were washed three times with sterile distilled water containing 200 µg/ml timentin, 300 µg/ml carbenicillin and 300 µg/ml cefotaxime, and three times with antibiotic-free distilled water. Then, plant

tissues were placed on solid selective shoot regeneration MS medium (0.2 mg/l NAA, 1mg/l BA, 200 µg/ml timentin with 80 µg/ml kanamycin and 250 µg/ml cefotaxime). Every 2 weeks, the callus explant was placed on fresh medium with sero-rotation of the antibiotics used (timentin, carbenicillin, cefotaxime). This method allows to reduce the risk of developing antibiotic resistant strains.

2.2. Proving the success of genetic transformation

Plant tissues were then plated on solid selective shoot regeneration MS medium (0.2 mg/l NAA, 1 mg/l BA, 200 µg/ml timentin, 80 µg/ml kanamycin and 250 µg/ml cefotaxime). Every two weeks, the explants were placed on fresh medium with sero-rotation of the antibiotics used (timentin, carbenicillin, cefotaxime). Genomic DNA was isolated from the leaves according to the manufacturer's protocol (DNeasy® Plant Mini Kit, Qiagen, Hilden, Germany), then the integration of the transgene into the plant genome was verified by PCR.. The designed primer pairs (Table 1.) were used to amplify the *FvADC* and *FvSPDS* sequences by PCR. Gel electrophoresis (TAE buffer, 1.2% agar) analysis was performed on the amplified sequences to confirm the amplification. Total RNA was isolated from plants showing positive results according to the manufacturer's protocol (Total RNA Mini Kit Plant, Geneaid®, New Taipei City, Taiwan). Oligo(dT)18 primer was used to synthesize cDNA with RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, USA). The transcription of the transgenes were verified by PCR using the cDNAs as described above.

2.3. Plant growing conditions and treatments

The segregation rate of T1 lines from self-fertilization was examined *in vitro* on MS medium containing 80 µg/ml kanamycin. The transgenic *Nicotiana tabacum* lines with a

segregation rate of 3:1 from different genetic events were pre-selected on MS medium supplemented with 80 µg/ml kanamycin, while wild-type (control) *Nicotiana tabacum* L. seeds were germinated on antibiotic-free MS medium. On day 21, the plants were placed on induction medium, which in all cases was MS medium supplemented with an organic molecule involved in polyamine metabolism. Transgenic lines (*FvADC*-5, *FvADC*-7, *FvADC*-37) carrying a novel candidate copy of the arginine decarboxylase enzyme gene were placed on medium containing 150 mg/l arginine or 10 mg/l putrescine. *FvSPDS*-2, *FvSPDS*-9, *FvSPDS*-82 lines transformed with the spermidine synthase gene were further grown on medium containing 10 mg/l putrescine or 10 mg/l spermidine (Bhatnagar et al., 2004; Veerasamy and Chinnagounder 2013). Wild-type plants were subjected to all three treatments (arginine, putrescine and spermidine). Wild-type and 3-3 transformed plant lines were also grown on MS medium without supplementation of the arginine, purescine, or spermidine as control treatment. Plants were grown under 16 h of illumination at 23ºC and samples were collected on day 90 after sowing.

2.4. Determination of chlorophyll content

Total chlorophyll content and chlorophyll a and b were determined according to the method of Porra et al. (1989). Of the leaf samples collected, 200 mg were ground in liquid nitrogen. The samples were then suspended in 2 ml of 80% (v/v) ice-cold acetone for 10 minutes. The samples in acetone were centrifuged at 12000 g for 20 min at 4ºC. The supernatant was centrifuged again at 12000 g for 5 min, also at 4ºC. To 1 ml of supernatant, 1 ml of 1 M Tris-HCl (pH 8) was added and the absorbance was measured at 645 nm (Abs.645) and 663 nm (Abs.663) using a NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, USA). The instrument was calibrated with

a solution prepared without plant sample. The chlorophyll a (Ca) and chlorophyll b (Cb) and total chlorophyll (Ct) contents were determined using the following formulae:

Ca=0,0127*(Abs.663)-0,00269*(Abs.645);

Cb=0,0229*(Abs.645)-0,00468*(Abs.663);

Ct=Ca+Cb.

2.5 Determination of lignin content

As a first step to determine the lignin content of the shoots, a protein-free cell wall extract was prepared. 200 mg of lyophilized shoots (stem and leaf mixed) were homogenized in 5 ml of 50 mM potassium phosphate buffer (pH 7) and centrifuged at 1400 g for 5 min. The supernatant was drained and centrifugation was repeated twice. To the bottom phase, 5 ml of 1% Triton-X-100 (pH 7) was added and centrifuged at 1400 g rpm for 5 min. The supernatant was drained and centrifugation was repeated twice. Then 5 ml of 1 M NaCl (pH 7) was added to the samples and centrifuged at 1400 g rpm for 5 min. The supernatant was drained and the step was repeated twice. 5 ml of distilled water was added to the precipitate and centrifuged at 1400 g for 5 minutes. The supernatant was poured off and the wash was repeated twice. Then 5 ml of acetone was added to the samples, centrifuged at 1400 g for 5 minutes and the supernatant was drained. Centrifuged two more times and dried at 60ºC for 12 hours. To quantify lignin, the method of Moreria-Vilar et al. (2014) was used. 0.5 ml of acetyl bromide dissolved in 25% (v/v) glacial acetic acid was added to 20 mg of protein-containing cell wall extract and incubated at 70ºC for 30 min. The samples were then cooled abruptly on ice. Then 0,9 ml of 2 M sodium hydroxide (NaOH), 0,1 ml of 5 M hydroxylamine hydrochloride (NH_2OH*HCl) and 4 ml of glacial acetic acid were added. The resulting solutions were centrifuged at 1400 g for 5 minutes. The

absorbance of the samples was measured with a WPA Biotech Photometer 1101 (Cambridge, UK) at 280 nm. For the standard curve, 10, 100 and 500 mg/ml solutions of alkaline lignin (Sigma-Aldrich, Saint Louis, USA) prepared as described above were used.

2.6. Determination of proline, putrescine, spermidine and spermine content using HPLC

Free polyamines and proline were determined by HPLC according to Németh et al. (2002). During sample preparation, 200 mg of plant sample was homogenized in liquid nitrogen, extracted with 2 ml of 0.2 M ice-cold perchloric acid ($HClO_4$). The prepared samples were placed on ice for 20 min and centrifuged at 10,000 g for 20 min at 4°C.According to the method of Smith and Davies (1985), the polyamine fraction was derivatized with dansyl chloride. To 100 µl of the supernatant, 200 µl of saturated sodium carbonate and 400 µl of freshly dissolved dansyl chloride (5 mg/ml) in acetone were added in a 2 ml Eppendorf tube. The samples were homogenized and incubated in the dark at 60°C for 60 min. Then, 100 µl of proline solution (100 mg/ml) was added and incubated for a further 30 min at room temperature in the dark. The dansyl derivatives were then extracted with 500 µl of toluene for 30 seconds and the upper, organic phase was transferred to 1,5 ml Eppendorf tubes using a Pasteur pipette. The samples were evaporated under vacuum. Danzylated polyamines and proline were dissolved in 1 ml of 100% methanol and filtered through a 0,2 µm pore size Teflon membrane filter. Danzylated polyamines Put, Spd, Spm) and proline were analyzed using acetonitrile carrier medium in a WATERS W 2690 (Milford, USA) HPLC instrument.

2.7. Microscopic analysis

Visual detection of the fusion green fluorescent proteins (*FvADC*::sGFP, *FvSPDS*::sGFP) was performed with a Leica TCS SP8 laser scanning confocal microscope and a Leica/Leitz fluorescence stereo microscope (DMRB 301-371.010, Leica, Wetzlar, Germany). Sections of 5*5 mm were made on the apical part of the examined leaf discs. The sections were examined in native form without fixation from the adaxial side. A 1:1 mixture of glycerol and distilled water was used to cover the sections. Leica LAS AF Lite 3.3.10134.0 software was used to process the images.

2.8. Statistical analysis

Three plant lines were tested in three treatments. The plant samples for the tests were leaves collected in equal proportions from 3-3 plants. The results were obtained from 9 measurements. One factor analysis of variance (ANOVA) was used to evaluate the data. The goodness of variance homogeneity was checked by Levene's test and the variance ratio test. Tukey's post hoc test and the Games-Howell test were used to determine significantly different groups. Correlation analysis was performed to examine the correlation of the data. Obtained values were analyzed by treatment and line using interaction analysis. IBM SPSS v.27 was used to evaluate the data.

Results and discussion

3.1. Subcellular localization of FvADC and FvSPDS enzymes

The segregation rate of T_1 lines from self-fertilization was examined *in vitro* on MS medium containing 80 μg/ml kanamycin. Lines were considered to be carrying a transgene copy if 75% of their progeny showed transgenic phenotype. Only the lines containing one copy of the transgenes were used for further investigation. For the *FvADC* and *FvSPDS* lines, the desired 3:1 cleavage ratio was obtained for lines derived from

several independent transformation events. As the integration was succesfull at several occasions (Figure 3.), it was unneccesarry to evaluate the proportion of these events. The transcription from the genes were verified by PCR, and the translation was observable with the sGFP fluorescence. The continuous presence of polyamines is most abundant in the nucleus and chloroplast, and in the sites most exposed to hazards (wounds, stomatal barrier cells, epidermal layer, *etc*.) Plant SPDS enzymes do not contain transit peptide-specific sequences, nor does *sGFP* modify the expression pattern. In epidermal cells, FvADC::sGFP detected sGFP in the same pattern as chlorophyll, thus a chloroplast localization was established, whereas in columnar parenchyma cells, the sGFP signal was detectable in the intercellular space (Figure 4-5.). The sGFP reporter gene, when coupled to a constitutive promoter, exhibits cytoplasmic expression by itself and thus cannot modify the subcellular localization of the fusion-induced sequence (Chiu et al., 1996). The endogenous arginine decarboxylase enzyme of *N. tabacum* is active in all plant organs, but the site of activity depends on the function of the tissue (Bortolotti et al., 2004). Previous studies have shown that the ADC enzyme functions mainly in the nucleus and chloroplast (Slocum, 1991). In photosynthetically active tissues, the ADC enzyme is present in the chloroplast, whereas in phptpsynthetically inactive tissues dominantly presents in nucleus. In *A. thaliana*, cytoplasmic and chloroplast localisation of ADC has also been observed using sGFP proteins (Maruri-López and Jiménez-Bremont, 2017). In addition to the nuceus and cytoplasmic localisation, all authors agree that polyamine biosynthesis and thus the enzymes involved in chloroplast biosynthesis are also highly active in chloroplasts (Gemperlová et al., 2006). Previous studies have adonucleated codons of chloroplast-specific transit proteins in ADC genes of *Arabidopsis*, tobacco,

rice, oat, rye, mustard and apple (Burtin and Michael, 1997; Peremarti et al., 2010; Urano et al., 2003).

FvSPDS::sGFP also showed fluorescent signal in the same locations as chloroplasts, thus a chloroplast localization was established. For columnar parenchyma cells, the signal of sGFP was also detectable in the cytoplasm in our experiment. The endogenous spermidine synthase enzyme of *N. tabacum* shows activity in all plant organs (Gomez-Jimenez et al., 2010), consistent with the results of studies showing that the SPDS enzyme functions mainly in the nucleus and chloroplast (Slocum, 1991). The role of the SPDS enzyme in chloroplasts has also been demonstrated by several studies (Gemperlová et al., 2006; Torrigiani et al., 1986). No signal peptide was found in the SPDS genes of *Morus spp.* Its localization was determined by its function. Plant SPDS genes do not contain transit peptide-specific sequences and sGFP does not modify the expression pattern. The enzyme spermidine synthase is synthesized in the cytoplasm and transported from there to the chloroplasts and nucleus (Liu et al., 2021).

3.2. Evaluation of the parameters studied

The values of the parameters tested (chlorophyll, lignin, proline, putrescine, spermidine, spermine, and total polyamine contents) are presented as the average of the nine lines already described for Wt plants, while for *FvADC* and *FvSPDS* plants, the averages of three biological replicates of three-to-three independent transformant lines are presented (Figure 6-8.).

In the case of chlorophyll, only arginine treatment resulted in a significantly higher value for wild-type plants, and the addition of putrescine caused a reduction in *FvSPDS* plants. The decrease in chlorophyll content from different origins can be prevented by externally applied polyamines (Duan, 2000). In addition to putrescine,

arginine also reduces membrane damage, thus protecting chloroplast integrity (Sun et al., 2018). Our measurements also showed that the *Ca/Cb* ratio was increased only with arginine treatment in wild-type and *FvADC*. Putrescine decreased this ratio in both transformant groups, whereas spermidine decreased it in wild type and *FvSPDS*.

In our experiment, the addition of putrescine did not affect, arginine and spermidine reduced the measurable lignin content in Wt plants. In *FvADC* and *FvSPDS* lines, arginine, putrescine and spermidine also increased lignin content. The metabolism of lignin utilizes the largest amount of SAM, which also provides an aminopropyl group for the biosynthesis of spermidine and spermine (Sánchez-Aguayo et al., 2004). Arginine added to the medium decreases lignin, while spermidine increases lignification in *Arabidopsis* plants grown *in vitro* (Xu et al., 2014).

Our measurements showed a significant decrease in proline content in spermidine-treated wild-type and *FvSPDS* plants, but an increase in proline content in *FvADC* lines after putrescine treatment (Figure 9-10.). Increased proline concentrations have been observed in several plant species in response to abiotic stress (Szabados and Savouré, 2010). In plant cells, arginine can directly increase proline levels through the urea cycle, which triggers putrescine production (Del Duca et al., 2014). In Islam's experiments, polyamine treatment increased proline content in stressed plants (Islam et al., 2022).

All three treatments reduced putrescine levels in wild-type plants used as controls. The addition of both putrescine and (in the case of *FvADC*) spermidine increased the measurable amount of putrescine in individuals of the two transformed lines. In Wt plants, endogenous spermidine levels were reduced by both arginine and spermidine treatment, putrescine was ineffective. Spermine levels in the wild type were slightly reduced by arginine. Spermine levels in *FvADC* lines were also increased by both treatments,

whereas only spermidine had an effect on *FvSPDS* plants. Spermidine and spermine levels were greatly increased by externally applied putrescine treatment in both untreated and osmotic stressed alfalfa plants (Zeid et al., 2007), while putrescine levels were slightly increased (Pál et al., 2018). Elevated putrescine levels were measured in *Arabidopsis* plants overproducing ADC enzyme without an increase in spermidine and spermine levels (Alcázar et al., 2010). This demonstrates that, although ADC enzyme activity is high, the arginine decarboxylase enzyme (and the transcriptional activity of the ADC gene encoding it) does not limit polyamine biosynthesis. In an experiment by Kasukabe et al. (2004), *Arabidopsis* plants overexpressing the SPDS enzyme showed increased arginine decarboxylation and elevated spermidine levels under stress. Overexpression of the SPDS gene in tobacco plants caused increased SPDS and SAMDC activity, while ADC enzyme activity was unchanged (Franceschetti et al., 2004).

In wild-type plants, it can be shown that the addition of arginine and putrescine to the medium significantly increased the ratio of spermidine to spermin compared to putrescine. The exogenous putrescine excess is converted to spermidine by the SPDS enzyme, thus confirming our measurements. However, the addition of arginine would be expected to increase putrescine content (via the ADC pathway), but the opposite was observed. The addition of spermidine reduced the proportion of more complex polyamines in favour of putrescine, also not entirely consistent with the expected effect (Figure 11.). *FvADC* and *FvSPDS* transgenic lines responded to treatments in the same way as Wt plants. The (Sper+Spm)/Put ratio was shown to be closely related to the different levels of stress tolerance. A higher ratio confers a higher degree of tolerance (Zhou et al., 2008). Spermidine exposure increased this ratio, and consequently the degree of salt tolerance, even more, suggesting that the combined amount of spermidine and

spermine is crucial for the development of tolerance (Li et al., 2016). In *Arabidopsis* and *Populus* plants producing excess putrescine, no significant changes in spermidine and spermin concentrations were measured (Shao et al., *2014*). Putrescine can only slightly increase spermidine and spermin, there is no strong correlation between the two. In contrast, spermidine and spermin levels show a tight regression, suggesting that there is a mutually positive interaction between SPDS and SPMS enzymes (and the genes encoding them) (Mattoo et al., 2010).

3.3. Relationships between the parameters studied

Based on the parameters of the treatments of the three lines, a correlation analysis was carried out, so that we can show which values of each trait are explained by the values of the other traits (Table 2).

The lignin content shows a medium negative correlation (-0.395 and -0.386) with the *Ca/Cb* ratio, so that a higher lignin content can be measured with a lower *Ca/Cb* ratio. Putrescine has a medium positive correlation with lignin content (0.492). Spermine, spermidine, total polyamine, and (Spd+Spm)/Put ratio have a negative medium strength correlation with *Ca/Cb* ratio. On average across all treatments and lines, higher polyamine contents and ratios increased the ratio of *Cb* to *Ca*. Spermine levels showed a medium positive correlation with lignin levels. Spermine is strongly correlated with lignin content (0.847), moderately strongly correlated with putrescine, and weakly correlated with spermidine. Total polyamine content has a very strong correlation with lignin content (0.920) and spermidine content (0.917), and a moderately strong correlation with putrescine and spermidine content. Total polyamine content is most strongly correlated with spermidine content and least strongly correlated with putrescine content. (Spd+Spm)/Put ratio shows a strong positive correlation with spermidine content (0.874)

and a medium strong correlation with lignin and total polyamine content. There is a negative medium-strong correlation between (Spd+Spm)/Put ratio and putrescine content, but spermidine content does not explain this value. These results suggest that total polyamine and the (Spd+Spm)/Put ratio are most strongly influenced by the amount of spermidine. This ratio influences cell division, cell elongation, tissue differentiation, seed germination, and tolerance to abiotic stresses (Minocha et al., 1996; Kakkar et al., 2000; Paul et al., 2017). Polyamines are involved in fruit ripening differently in non-climacteric strawberry and climacteric fruits. While spermidine and spermine levels in strawberry increase greatly with ripening, putrescine levels gradually decrease (Guo et al., 2018). The independence of the chlorophyll and proline contents from the other parameters demonstrates that the measured differences are not due to changes in polyamine metabolism.

4. Conclusions

The *FvADC* and *FvSPDS* genes of *Fragaria vesca* L. cv. 'Rügen' were successfully transferred into tissues of *Nicotiana tabacum* L. plants, and transcriptional activity of the genes was demonstrated in the progeny. In the epidermal cells of stable transformants, chloroplast localization was observed for the FvADC::sGFP fusion proteins, and chloroplast and cytoplasmic localization for the FvSPDS::sGFP fusion proteins.

Based on physiological parameters and polyamine levels, it can be concluded that constitutive overexpression of the *FvADC* gene has a greater effect on vigor than overexpression of the *FvSPDS* gene compared to Wt plants. In contrast, the addition of arginine to the medium induced a smaller effect than putrescine and spermidine treatment. It seems that arginine, because of its role in other biosynthetic pathways (amino acid,

proline, GABA biosynthesis, citrate cycle, urea cycle, *etc*.), cannot have the same effect on the parameters studied as polyamines.

The effect of the *FvSPDS* gene is more likely to be seen in the conversion of polyamine forms. The added putrescine and spermidine help to convert it directly, but it also has a beneficial effect on putrescine levels. It seems that the amount of different polyamine forms alone does not indicate an improved physiological state, but rather the ratio of diamine putrescine to the longer chain spermidine and spermin is crucial.

The relative proportions of polyamine forms have a greater effect than their relative amounts. In our tests, the (Spd+Spm)/Put ratio did not show a significant interaction with respect to the lines in any of the cases. The evolution of this value is not affected by overexpression of *FvADC* or *FvSPDS* genes, but it is increased by the addition of arginine or putrescine to the medium. This ratio is tightly regulated and some authors have suggested that it plays a crucial role in controlling tissue and organ differentiation.

Literature cited

Ahmad MSA, Ali Q, Ashraf M, Haider MZ, Abbas Q. (2009). Involvement of polyamines, abscisic acid and anti-oxidative enzymes in adaptation of Blue Panicgrass (*Panicum antidotale* Retz.) to saline environments. Environmental and Experimental Botany 66: 409-417. https://doi.org/10.1016/j.envexpbot.2009.02.011

Akiyama T, Jin S. (2007). Molecular cloning and characterization of an arginine decarboxylase gene up-regulated by chilling stress in rice seedlings. Journal of plant physiology, 164 (5): 645-654. https://doi.org/10.1016/j.jplph.2006.04.006

Alabadí D, Carbonell J. (1999). Differential expression of two spermidine synthase genes during early fruit development and in vegetative tissues of pea. Plant Molecular Biology, 39: 933–943. https://doi.org/10.1023/A:1006158819849

Alcázar R, Planas J, Saxena T, Zarza X, Bortolotti C et al. (2010). Putrescine accumulation confers drought tolerance in transgenic *Arabidopsis* plants over-expressing the homologous *Arginine decarboxylase 2* gene. Plant Physiology and Biochemistry 48 (7): 547-52. https://doi.org/10.1016/j.plaphy.2010.02.002

Ben HA, Ghanem Me, Bouzid S, Lutts S (2009). Abscisic acid has contrasting effects on salt excretion and polyamine concentrations of an inland and a coastal population of the Mediterranean xero-halophyte species *Atriplex halimus*. Annals of Botany 104: 925-936. https://doi.org/10.1093/aob/mcp174

Bhatnagar P, Bhatnagar M, Amarjit K (2004). Production of solasodine by *Solanum laciniatum* using plant tissue culture technique. Indian Journal of Experimental Biology 42: 1020-1023.

Biastoff S, Brandt W, Dräger B (2009). Putrescine N-metyltranferase – The start for alkaloids. Phytochemistry 70: 1708-1718. https://doi.org/10.1016/j.phytochem.2009.06.012

Block MA, Tewari Ak, Albrieux C, Marechal E, Joyard J (2002). The plant S-adenosyl-L-methionine: Mg-protoporphyrin IX methyltransferase is located in both envelope and thylakoid chloroplast membranes. European Journal Biochemistry 269: 240-248. https://doi.org/10.1046/j.0014-2956.2001.02643.x

Boerjan W, Bauw G, van Montagu M, Inzé D (1994). Distinct phenotypes generated by overexpression and suppression of S-adenosyl-L-methionine synthetase reveal developmental patterns of gene silencing in tobacco. Plant Cell 6: 1401-1414. https://doi.org/10.1105/tpc.6.10.1401

Borgo L, Marur CJ, Vieira LGE (2015). Effects of high proline accumulation on chloroplast and mitochondrial ultrastructure and on osmotic adjustment in tobacco plants. Acta Scientiarum Agronomy 37: 191-199. https://doi.org/10.4025/actasciagron.v37i2.19097

Bortolotti C, Cordeiro A, Alcázar R, Borrell A, Culiañez-Macià FA, et al. (2004). Localization of arginine decarboxylase in tobacco plants. Physiologia Plantarum, 120: 84-92. https://doi.org/10.1111/j.0031-9317.2004.0216.x

Bouchereau A, Aziz A, Larher F, Martin-Tanguy J (1999). Polyamines and environmental challenges: recent development. Plant Science 140: 103-125. https://doi.org/10.1016/S0168-9452(98)00218-0

Burtin D, Michael AJ. (1997). Overexpression of arginine decarboxylase in transgenic plants. Biochemical Journal, 325 (2): 331-337. https://doi.org/10.1007/s11248-009-9354-0

Bregoli AM, Scaramagli S, Costa G, Sabatini E, Ziosi V et al. (2002). Peach (*Prunus persica*) fruit ripening: aminoethoxyvinylglycine (AVG) and exogenous polyamines affect ethylene emission and flesh firmness. Physiologia Plantarum 114: 472-481. https://doi.org/10.1034/j.1399-3054.2002.1140317.x

Cantoni GL (1975). Biological methylation: selected aspects. Annual Review of Biochemistry 44: 435-451. https://doi.org/10.1146/annurev.bi.44.070175.002251

Chen D, Ma X, Li C, Zhang W, Xia G et al. (2014). A wheat aminocyclopropane-1-carboxylate oxidase gene, *TaACO1*, negatively regulates salinity stress in *Arabidopsis thaliana*. Plant Cell Reports 33: 1815-1827. https://doi.org/10.1007/s00299-014-1659-7

Chiu WL, Niwa Y, Zeng W, Hirano T, Kobayashi H et al. (1996). Engineered GFP as a vital reporter in plants. Current biology, 6 (3): 325-330. https://doi.org/10.1016/S0960-9822(02)00483-9

Del Duca S, Serafini-Fracassini D, Cai G. (2014). Senescence and programmed cell death in plants: polyamine action mediated by trans-glutaminase. Frontiers in plant science 5:1–17. https://doi.org/10.3389/fpls.2014.00120

Du J, Shu S, An Y, Zhou H, Guo S et al. (2017). Influence of exogenous spermidine on carbon–nitrogen metabolism under $Ca(NO_3)_2$ stress in cucumber root. Plant Growth Regulation,81: 103-115. https://doi.org/10.1007/s10725-016-0193-8

Duan G. (2000). Effect of speridine on protein contents and protease during senescence of fxicsed wheat leaves. Journal of Sichuan Teaching Collegium 21: 44–47.

Franceschetti M, Fornalé S, Tassonia A, Zuccherelli K, Mayer MJ et al. (2004). Effects of spermidine synthase overexpression on polyamine biosynthetic pathway in tobacco plants. Journal of Plant Physiology, 161 (9):989-1001. https://doi.org/10.1016/j.jplph.2004.02.004

Galloway GL, Malmberg RL, Price RA. (1998). Phylogenetic utility of the nuclear gene arginine decarboxylase: an example from *Brassicaceae*. Molecular Biology and Evolution, 15: 1312–1320. https://doi.org/10.1093/oxfordjournals.molbev.a025859

Gemperlová L, Nováková M, Vaňková R, Eder J, Cvikrová M. (2006). Diurnal changes in polyamine content, arginine and ornithine decarboxylase, and diamine oxidase in tobacco leaves, Journal of Experimental Botany, 57 (6): 1413–1421. https://doi.org/10.1093/jxb/erj121

Given NK, Venis MA, Grierson D. (1988). Hormonal regulation of ripening in the strawberry, a non-climacteric fruit. Planta, 174: 402-406. https://doi.org/10.1007/BF00959527

Gomez-Jimenez MC, Paredes MA, Gallardo M, Fernandez-Garcia N, Olmos E et al. (2010). Tissue-specific expression of olive S-adenosyl methionine decarboxylase and spermidine synthase genes and polyamine metabolism during flower opening and early fruit development. Planta, 232 (3): 629-47. https://doi.org/10.1007/s00425-010-1198-6

Guo J, Wang S, Yu X, Dong R, Li Y et al. (2018). Polyamines regulate strawberry fruit ripening by abscisic acid, auxin, and ethylene. Plant Physiology 177 (1): 339-351. https://doi.org/10.1104/pp.18.00245

Gupta K, Gupta B, Ghosh B, Sengupta DN (2012). Spermidine and abscisic acid-mediated phosphorylation of a cytoplasmic protein from rice root in response to salinity stress. Acta Physiologiae Plantarum 34: 29-40. https://doi.org/10.1007/s11738-011-0802-0

Hanson AD, Rivoal J, Burnet M, Rathinasabapathi B (1994). Biosynthesis of quaternary ammonium and tertiary sulphonium compounds in response to water deficit. In: Smirnoff N. (ed) Environment and plant metabolism. Bios, Oxford 189-198.

Hanzawa Y. Imai A. Michael AJ, Komeda Y, Takahashi T. (2002). Characterization of the spermidine synthase-related gene family in *Arabidopsis thaliana*. Febs Letters, 527(1-3): 176-180. https://doi.org/10.1016/S0014-5793(02)03217-9

Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ . (2000). Plant cellular and molecular responses to high salinity. Annual Review of Plant Physiology and Plant Molecular Biology, 51: 463-99. https://doi.org/10.1146/annurev.arplant.51.1.463

Hummel I, Bourdais G, Gouesbet G, Couée I, Malmberg RL et al. (2004). Differential gene expression of arginine decarboxylase *ADC1* and *ADC2* in *Arabidopsis thaliana*: characterization of transcriptional regulation during seed germination and seedling development. New Phytologist, 163 (3): 519-531. https://doi.org/10.1111/j.1469-8137.2004.01128.x

Islam MJ, Uddin MJ, Hossain MA, Henry R, Begum MK et al. (2022) Exogenous putrescine attenuates the negative impact of drought stress by modulating physio-biochemical traits and gene expression in sugar beet (*Beta vulgaris* L.). PLoS One. 17 (1): e0262099. https://doi.org/10.1371/journal.pone.0262099

Jia YX, Sun J, Guo SR, Li J, Hu XH et al. (2010). Effect of root-applied spermidine on growth and respiratory metabolism in roots of cucumber (*Cucumis sativus*) seedlings under hypoxia. Russian Journal of Plant Physiology 57: 648-655. https://doi.org/10.1134/S1021443710050079

Jia HF, Chai YM, Li CL, Lu D, Luo JJ et al. (2011). Abscisic acid plays an important role in the regulation of strawberry fruit ripening. Plant Physiology, 157:188–199. https://doi.org/10.1104/pp.111.177311

Jiu S, Haider MS, Kurjogi MM, Zhang K, Zhu X et al. (2018). Genome-wide characterization and expression analysis of sugar transporter family genes in

woodland strawberry. The Plant Genome, 11 (3): 170103. https://doi.org/10.3835/plantgenome2017.11.0103

Kakkar RK, Nagar PK, Ahuja P (2000). Polyamines and plant morphogenesis. Biologia Plantarum 43: 1-11. https://doi.org/10.1023/a:1026582308902

Kasukabe Y, He L, Nada K, Misawa S, Ihara I et al. (2004). Overexpression of spermidin synthase enhances tolerance to multiple environmental stresses and up-regulates the expression of various stress-regulated genes in transgenic *Arabidopsis thaliana*. Plant Cell Physiology 45 (6): 712-722. https://doi.org/10.1093/pcp/pch083

Kavi Kishore PB, Hong Z, Miao GH, Hu CAA, Verma DPS (1995). Overexpression of Δ1-pyrolline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. Plant Physiology 108 (4): 1387-1394.

Khan AS, Singh Z (2010). Pre-harvest application of putrescine influences Japanese plum fruit ripening and quality. Food Science and Technology International 16: 53-64. https://doi.org/10.1177/1082013209353242

Kim JS, Shim IS, Kim MJ (2010). Physiological response of Chinese cabbage to salt stress. Korean Journal of Horticultural Science and Technology 28: 343-352.

Kovács L, Mendel Á, Szentgyörgyi A, Fekete S, Söre F et al. (2020). Comparative analysis of overexpressed *Fragaria vesca* S-adenosyl-L-methionine synthase (FvSAMS) and decarboxylase (FvSAMDC) during salt stress in transgenic *Nicotiana benthamiana*. Plant Growth Regulation 91:53-73. https://doi.org/10.1007/s10725-020-00587-3

Kovács V, Gondor OK, Szalai G, Majláth I, Janda T et al. (2014). UV-B radiation modifies the acclimation processes to drought or cadmium in wheat. Environmental and Experimental Botany 100: 122-131. https://doi.org/10.1016/j.envexpbot.2013.12.019

Lazzarato L, Trebbi G, Pagnucco C, Franchin C, Torrigiani P et al. (2009). Exogenous spermidine, arsenic and β-aminobutyric acid modulate tobacco resistance to tobacco mosaic virus, and affect local and systemic glucosylsalicylic acid levels and arginine decarboxylase gene expression in tobacco leaves. Journal of Plant Physiology, 166: 90–100. https://doi.org/10.1016/j.jplph.2008.01.011

Lechowska K, Wojtyla L, Quinet M, Kubala Sz, Lutts S et al. (2021). Endogenous polyamines and ethylene biosynthesis in relation to germination of osmoprimed brassica napus seeds under salt stress. International Journal of Molecular Sciences 23: 349. https://doi.org/10.3390/ijms23010349

Legocka J, Sobieszczuk-Nowicka E (2012). Sorbitol and NaCl stresses affect free, microsomeassociated and thylakoid-associated polyamine content in *Zea mays* and *Phaseolus vulgaris*. Acta Physiologiae Plantarum 34: 1145-1151. https://doi.org/10.1007/s11738-011-0911-9

Li C, Jiao J, Wang G (2004). The important roles of reactive oxygen speciesinthe relationship between ethylene and polyamines in leaves of spring wheat seedlings under root osmotic stress. Plant Science 166: 303-315. https://doi.org/10.1016/j.plantsci.2003.09.019

Li S, Jin H. Zhang Q. (2016). The effect of exogenous spermidine concentration on polyamine metabolism and salt tolerance in Zoysiagrass (*Zoysia japonica* Steud.) subjected to short-term salinity stress. Frontiers of plant sciences, 7: 1221. https://doi.org/10.3389/fpls.2016.01221

Liu JH, Inoue H, Moriguchi T (2008). Salt stress-mediated changes in free polyamine titers and expression of genes responsible for polyamine biosynthesis of apple *in vitro* shoots. Enviromental and Experimental Botany 62: 28-35. https://doi.org/10.1016/j.envexpbot.2007.07.002

Liu Q, Nishibori N, Imai I, Al E (2016). Response of polyamine pools in marine phytoplankton to nutrient limitation and variation in temperature and salinity. Marine Ecology Progress Series 544: 93-105. https://doi.org/10.3354/meps11583

Liu W, Tan M, Zhang C, Al E (2017). Functional characterization of murB-potABCD operon for polyamine uptake and peptidoglycan synthesis in *Streptococcus suis*. Microbioogical research 207: 177-187. https://doi.org/10.1016/j.micres.2017.11.008

Liu D, Zeng Y, Qiu C, Lin Q (2021). Molecular cloning and adversity stress expression analysis of spds genes in mulberry (*Morus notabilis*). Russian Journal of Plant Physiology, 68: 1186–1193. https://doi.org/10.1134/S1021443721060108

Mahajan S, Tuteja N (2005). Cold, salinity and drought stresses: an overview. Archives of Biochemistry and Biophysics, 444: 139-158. https://doi.org/10.1016/j.abb.2005.10.018

Maruri-López I, Jiménez-Bremont JF (2017). Hetero- and homodimerization of *Arabidopsis thaliana* arginine decarboxylase AtADC1 and AtADC2, Biochemical and Biophysical Research Communications, 484 (3): 508-513. https://doi.org/10.1016/j.bbrc.2017.01.083

Mattoo AK, Minocha SC, Minocha R, Handa AK (2010). Polyamines and cellular metabolism in plants: transgenic approaches reveal different responses to diamine putrescine versus higher polyamines spermidine and spermine. Amino Acids 38 (2): 405-13. https://doi.org/10.1007/s00726-009-0399-4

Mattoo AK, Fatima T, Upadhyay RK, Handa AK (2015). Polyamines in plants: biosynthesis from arginine, and metabolic, physiological and stress-response roles. Amino Acids in Higher Plants, 177-194. HTTPS://DOI.ORG/10.1079/9781780642635.0177

Mehta RA, Cassol T, Li N, Ali N, Handa AK et al. (2002). Engineered polyamine accumulation in tomato enhances phytonutrient content, juice quality and vine life. Nature Biotechnology 20:613-618. https://doi.org/10.1038/nbt0602-613

Mendel Á, Kovács L, Szentgyörgyi A, Fekete S, Posta K et al. (2018). Expression patterns of ethylene and polyamine biosynthetic genes during fruit ripening in strawberry. Studia Universitatis „Vasile Goldiș" Seria Stiintele Vietii 28 (4): 174-182.

Mendel Á, Kovács L, Kiss E (2021). Effect of arginine, putrescine and spermidine on the polyamine, proline and chlorophyll content of tobacco (*Nicotiana tabacum* L.). Acta Agraria Debreceniensis 2: 39-43. https://doi.org/10.34101/actaagrar/2/9572

Minocha SC (1988). Relationship between polyamine and ethylene biosynthesis in plants and its significance for morphogenesis in cell cultures. Advances in Experimental Medicine and Biology 250: 601-616.

Minocha S, Shortle WC, Coughin DJJ, Minocha SC (1996). Effects of aluminium on growth, polyamine metabolism, and inorganic ions in suspension cultures of red spruce (*Picea rubens*). Canadian Journal for Forest Research 26: 550-559. https://doi.org/10.1139/x26-063

Miura K (2013). Nitrogen and phosphorus nutrition under salinity stress. In: Ahmad P, Prasad MNV, Azooz MM Eds. Ecophysiology and responses of plants under salt stress. Springer, New York 425-441.

Mo H. Pua EC. (2002). Up-regulation of arginine decarboxylase gene expression and accumulation of polyamines in mustard (*Brassica juncea*) in response to stress. Physiologia Plantarum 114: 439–449. https://doi.org/10.1034/j.1399-3054.2002.1140314.x

Mustafavi SH, Badi HN, Sękara A, Mehrafarin A, Janda T et al. (2018). Polyamines and their possible mechanisms involved in plant physiological processes and elicitation of secondary metabolites. Acta Physiologiae Plantarum 40: 102. https://doi.org/10.1007/s11738-018-2671-2

Nam KH, Lee SH, Lee J. (1997). Differential expression of ADC mRNA during development and upon acid stress in soybean (*Glycine max*) hypocotyls. Plant and Cell Physiology 38: 1156–1166. https://doi.org/10.1093/oxfordjournals.pcp.a029101

Nambeesan S, Abuqamar S, Laluk K, Mattoo AK, Mickelbart MV et al. (2012). Polyamines attenuate ethylene-mediated defense responses to abrogate resistance to *Botrytis cinerea* in tomato. Plant Physiology 158: 1034-1045. https://doi.org/10.1104/pp.111.188698

Németh M, Janda T, Horváth E, Páldi E, Szalai G (2002). Exogenous salicylic acid increases polyamine content but may decrease drought tolerance in maize. Plant Science 162 (4): 569-574. https://doi.org/10.1016/S0168-9452(01)00593-3

Ondo Ovono P, Kevers C, Dommes J. (2010). Tuber formation and development of *Dioscorea cayenensis–Dioscorea rotundata* complex *in vitro* effect of polyamines. *In Vitro* Cellular and Developmental Biology - Plant, 46 (1): 81-88. https://doi.org/10.1007/s11627-009-9256-0

Pál M, Tajti J, Szalai G, Peeva V, Végh B et al. (2018). Interaction of polyamines, abscisic acid and proline under osmotic stress in the leaves of wheat plants. Scientific reports, 8 (1): 12839. https://doi.org/10.1038/s41598-018-31297-6

Paul S, Roychoudhury A (2017). Seed priming with spermine and spermidine regulates the expression of diverse groups of abiotic stress-responsive genes during salinity

stress in the seedlings of Indica rice varieties. Plant Gene 11: 124-132. https://doi.org/10.1016/j.plgene.2017.04.004

Pedros AR, Macleod MR, Ross HA, McRae D, Tiburcio AF et al. (1999). Manipulation of s-adenosylmethionine decarboxylase activity in potato tubers. Planta, 209 (1): 153-160. https://doi.org/10.1007/s004250050617

Peng J, Li Z, Wen X, Li W, Shi H et al. (2014). Salt-induced stabilization of EIN3/EIL1 confers salinity tolerance by deterring ROS accumulation in *Arabidopsis*. PLoS Genetics,10 (10): e1004664. https://doi.org/10.1371/journal.pgen.1004664

Peremarti A, Bassie L, Zhu C, Christou P, Capell T (2010). Molecular characterization of the arginine decarboxylase gene family in rice. Transgenic Research, 19: 785–797. https://doi.org/10.1007/s11248-009-9354-0

Perkins-Veazie P. (1995). Growth and ripening of strawberry fruit. Horticultural Reviews, 17: 267-297. https://doi.org/10.1002/9780470650585.ch8

Podwyszyńska M, Kosson R, Treder J. (2015). Polyamines and methyl jasmonate in bulb formation of *in vitro* propagated tulips. Plant Cell, Tissue and Organ Culture (PCTOC), 123 (3): 591-605. https://doi.org/10.1007/s11240-015-0863-7

Porra RJ, Thompson WA, Kriedemann PE (1989). Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochimica et Biophysica Acta 975: 384-394. https://doi.org/10.1016/S0005-2728(89)80347-0

Primikirios NI, Roubelakis-Angelakis KA. (1999). Cloning and expression of an arginine decarboxylase cDNA from *Vitis vinifera* L. cell-suspension cultures. Planta 208: 574–582. https://doi.org/10.1007/s004250050595

Rastogi R, Dulson J, Rothstein SJ. (1993). Cloning of tomato (*Lycopersicon esculentum* Mill.) arginine decarboxylase gene and its expression during fruit ripening. Plant Physiology, 103: 829-834. Plant Physiology, Volume 103, Issue 3, November 1993, Pages 829–834, https://doi.org/10.1104/pp.103.3.829

Rodríguez-Kessler M, Alpuche-Solís AG, Ruiz OA, Jimenez-Bremont JF (2006). Effect of salt stress on the regulation of maize (*Zea mays* L.) genes involved in polyamine biosynthesis. Plant Growth Regulation, 48 (2): 175-185. https://doi.org/10.1007/s10725-005-5990-4

Romero FM, Maiale SJ, Rossi FR, Marina M, Ruíz OA et al. (2018). Polyamine metabolism responses to biotic and abiotic stress. In: Alcazar R, Tiburcio AF. Eds. Polyamines: methods and protocols, methods in molecular biology, 1694: 37-49. https://doi.org/10.1007/978-1-4939-7398-9_3

Roy P, Niyogi K, Sengupta DN, Ghosh B (2005): Spermidine treatment to rice seedlings recovers salinity stress-induced damage of plasma membrane and PM-bound H^+-ATPase in salt-tolerant and salt-sensitive rice cultivars. Plant Science 168: 583-591. https://doi.org/10.1016/j.plantsci.2004.08.014

Sánchez-Aguayo I, Rodríguez-Galán JM, García R, Torreblanca J, Pardo JM. (2004). Salt stress enhances xylem development and expression of S-adenosyl-L-methionine synthase in lignifying tissues of tomato plants. Planta, 220: 278–285. https://doi.org/10.1007/s00425-004-1350-2

Serafini-Fracassini D, Sandro AD, Duca SD (2010). Spermine delays leaf senescence in *Lactuca sativa* and prevents the decay of chloroplast photosystems. Plant Physiology and Biochemistry, 48: 602-611. https://doi.org/10.1016/j.plaphy.2010.03.005

Sequera-Mutiozabal M, Antoniou C, Tiburcio AF, Alcázar R, Fotopoulos V (2017). Polyamines: emerging hubs promoting drought and salt stress tolerance in plants. current molecular biology reports 3 (1): 28-36. https://doi.org/10.1007/s40610-017-0052-z

Shen W, Kazuyoshi N, Shoji T (2000). Involvement of polyamines in the chilling tolerance of cucumber cultivars. Plant Physiology 124 (1): 431-440. https://doi.org/10.1104/pp.124.1.431

Shen YY, Rose JKC (2014). ABA metabolism and signaling in fleshy fruits. In: Zhang DP Ed. Abscisic acid: betabolism, transport and signaling 271-286.

Slocum RD. (1991). Tissue and subcellular localization of polyamines and enzymes of polyamine metabolism. In: Slocum RD, Flores HE. Eds. Biochemistry And Physiology of Polyamines in Plants, 93–105.

Smith MA, Davies PJ (1985). Separation and quantitation of polyamines in plant tissue by High Performance Liquid Chromatography of their dansyl derivatives. Plant Physiology 78: 89-91. https://doi.org/10.1104/pp.78.1.89

Sun X, Wang Y, Tan J, Al E. (2018). Effects of exogenous putrescine and D-Arg on physiological and biochemical indices of anthurium under chilling stress. Jiangsu Journal of Agricultural Sciences, 34: 152–157.

Szabados L, Savouré A. (2010). Proline: A multifunctional amino acid. Trends in Plant Science, 15: 89–97. https://doi.org/10.1016/j.tplants.2009.11.009

Tabor CW, Tabor H (1984). Polyamines. Annual Review of Biochemistry 53: 749-790. https://doi.org/10.1146/annurev.bi.53.070184.003533

Torrigiani P, Serafini-Fracassini D, Biondi S, Bagni N. (1986). Evidence for the subcellular localization of polyamines and their biosynthetic enzymes in plant cells. Journal of Plant Physiology, 124: 23-29. https://doi.org/10.1016/S0176-1617(86)80174-2

Upreti KK, Murti GSR (2010). Response of grape rootstocks to salinity: changes in root growth, polyamines and abscisic acid. Biologia Plantarum 54: 730-734. https://doi.org/10.1007/s10535-010-0130-z

Urano K, Yoshiba Y, Nanjo T, Igarashi Y, Seki M et al. (2003). Characterization of *Arabidopsis* genes involved in biosynthesis of polyamines in abiotic stress responses and developmental stages. Plant, Cell and Environment, 26 (11): 1917-1926. https://doi.org/10.1046/j.1365-3040.2003.01108.x

Veerasamy G, Chinnagounder S (2013). Effect of polyamines on *in vitro* organogenesis using shoot tip explants of *Stevia rebaudiana* Bert. International Journal of Current Biotechnology,1 (1): 16-18.

Wang J, Sheehan M, Brookman H, Timko MP. (2000). Characterization of cDNAs differentially expressed in roots of tobacco (*Nicotiana tabacum* cv. Burley 21) during the early stages of alkaloid biosynthesis. Plant Science, 158 (1-2): 19-32. https://doi.org/10.1016/S0168-9452(00)00293-4

Wang X, Shi G (2004). Effect of exogenous spermidine on anti-Hg^{2+} stress ability of amaranth. Journal of Plant Physiolog and Molecular Biology 30: 69-74.

Wu M, Tian T, Liu H, Zhang H, Qiu Z et al.(2020). Shelter covering altered polyamines accumulation of cherry (*Prunus pseudocerasus* Lindl.) via elevating the expression of *spermidine synthase* (*SPDS*) gene. Scientia Horticulturae, 270: 109440. https://doi.org/10.1016/j.scienta.2020.109440

Xu D, Guo J, Xu L. (2014). The relationship between polyamine oxidase activity and lignin deposition and chrysanthemum flower bud differentiation. Acta Agricultureae Boreali Sinica 29: 164-169.

Yang SF, Hoffman NE (1984). Ethylene biosynthesis and its regulation in higher plants. Annual Review of Plant Physiology 35: 155-189. https://doi.org/10.1146/annurev.pp.35.060184.001103

Yu Y, Jin C, Sun C, Wang J, Ye Y et al. (2016). Inhibition of ethylene production by putrescine alleviates aluminium-induced root inhibition in wheat plants. Scientific Reports 6: 18888. https://doi.org/10.1038/srep18888

Yuan F, Yang H, Xue Y, Kong D, Ye R et al. (2014). OSCA1 mediates osmotic-stressevoked Ca^{2+} increases vital for osmosensing in *Arabidopsis*. Nature 514: 367-371. https://doi.org/10.1038/nature13593

Zeid IM, Shedeed ZA. (2007). Alterations in nitrogen metabolites after putrescine treatment in alfalfa under drought stress. Pakistan Journal of Biological Sciences: PJBS, 10 (9): 1513-1518. https://doi.org/10.3923/pjbs.2007.1513.1518

Zhou JG, Hu HL, Zhu YL, Zhang GW, Liu ZL. (2008). Effects of NaCl stress on free polyamines content and reactive oxygen species level in pumpkin roots. (in Chineese) Ying Yong Sheng Tai Xue Bao, 19 (9):1989-94.

Zhuo C, Liang L, Zhao Y, Guo Z, Lu S. (2018). A cold responsive ethylene responsive factor from *Medicago falcata* confers cold tolerance by up-regulation of polyamine turnover, antioxidant protection, and proline accumulation. Plant, Cell and Environment, 41 (9): 2021-2032. https://doi.org/10.1111/pce.13114

Figures and Tables

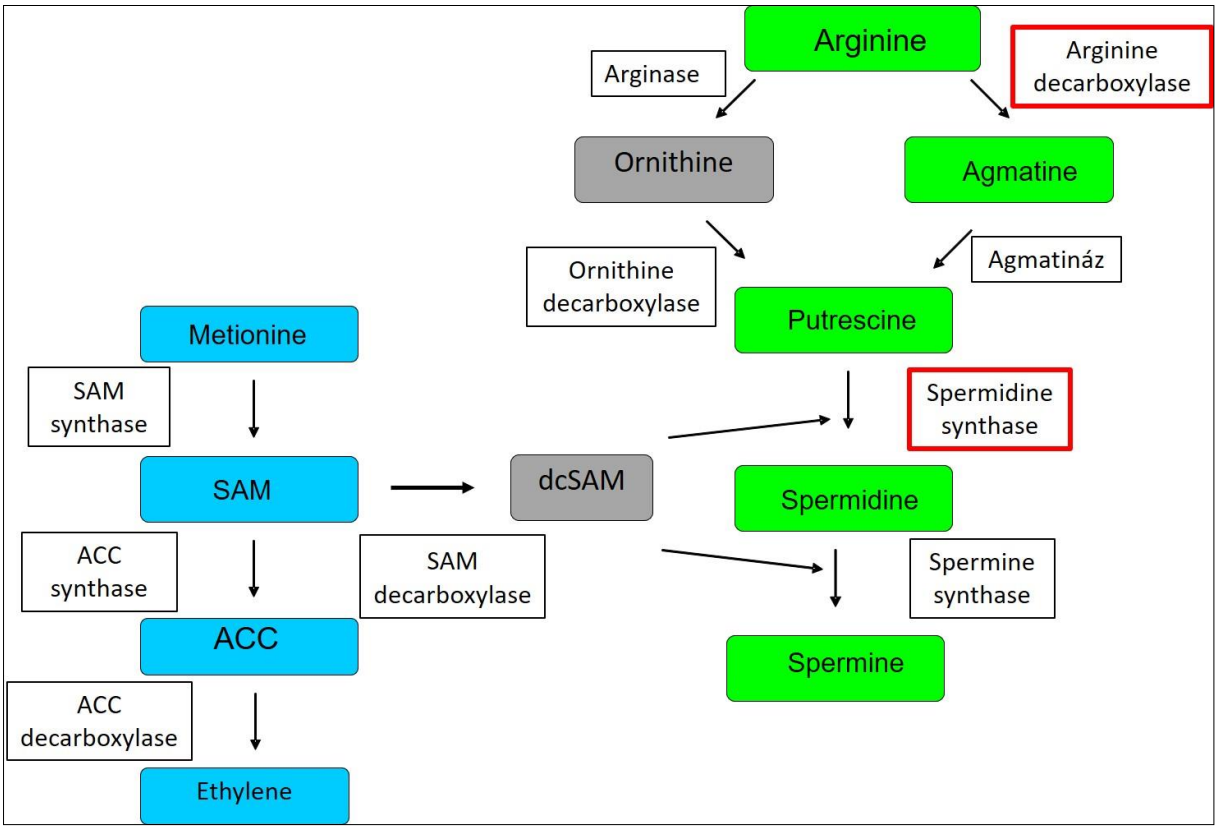

Figure 1: Biosynthesis of polyamines (green boxes) and ethylene (blue boxes), and the key enzymes (white boxes) (Mendel *et al*., 2018).

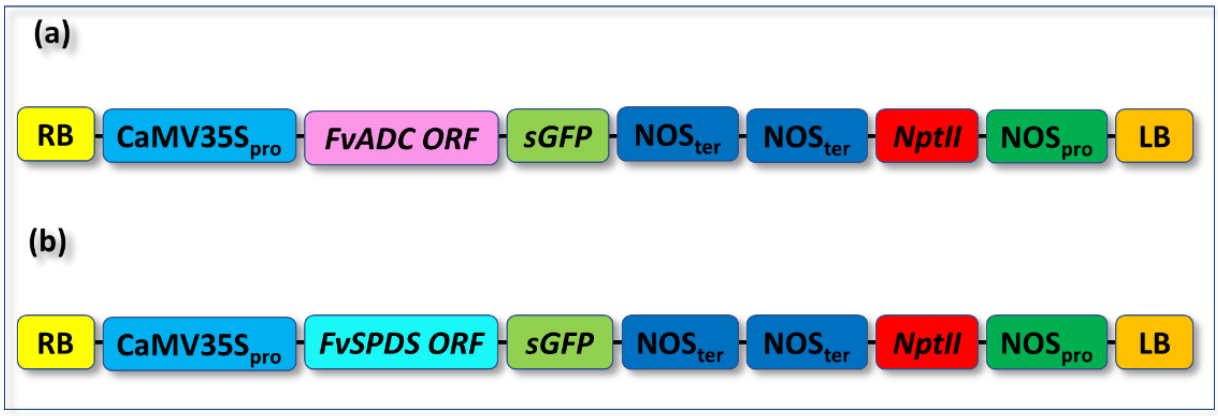

Figure 2: The T-DNA regions of pGWB405::*FvADC* (a) and pGWB405::*FvSPDS* (b) transformation vectors. RB- right border region; CaMV35Spro - cauliflower mosaic virus 35S constitutive promoter; sGFP - gene encoding synthetic green fluorescent protein; NOSter - nopaline synthase terminator region; NptII - neomycin phosphotransferase II (kanamycin resistance gene); NOSpro - nopaline synthase promoter; LB - left border region.

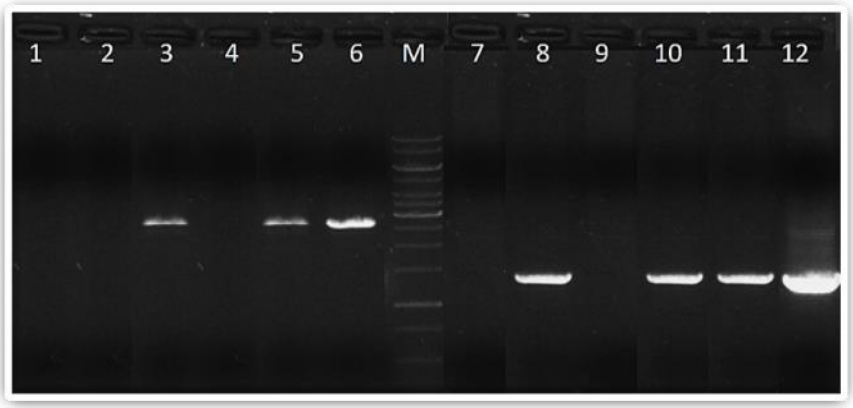

Figure 3: DNS-level testing of T_0 lines containing the pGWB405::FvADC and pGWB405::FvSPDS vector constructs. Fragments of the desired size are marked with white arrows. 1-5: T_0 1-5 lines transformed with pGWB405::FvADC vector construct; 6: pGWB405::FvADC vector construct (positive control); 7-11: T_0 1-5 lines transformed with pGWB405::FvSPDS vector construct; 12: pGWB405::FvSPDS vector construct: FvSPDS vector reconstruction (positive control); M: Molecular weight marker, ThermoFischer Scientific DNA Ladder 100 bp Plus.

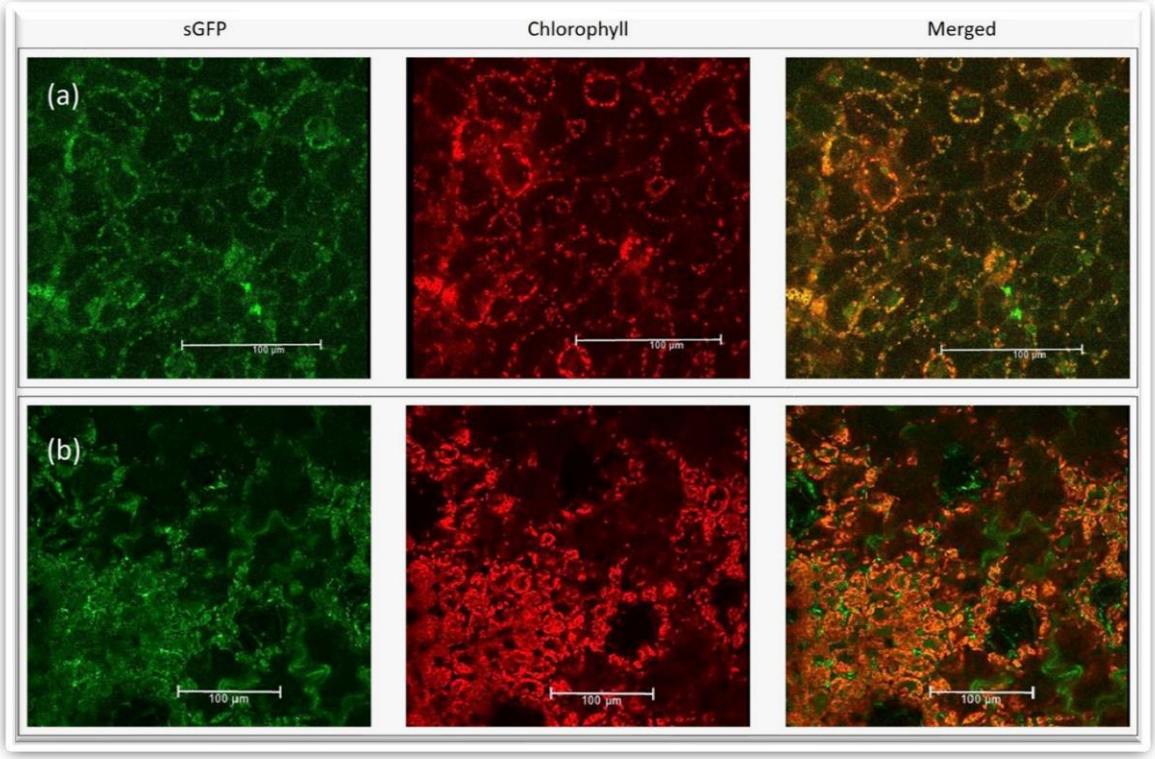

Figure 4: Expression pattern of the *FvADC*::sGFP construct in epidermal (a) and columnar parenchyma (b) cells of stable transformants. The line indicates 100 µm.

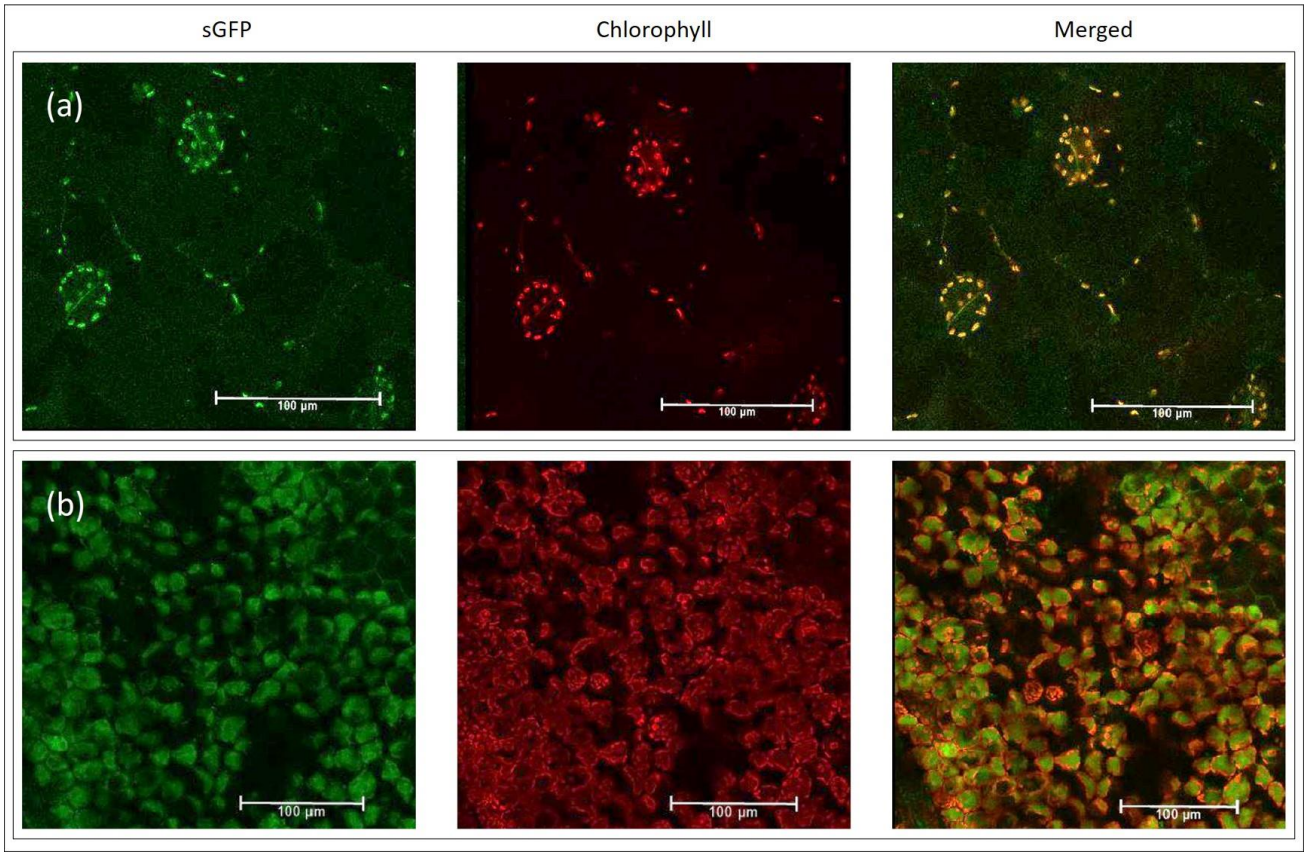

Figure 5: Expression pattern of the FvSPDS::sGFP construct in epidermal (a) and columnar parenchyma (b) cells of stable transformants. The line indicates 100 µm.

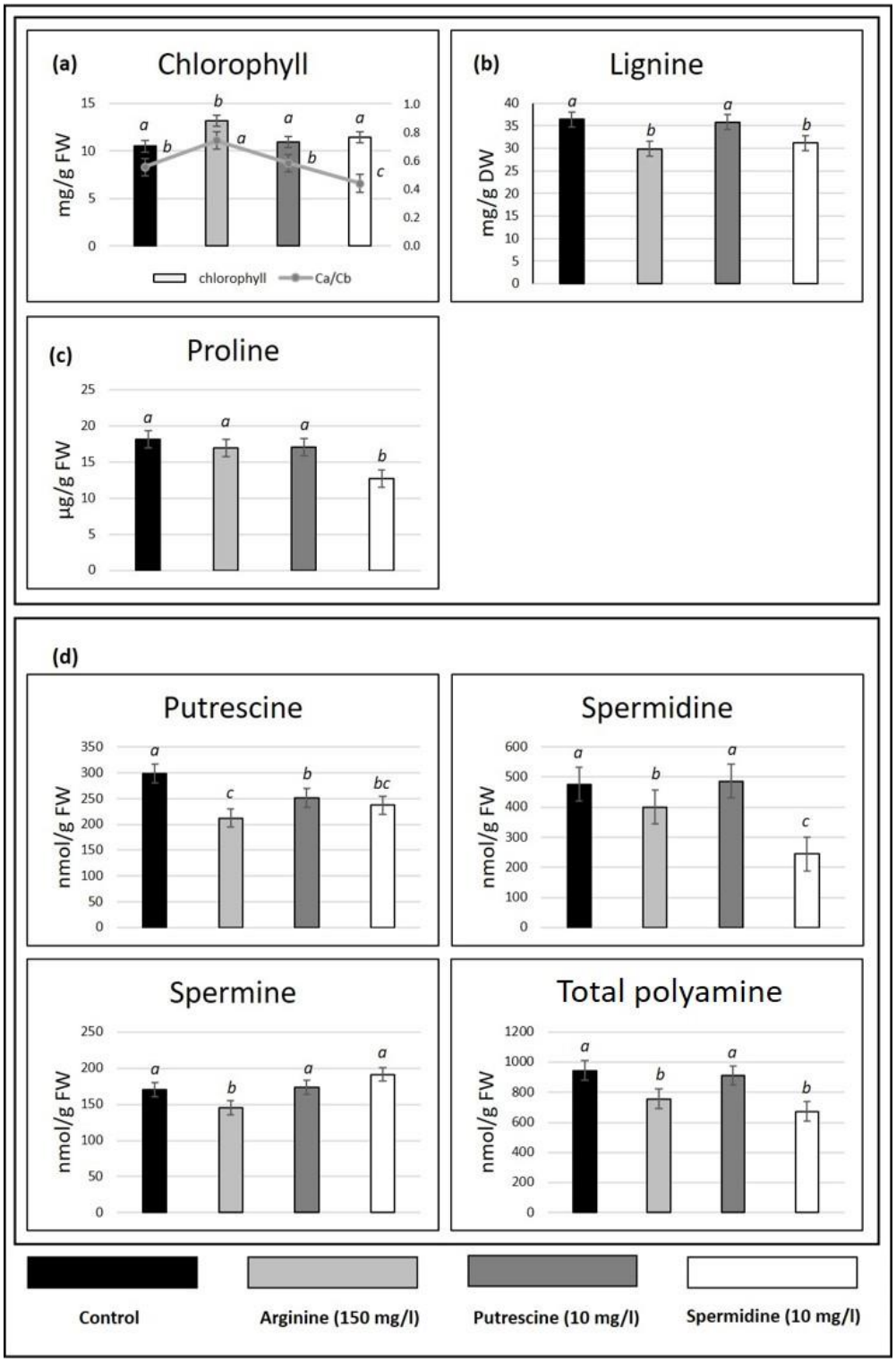

Figure 6: Chlorophyll *a* and *b* content and their relative ratios, lignin, proline, putrescine, spermidine, spermidine and total polyamine content of wild-type *Nicotiana tabacum* plants under control conditions and in response to 150 mg/l arginine, 10 mg/l putrescine and 10 mg/l spermidine (SD ±, n = 3), the significantly identical groups are indicated in italics ($p < 0.05$).

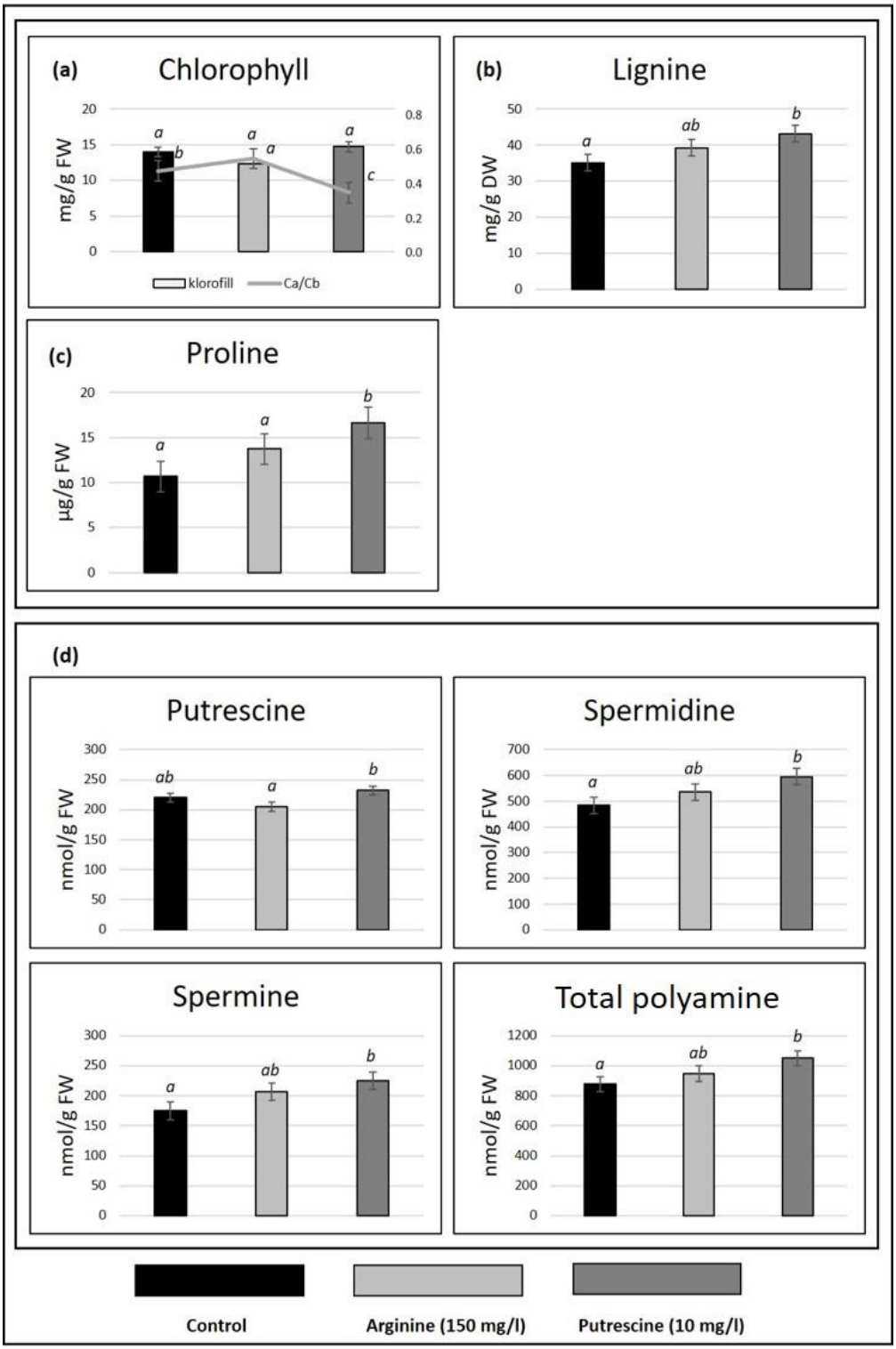

Figure 7: Chlorophyll *a* and *b* content and their relative ratios, lignin, proline, putrescine, spermidine, spermidine and total polyamine content of *FvADC Nicotiana tabacum* plants under control conditions and in response to 150 mg/l arginine, 10 mg/l putrescine and 10 mg/l spermidine (SD ±, n = 3), the significantly identical groups are indicated in italics ($p < 0.05$).

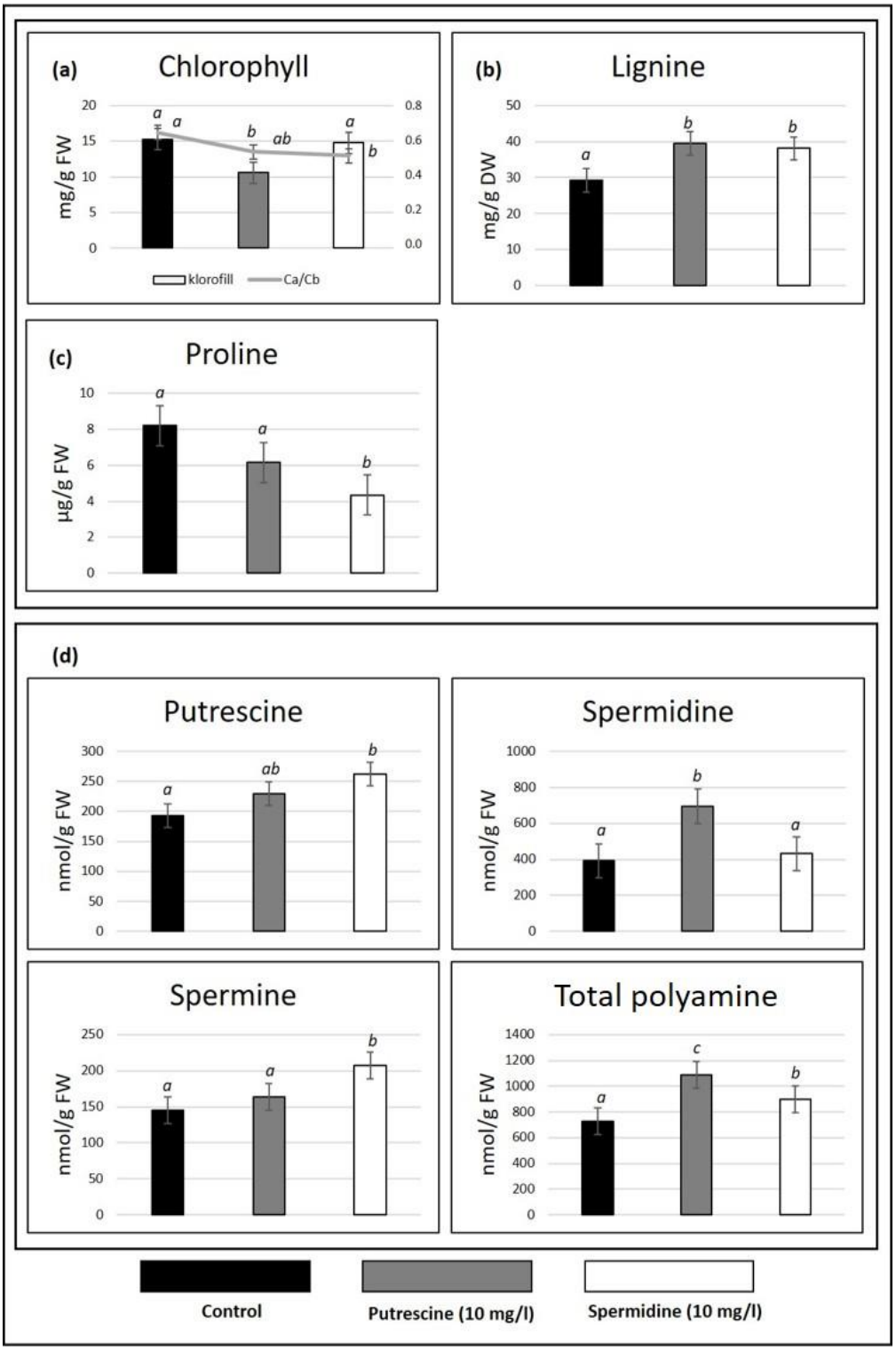

Figure 8: Chlorophyll *a* and *b* content and their relative ratios, lignin, proline, putrescine, spermidine, spermidine and total polyamine content of *FvSPDS Nicotiana tabacum* plants under control conditions and in response to 150 mg/l arginine, 10 mg/l putrescine and 10 mg/l spermidine (SD ±, n = 3), the significantly identical groups are indicated in italics ($p < 0.05$).

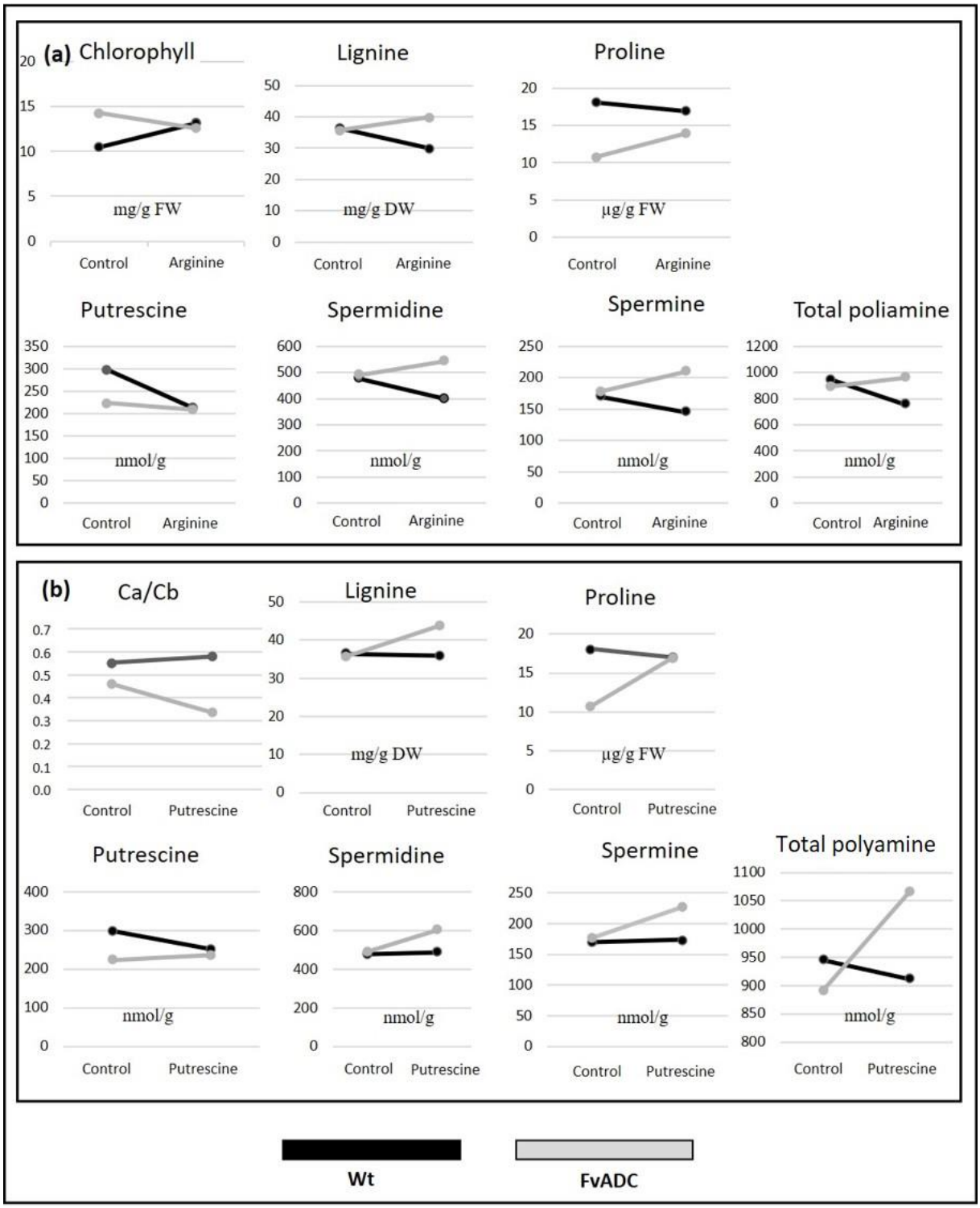

Figure 9: Comparison of parameter changes in wild-type (Wt) and *FvADC* *Nicotiana tabacum* plants after treatment with 150 mg/l arginine (a) and 10 mg/l putrescine (b) n = 9.

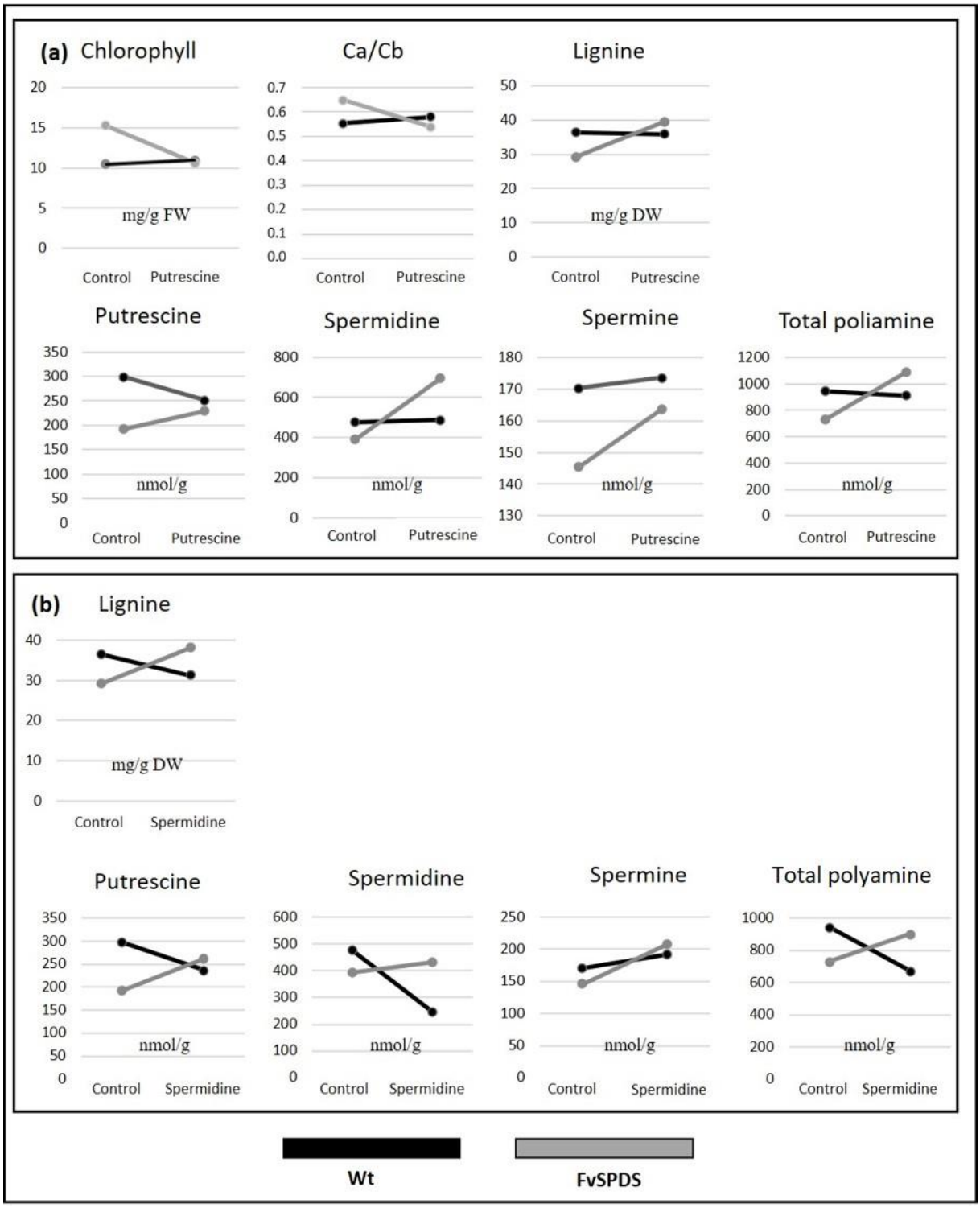

Figure 10: Comparison of parameter changes in wild-type (Wt) and *FvSPDS* *Nicotiana tabacum* plants treated with 10 mg/l putrescine (a) and 10 mg/l spermidine (b) n = 9.

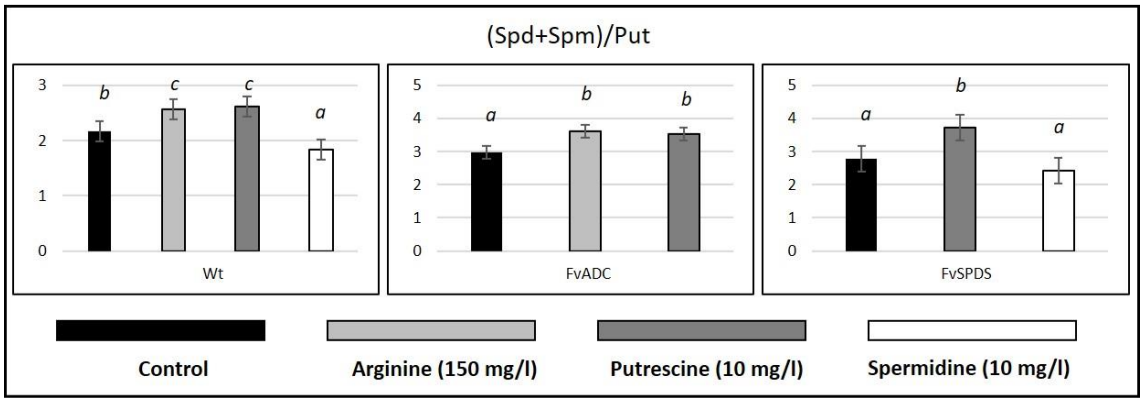

Figure 11: Proportions of polyamine forms of wild type, *FvADC* and *FvSPDS* *Nicotiana tabacum* plants, SD ±, n = 9, the significantly identical groups are indicated in italics ($p < 0.05$).

Table 1: Priners used for cloning *FvADC* and *FvSPDS* genes

Primer name	Sequence	Fragment length (bp)	$T_{annealing}$(°C)
FvADC_RT_F	CTTC**CAC**AACATGCCGTATCTG		54.8
FvADC_RT_R	TCAACCACTGCAGTATGACCACT		55.3
FvSPDS_RT_F	CAGAGAGTATATGGCTT**CAC**ATG**CAC**AT		58.5
FvSPDS_RT_R	GGTCCCTCAGTAGAACAGAGCAT		57.1

Table 2: Correlation between the measured parameters (n=66, p<0.01).

	Ca / Cb	Lignine	Putrescine	Spermidine	Spermine	Total polyamine
Lignine	-0.395					
Putrescine		0.492				
Spermidine	-0.343	0.712				
Spermine	-0.344	0.847	0.524	0.247		
Total polyamine	-0.357	0.920	0.373	0.917	0.569	
(Spd + Spm) / Put	-0.410	0.491	-0.413	0.874		0.679