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

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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 contents of the samples were measured spectrophotometrically, while proline, putrescine, spermidine, and spermine contents were determined using high-performance liquid chromatography methods. The experimental results showed that the arginine decarboxylase enzyme affects 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 (spermidine and spermine) 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 nonclimacteric fruit ripening. This compact, perennial model plant fruits early, can be propagated both generatively and vegetatively, and tolerates in vitro conditions well (Jiu et al., 2018). In strawberries, ethylene levels are highest during the green ripening phase, decline during the white phase, and then increase slightly again until the red ripening phase (Kovács et al., 2020), with no ethylene peak at full fruit ripening. Thus, strawberry fruit ripening is regulated not only by ethylene but also 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). IAA alone induces elongation of the receptor cells, simultaneously inhibiting the progress of ripening (Given et al., 1988) In contrast, elevated ABA 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

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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 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 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 posttranslational processes, but they 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 of great help in the in vitro cultivation of fruit species with poor rooting. It also increases tuber development, tuber size, and yield in several species (Pedros et al., 1999; Ondo Ovono et al., 2010). Additionally, Rhizome mass can be increased in vitro by polyamines. Bulb yield of tulip cultivars has 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 lignin accumulation between the primary and secondary cell walls is observed only in certain cells and at specific developmental stages (Hanson et al., 1994). SAM is used for the methylation of putrescine, nicotine, tropane, and nortropane alkaloids (Biastoff et al., 2009). 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.,

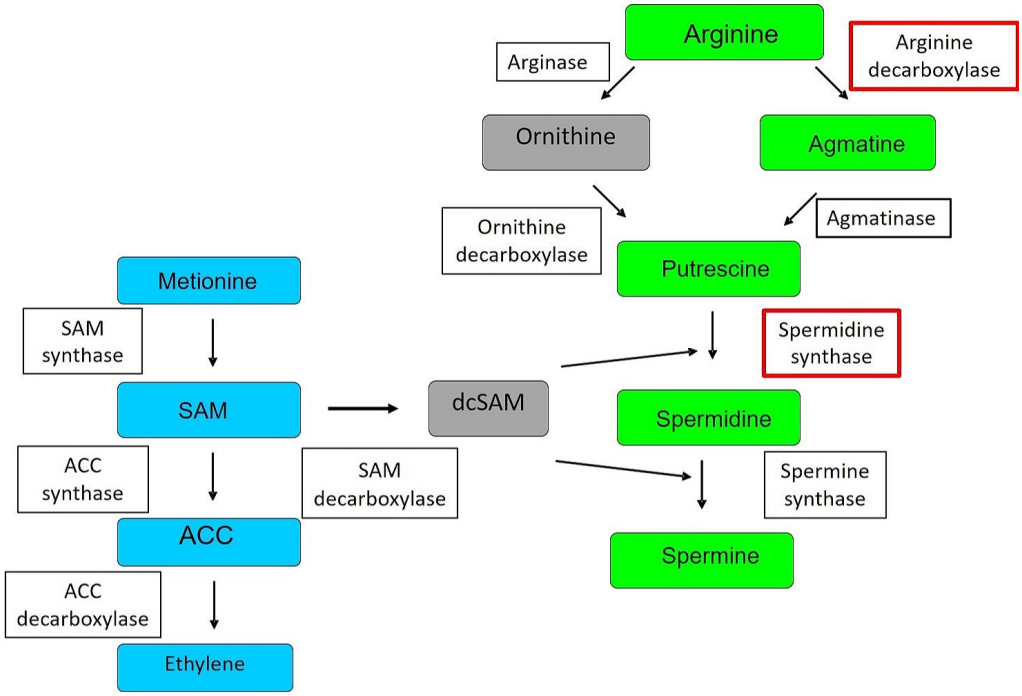

Figure 1. Biosynthesis of polyamines (green boxes) and ethylene (blue boxes), and the key enzymes (white boxes) (Mendel et al., 2018).

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). 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*, and *Oryza sativa* have reported 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 family *Brassicaceae*. The expression of the 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 tomatoes, the same enzyme is derived from a 55 kDa (502 amino acids) 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, and *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 and Carbonell, 1999). Similar expression patterns have been described in *Nicotiana tabacum*, *Medicago falcata*, and *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%, and *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 positively affects 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, MA, 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 reisolated from the agarose gel (Promega Wizard® SV Gel and PCR Clean-Up System Kit, Promega, Madison, WI, 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 modifications, we successfully fused the cDNA ORFs to the sGFP reporter gene at the C-terminus 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 28 °C.

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 cocultured for 4 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

Table 1. Primers 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

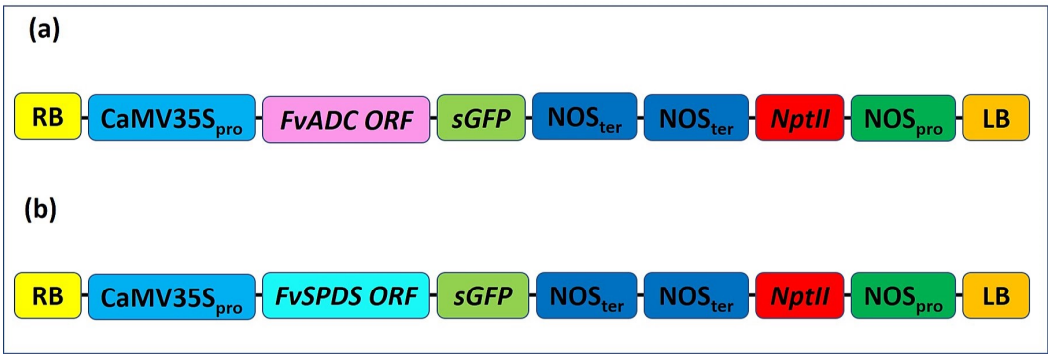

Figure 2. The T-DNA regions of pGWB405::*FvADC* (a) and pGWB405::*FvSPDS* (b) transformation vectors. RB, right border region; CaMV35Spro, cauliflower mosaic virus 35S constitutive promoter; sGFP, gene encoding synthetic green fluorescent protein; NOSter, nopaline synthase terminator region; NptII, neomycin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

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 preselected on Murashige and Skoog (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, and *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 23 °C, and samples were collected on day 90 after sowing.

2.4. Determination of chlorophyll content

Total chlorophyll, chlorophyll a, and chlorophyll b contents were determined according to the method described by 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 min. The samples in acetone were centrifuged at 12,000 × *g* for 20 min at 4 °C. The supernatant was centrifuged again at 12,000 × *g* for 5 min, also at 4 °C. One milliliter of 1 M Tris-HCl (pH 8) was added to 1 mL of supernatant, 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), 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. Two hundred milligrams of lyophilized shoots (stem and leaf mixed) were homogenized in 5 mL of 50 mM potassium phosphate buffer (pH 7) and the mixture was centrifuged at 1400 × *g* for 5 min. The supernatant was drained and centrifugation was repeated twice. Five milliliters of 1% Triton-X-100 (pH 7) was added to the bottom phase and the mixture was centrifuged at 1400 × *g* for 5 min. The supernatant was drained and centrifugation was repeated twice. Subsequently, 5 mL of 1 M NaCl (pH 7) was added to the samples and the mixture was centrifuged at 1400 × *g* for 5 min. The supernatant was drained and the step was repeated twice. Next, 5 mL of distilled water was added to the precipitate and the mixture was centrifuged at 1400 × *g* for 5 min. The supernatant was poured off and the wash was repeated twice. Subsequently, 5 mL of acetone was added to the samples, centrifuged at 1400 × *g* for 5 min and the supernatant was drained, centrifuged two more times, and dried at 60 °C for 12 h. To quantify lignin, the method described by Moreira-Vilar et al. (2014) was used. First, 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 70 °C for 30 min. The samples were then cooled abruptly on ice. Subsequently, 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 min. 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 high-performance liquid chromatography (HPLC)

Free polyamines and proline were determined with 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 described by 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. Subsequently, 100 µL of proline solution (100 mg/mL) was added and the mixture was 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 s 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 stereomicroscope (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 using 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.

3. 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 of 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 successful at several occasions (Figure 3), it was unnecessary to evaluate the proportion of these events. The transcription from the genes was 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 (Figures 4 and 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 photosynthetically inactive tissues, it is dominantly present in the nucleus. In *A. thaliana*, cytoplasmic and chloroplast localization of ADC has also been observed using sGFP proteins (Maruri-López and Jiménez-Bremont, 2017). In addition to the nucleus and cytoplasmic localization, 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

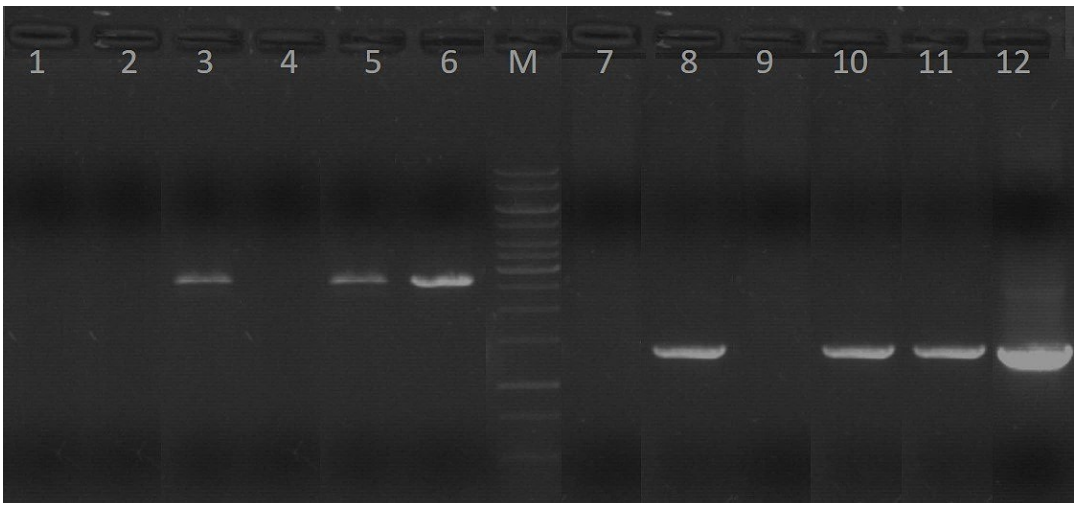

Figure 3. DNS-level testing of T_0 lines containing the pGWB405::FvADC and pGWB405::FvSPDS vector constructs. Fragments of the desired size are marked with white arrows. 1-5: T_0 1-5 lines transformed with pGWB405::FvADC vector construct; 6: pGWB405::FvADC vector construct (positive control); 7-11: T_0 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.

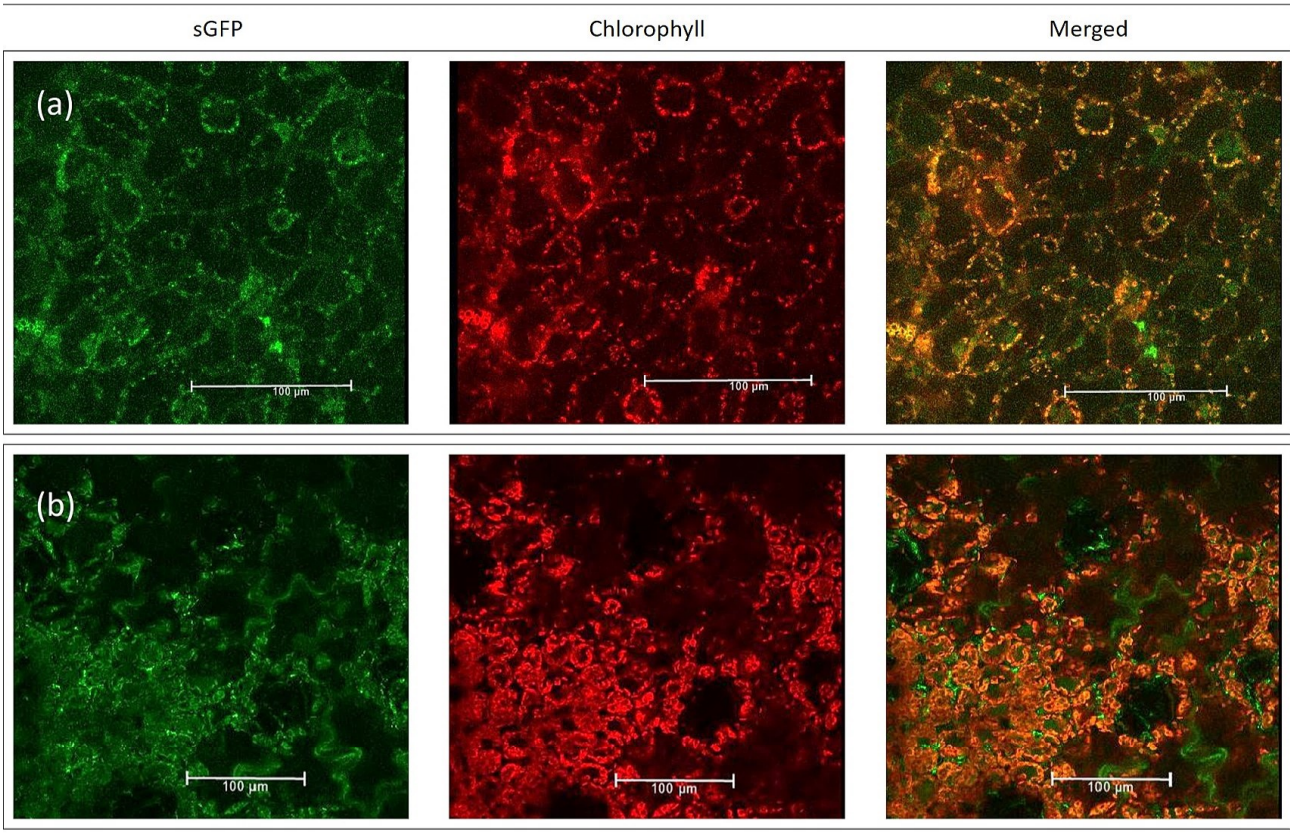

Figure 4. Expression pattern of the *FvADC*::sGFP construct in epidermal (a) and columnar parenchyma (b) cells of stable transformants. The line indicates 100 µm.

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 the 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 in Figures 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 the arginine and spermidine but reduced the measurable lignin content in Wt plants. In *FvADC* and *FvSPDS* lines, arginine, putrescine, and spermidine also increased the 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 (Figures 9 and 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

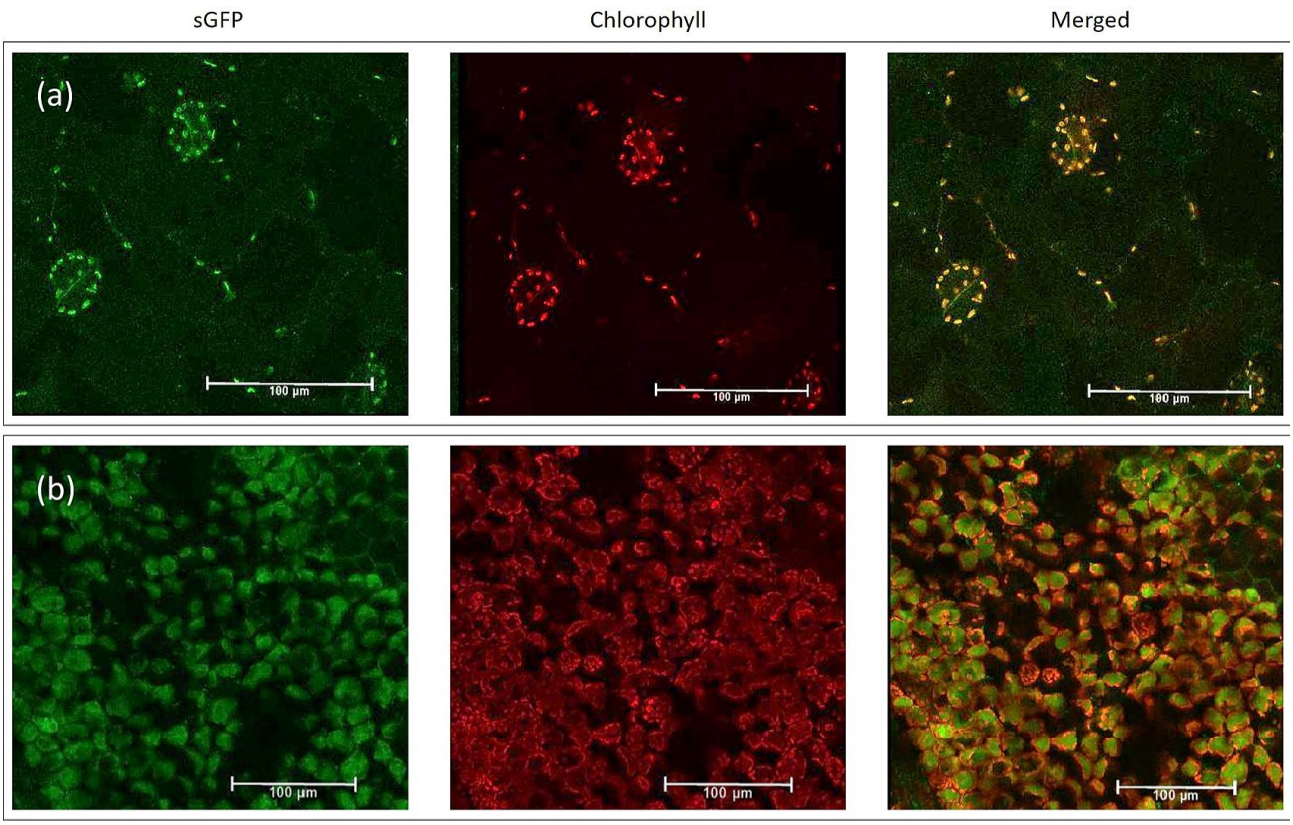

Figure 5. Expression pattern of the FvSPDS::sGFP construct in epidermal (a) and columnar parenchyma (b) cells of stable transformants. The line indicates 100 µm.

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 arginine and spermidine treatment, while 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 and Shedeed, 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 spermine 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 favor 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

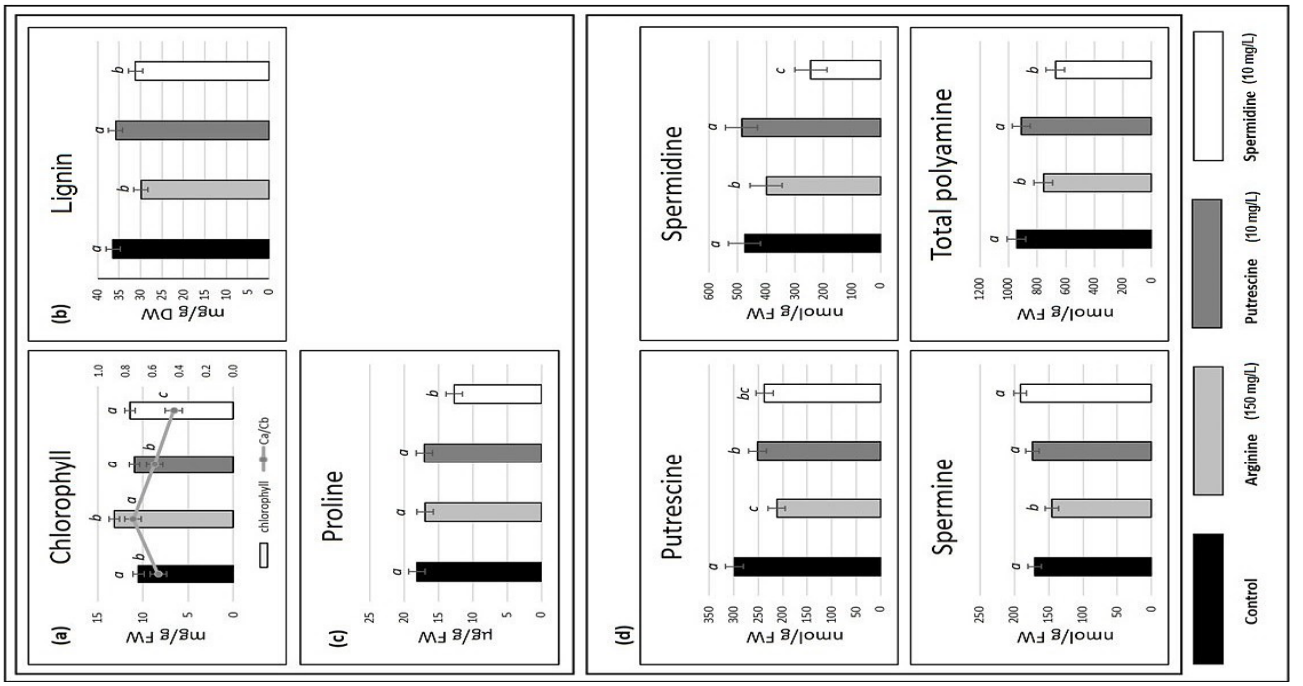

Figure 6. (a) Chlorophyll *a* and *b* contents and their relative ratios. (b–d) Lignin, proline, putrescine, spermidine, spermidine, and total polyamine contents of wild-type *Nicotiana tabacum* plants under control conditions and in response to 150 mg/L arginine, 10 mg/L putrescine, and 10 mg/L spermidine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

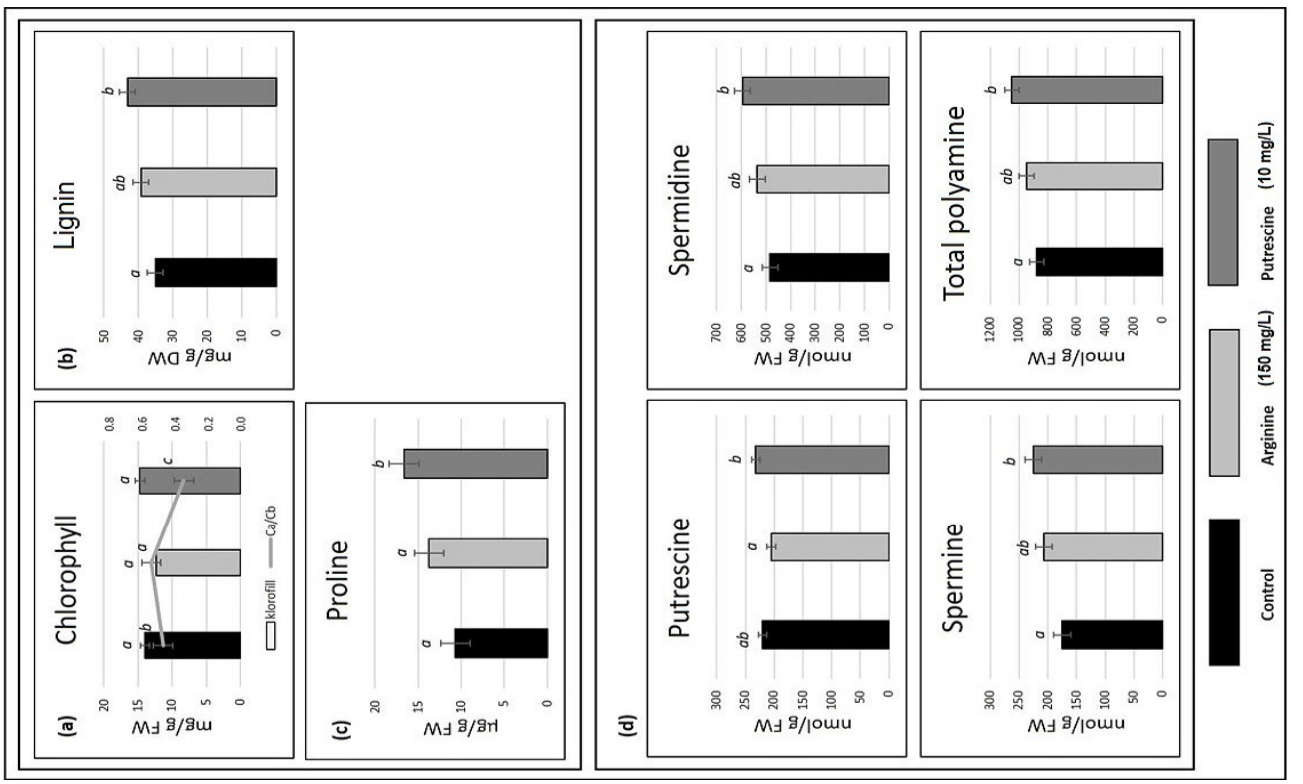

Figure 7. (a) Chlorophyll *a* and *b* contents and their relative ratios. (b–d) Lignin, proline, putrescine, spermidine, spermidine, and total polyamine contents of *FvADC Nicotiana tabacum* plants under control conditions and in response to 150 mg/L arginine, 10 mg/L putrescine, and 10 mg/L spermidine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

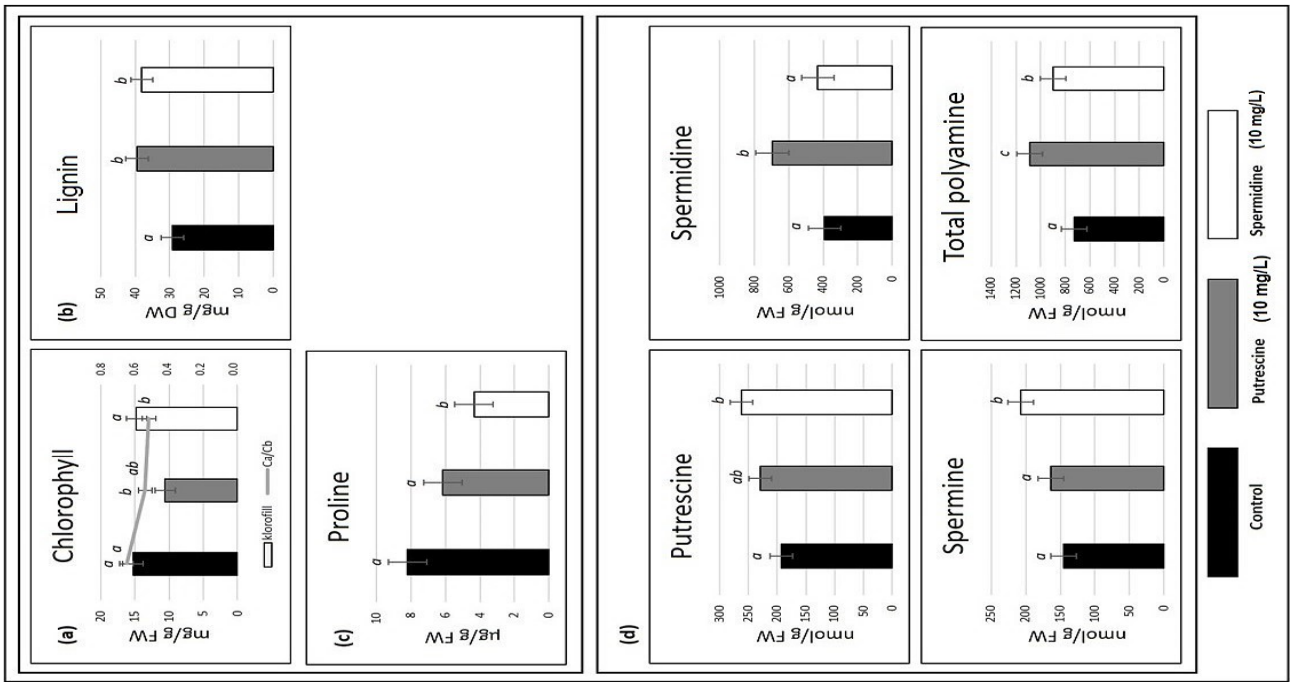

Figure 8. (a) Chlorophyll *a* and *b* contents and their relative ratios. (b–d) lignin, proline, putrescine, spermidine, spermidine, and total polyamine contents of *FvSPDS Nicotiana tabacum* plants under control conditions and in response to 150 mg/L arginine, 10 mg/L putrescine, and 10 mg/L spermidine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

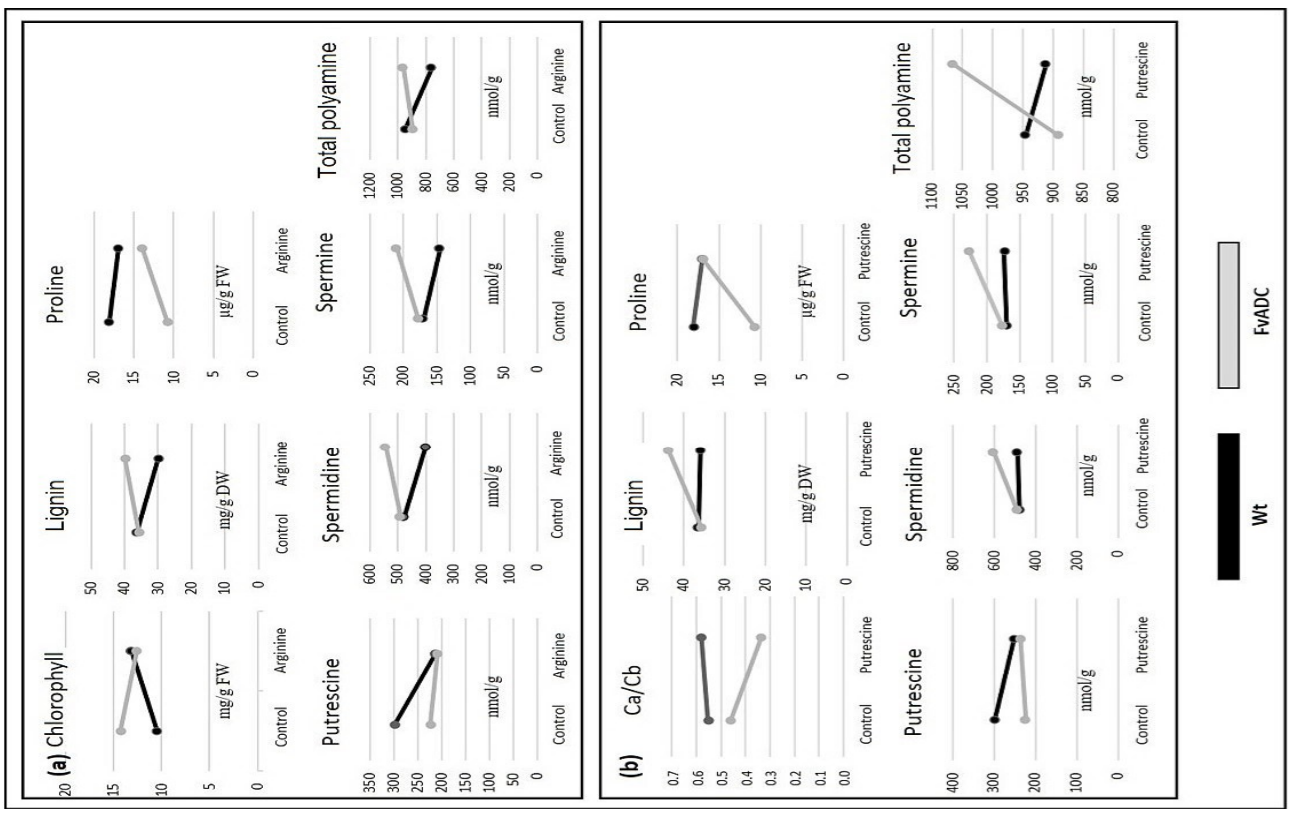

Figure 9. Comparison of parameter changes in wild-type (Wt) and *FvADC Nicotiana tabacum* plants after treatment with 150 mg/L arginine (a) and 10 mg/L putrescine (b) n = 9.

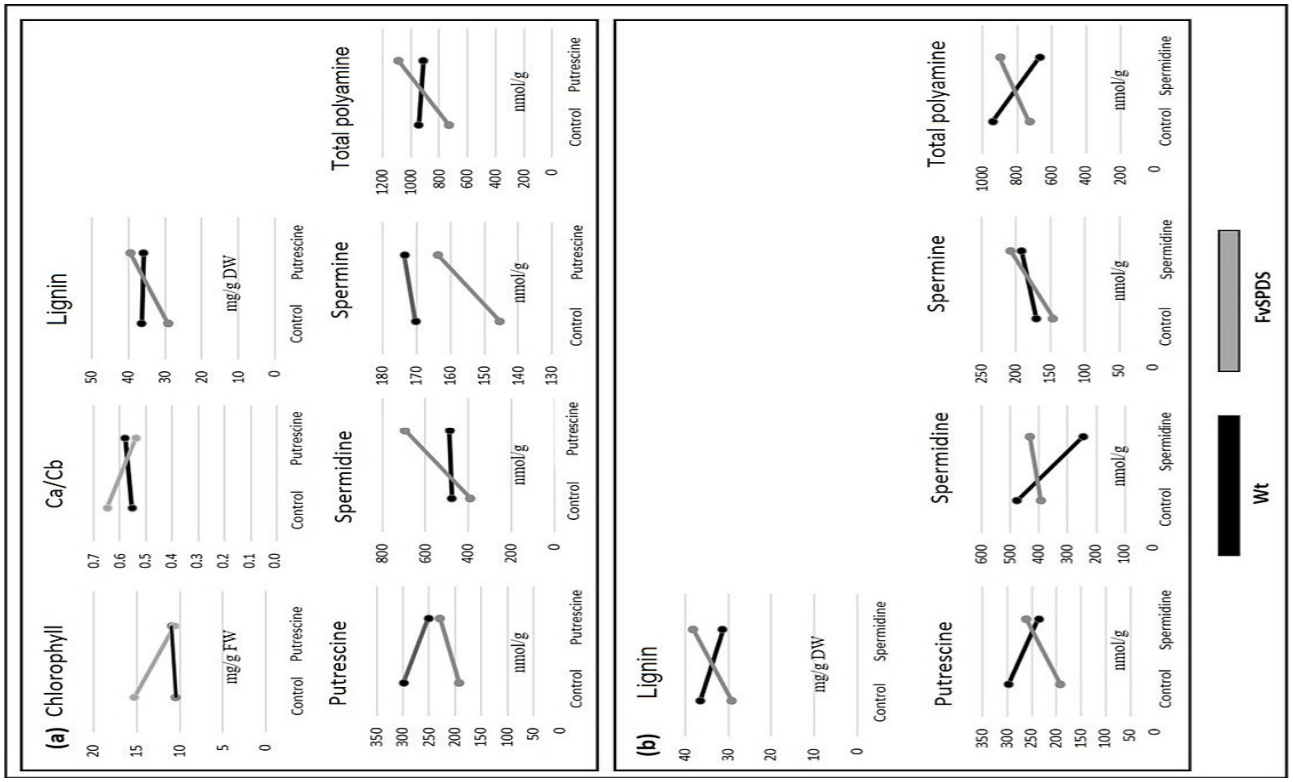

Figure 10. Comparison of parameter changes in wild-type (Wt) and *FvSPDS Nicotiana tabacum* plants treated with 10 mg/L putrescine (a) and 10 mg/L spermidine (b) n = 9.

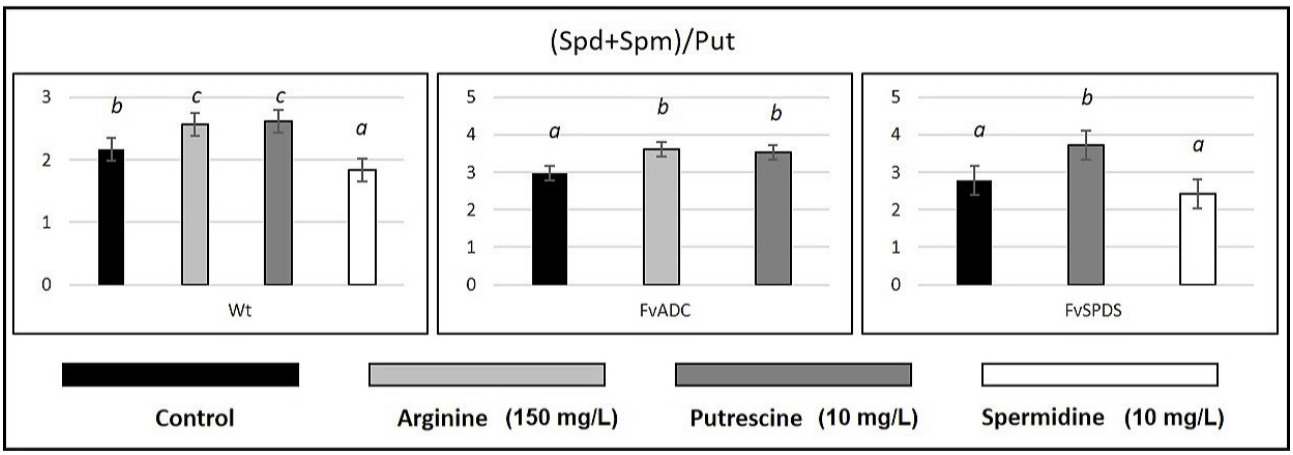

Figure 11. Proportions of polyamine forms of wild type, *FvADC*, and *FvSPDS Nicotiana tabacum* plants, SD ±, n = 9, the significantly identical groups are indicated in italics ($p < 0.05$).

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 spermine concentrations were measured (Shao et al., 2014). Putrescine can only slightly increase spermidine and spermine, there is no strong correlation between the two. In contrast, spermidine and spermine 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 nonclimacteric 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.

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

	Ca / Cb	Lignin	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

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