HUNGARIAN UNIVERSITY OF AGRICULTURE AND LIFE SCIENCES

Doctoral School of Plant Sciences

Functional evaluation of arginine decarboxylase (*FvADC*) and spermidine synthase (*FvSPDS*) genes of woodland strawberry (*Fragaria vesca* L. *cv.* 'Rügen') in tobacco (*Nicotiana tabacum* L.)

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1. SCIENTIFIC BACKGROUND AND OBJECTIVES

In researches conducted at Hungarian University of Agriculture and Life Sciences, Institute of Genetics and Biotechnology Balogh *et al*. (2005) were carried out RNA fingerprinting of receptacle and achene tissues of strawberry (*Fragaria x ananassa* Duch. cv. Elsanta). 130 transcript-derived fragment and partial cDNA were isolated and sequenced from genes showing altered expression patterns during the green, white, pink and red stages of ripening. Among the sequenced transcripts was the the spermidine synthase (DQ074728.1) which is involved in polyamine metabolism.

At the early stage of our researches, the genes and their promoters involved in polyamine metabolism were in focus. As a first step, sequences of putative *SPDS* genes of 12 fruit species were determined bioinformatically (Kovács *et al*., 2015). The theory was confirmed by PCR, using degenerated primers, then the sequence of *RiSPSD* gene of raspberry (*Rubus idaeus* cv. Blissy) was uploaded to the NCBI database (KP980552.1). High similarity was found between the genes, including with their promoters with the same function species belonging to *Rosaceae* family. The promoter regions contained fairly similar binding regions (Mendel *et al*., 2013).

The 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 in previous studies (Kovács, 2018; 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. This biosynthetic pathway is regulated by arginine -decarboxylase (ADC), ornithine-decarboxylase (ODC), a SAM-decarboxylase (SAMDC) and spermidine-synthase (SPDS) enzymes in plants (**Figure 1.**) (Hasegawa *et al*., 2000).

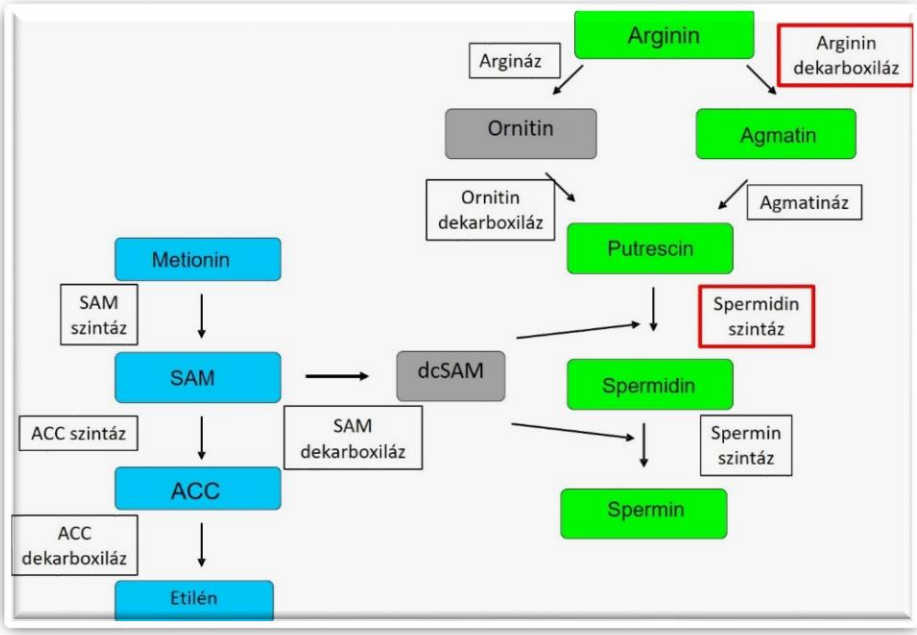

Figure 1: Biosynthesis of polyamines (green) and ethylene (blue), and the key enzymes (white) (Mendel *et al*., 2013).

Polyamines provide the stability of internal and external membranes of plant cells, attenuate the scale of oxidative injuries, and buffer the osmotic conditions. Moreover, some polyamine-forms help the production of antioxidant enzymes, which are responsible for the elimination of free radicals.

- Our primary goal was to gain a deeper understanding of the genes and their mode of action encoding the arginine decarboxylase and spermidine synthase enzymes.
- A comparative study was be performed between control and *Fragaria vesca* lines overexpressing the arginine decarboxylase (FvADC) and spermidine synthase (FvSPDS) enzymes in control and wild-type *Fragaria vesca* exposed to long-term arginine, putrescine and spermidine treatment.
- A further aim was to determine the subcellular localization of FvADC::sGFP and FvSPDS::sGFP enzymes.
- Finally, we aimed to evaluate the results and reveal to map the correlations between the values of the parameters studied and to detect the effect of arginine, putrescine and spermidine supplements and transgenes.

2. MATERIALS AND METHODS

2.1. Plant material and genetic transformation

The whole genome sequence of the *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*. ORF of *FvADC* is 2856 bp (putative FvADC - XM_004306397.2), ORF of *FvSPDS* is 1378 bp (putative FvSPDS - XM_004297595.2) long.

FvADC and *FvSPDS* sequences were ligated into pGWB405 binary vector. The binary vector pGWB405 contains a constitutive CaMV35S promoter and sGFP reporter gene, which is suitable for C-terminal fusion of the sequences (**Figure 2.**).

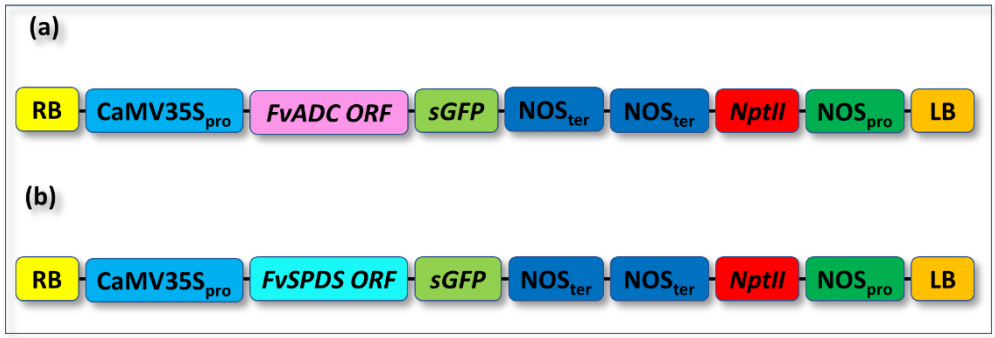

Figure 2: The binary vector constructs pGWB405::FvADC (a) and pGWB405::FvSPDS (b). RB- right border region; CaMV35Spro - cauliflower mosaic virus 35S constitutive promoter; sGFP - gene encoding synthetic green fluorescent protein; NOSter - nopaline synthase terminator region; NptII - neomycin phosphotransferase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

2.2. Proving the success of genetic transformation

The integration of the transgene into the plant genome was verified by PCR from plants growing normally on selective MS medium, then total RNA was isolated from plants with positive results and cDNA was synthesized using oligo(dT)18 primer. Transcription from the transgene was assayed by PCR using the aforementioned method using the cDNA samples.

The segregation rate of T1 lines from self-fertilization was examined in vitro on MS medium containing 80 µg/ml kanamycin. For the FvADC and FvSPDS lines, the desired 3:1 cleavage ratio was obtained for lines derived from several independent transformation events. From these, the following

three to three lines were selected for further studies: FvADC-5, FvADC-7, FvADC-37 FvSPDS-2, FvSPDS-9, FvSPDS-82.

2.3. Crop production parameters

Lines carrying a new marked copy of the arginine decarboxylase enzyme gene were placed on medium containing 150 mg/l arginine or 10 mg/l putrescine. The 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).

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). 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. To determine the amount of lignin, the acetyl-bromide method of Moreria-Vilar *et al*. (2014) was used.

2.6. Determination of proline and polyamine content using HPLC

According to the method of Smith and Davies (1985), the free polyamine fraction and the proline were converted to dansyl-chloride derivative. The determination was carried out by HPLC according to Németh *et al*. (2002).

2.7. Microscopic analysis

Visual detection of the green fluorescent fusion proteins (FvADC::sGFP, FvSPDS::sGFP) was performed using a Leica TCS SP8 laser scanning confocal microscope and a Leica/Leitz fluorescence stereo microscope without fixation from the adaxial side of the leaves.

2.8. Statistical analysis

For the tests the leaf samples were collected in equal proportions from 3-3 plants. The results were obtained from 9 measurements. Monofactorial

variance analysis (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.

3. RESULTS AND DISCUSSION

3.1. Expression pattern of FvADC, FvSPDS during fruit ripening in *Fragaria x ananassa* Duch.

Primers designed for the *Fragaria vesca* genome were 100% functional with the genomic DNA of *Fragaria x ananassa* Duch. cv. 'Asia', and qPCR was performed on cDNA reverse transcribed from RNA isolated from the receptaculum and ashene tissues of green, white, pink and red ripening stage fruits. For the *FaADC* gene, we measured a 14-fold higher relative expression in green ripening stage fruit than in white stage, and nearly 40-fold higher than in pink and red stages. A similar expression pattern of *FaADC* was obtained for the *FaSPDS* gene, with more than 10-fold higher relative expression detected in green fruit than in white and pink ripening stages. The relative expression of both genes was only prominent in the green ripening stage (Mendel *et al*., 2018).

3.2. Subcellular localization of FvADC and FvSPDS enzymes

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.

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 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*.) (Maruri-López and Jiménez-Bremont *et al*., 2014). No signal peptide coding sequence was found in the *SPDS* genes of *Morus spp*. Its localization was determined by its function (Liu *et al*., 2021). Plant SPDS enzymes do not contain transit peptide-specific sequences, nor does *sGFP* modify the expression pattern.

3.3. Evaluation of the parameters studied in the light of the treatments

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 the case of chlorophyll, only arginine treatment resulted in a significantly higher value for wild-type, while the addition of putrescine caused a significant decrease in FvSPDS plants. In the other cases no change could be detected. Our measurements also showed that the *Ca/Cb* ratio increased only in the arginine treatment in wild type and FvADC. Putrescine decreased this value in both transformant groups, whereas spermidine decreased it in the 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.

A significant decrease in proline content could be detected in spermidine-treated wild-type and FvSPDS plants, but proline content of FvADC lines increased with putrescine treatment. The other treatments had no measurable effect.

The addition of arginine, putrescine and spermidine to the medium had an impact on the concentration of different forms of polyamines. 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 was reduced by both arginine and spermidine treatment, putrescine was ineffective. Spermidine concentrations of FvADC lines increased with both treatments, while those of FvSPDS plants responded positively only to putrescine treatment. Spermine levels in the wild type were slightly reduced by arginine. Spermine levels in FvADC lines also increased in response to both treatments, but only spermidine had an effect on FvSPDS plants. The total polyamine content reflected the spermidine content in all three tested plant lines.

For wild-type plants, it was shown that the addition of arginine and putrescine to the medium significantly increased the (Spd+Spm)/Put ratio. 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. FvADC and FvSPDS lines responded to treatments in the same way as Wt plants.

3.4. 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. 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 high correlation with lignin (0.920) and spermidine content (0.917), and a moderately strong correlation with putrescine and spermidine content. Total polyamine content strongly correlated with spermidine content and weakly correlated with putrescine content. (Spd+Spm)/Put ratio shows a high 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. 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.

3.5. Comparison of responses to treatments in FvADC and FvSPDS enzyme overproducing and wild-type plants

The chlorophyll content of tobacco plants overproducing the enzyme arginine decarboxylase decreased on the addition of arginine, while that of the wild type increased. The excess arginine was decarboxylated by increased ADC activity and reduced chlorophyll content. In contrast, lignin and proline content increased compared to the wild type. Plant health and stress status parameters (except of chlorophyll content) were improved. The putrescine, spermidine and spermine content of FvADC plants showed an upward trend as expected after arginine treatment, and thus their total content increased. Plants overproducing the enzyme arginine decarboxylase responded to

putrescine treatment in a completely similar way as to arginine. The increase in the amount of polyamines was more pronounced in this case, increasing to a greater extent than in the previous treatment. Increased polyamine content results in increased stress tolerance.

The chlorophyll content and the proportion of chlorophyll forms of tobacco plants overexpressing the enzyme strawberry spermidine synthase also decreased to a greater extent in response to putrescine treatment than in wild-type plants. More intensive polyamine production did not contribute to an improvement in photosynthetic efficiency. As a substrate for the SPDS enzyme, putrescine also increased lignin content in parallel with the abundance of polyamine-forms. In addition to the expected increase in spermidine and spermine synthesis, the amount of putrescine also increased. Spermidine treatment increased the lignin and polyamine content of FvSPDS plants to a greater extent compared to the same parameters in wild-type plants. This means that increased production of spermidine synthase enzyme has a positive effect on plant health and stress tolerance. The addition of spermidine did not reduce the endogenous spermidine levels in transformant plants. The proline content of FvSPDS plants indicating stress status did not differ from that of wild type.

In our tests, the (Spd+Spm)/Put ratio did not show a significant interaction with respect to lines in any of the cases. The evolution of this value is completely independent of the transcriptional activity of the stronger ADC or *SPDS* gene.

4. CONCLUSIONS AND SUGGESTIONS

We demonstrated that *FaADC* and *FaSPDS* showed variable expression during early ripening of strawberry fruit. Based on our previous experiments, we have already described that several genes responsible for polyamine biosynthesis are also involved in fruit ripening, thus polyamines play an important role in quality changes. Polyamines enhance the activity of enzymes in the antioxidant system, reduce Na^+, H_2O_2 and O^{2-} toxicity, and enhance membrane stability, thus helping to reduce and counteract the effects of intracellular stresses during fruit development and ripening.

The *FvADC* and *FvSPDS* genes of *Fragaria vesca* L. cv. 'Rügen' were successfully introduced into tissues of *Nicotiana tabacum* L. plants, and transcriptional activity of the genes was demonstrated in the progeny. The resulting plant lines were subjected to further studies. 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.

All assays and measurements were performed on wild-type *N. tabacum* L. plants as well as on samples of transformant lines overexpressing FvADC and FvSPDS enzymes. 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. Arginine is consumed by others and therefore enhances polyamine biosynthesis to a lesser extent. In the case where arginine was diverted towards the production of polyamines (by overexpression of the *ADC* gene), its effect was more pronounced in the changes observed in the parameters studied.

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. In our tests, the (Spd+Spm)/Put ratio did not show a significant interaction with respect to the lines in any of the cases. 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.

Detection of which other biosynthetic pathways are affected by the molecular changes induced by overexpression of *FvADC* and *FvSPDS* genes is best achieved by qPCR assays following reverse transcription of RNA samples isolated from treated samples of wild-type and transformant lines. A move towards applied research would be to adapt the in vivo experimental system to greenhouse and field conditions for annual or even perennial horticultural crops. Plant conditioning agents containing polyamines are already commercially available. Their application by nutrient solution or spraying enhances root and shoot development and resistance to various stresses (herbicide, water deficit, cold, heat shock) and shortens the time of regeneration, but their usefulness is less well studied.

5. NEW SCIENTIFIC RESULTS

1. We have shown that *FaADC* and *FaSPDS* show variable expression during fruit ripening in *Fragaria x annanassa* Duch. cv. 'Asia' strawberry.
2. We have demonstrated that *FvADC* and *FvSPDS* genes are expressed in transgenic *Nicotiana tabacum* L. plants.
3. Intercellular localization of FvADC was determined using GFP fusion proteins.
4. We showed that *N. tabacum* L. plants overexpressing the FvADC enzyme were modified in more physiological parameters by putrescine than by arginine in the medium.
5. Based on the data measured in Wt, FvADC and FvSPDS lines, we found that chlorophyll content was independent of all parameters studied, with lignin, spermidine, spermidine, total polyamine, and (Spd+Spm)/Put ratio showing a negative correlation with *Ca/Cb* ratio. The amount of polyamines and the (Spd+Spm)/Put ratio are positively correlated with lignin content.
6. It has been shown that the ratio of polyamines to each other is not affected by the overproduction of FvADC or FvSPDS enzymes, but is increased by the addition of arginine or putrescine to the medium.

6. THESIS RELATED SCIENTIFIC PUBLICATIONS

Scientific article (in English)

Mendel Á., Kovács L., Szentgyörgyi A., Fekete S., Posta K., Kiss E. (2018). Expression patterns of ethylene and polyamine biosynthetic genes during fruit ripening in strawberry. Studia Universitatis „Vasile Goldiș" Seria Stiintele Vietii (Life Science Series), 28: (4) **IF: 0,199**

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Scientific article (in Hungarian)

Kovács L., **Mendel Á**., Kiss E. (2015). A málna (*Rubus idaeus* L.) és a fekete eperfa (*Morus nigra* L.) spermidin-szintáz gén szekvenciájának meghatározása. Kertgazdaság, 47 (2): 72-78.

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