SZENT ISTVÁN UNIVERSITY

Comparative analysis of overexpressed *Fragaria vesca* L. cv. Rügen S-adenosyl-L-methionine synthase (*FvSAMS*) and decarboxylase (*FvSAMDC*) during salt stress in transgenic *Nicotiana benthamiana*

Ph.D. thesis

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SCIENTIFIC BACKGROUND AND OBJECTIVES

In the Institute of Genetics, Microbiology and Biotechnology of Szent István University while carrying out the RNA fingerprinting of receptacle and achene tissues of strawberry (*Fragaria x ananassa* Duch. cv. Elsanta) genes showing altered expression patterns during the green, white, pink and red stages of ripening were identified. 130 transcript-derived fragment and partial cDNA were isolated and sequenced. Among the sequenced transcripts were the spermidine synthase (DQ074728.1) involved in polyamine metabolism and S-adenosyl-L-methionine (SAM)-dependent methyltransferase (AY873806). The SAM synthase (*SAMS*) and SAM decarboxylase (*SAMDC*) we chose to study were not among the sequenced transcripts. However, S-adenosyl-L-methionine, the common precursor molecule of ethylene and polyamine metabolism, is synthesized by SAMS, while decarboxylated SAM (dcSAM) the precursor molecule of polyamine metabolism is produced by SAMDC. Being a key signal molecule, ethylene takes part along with polyamines in numerous regulatory mechanisms involved in plant developmental and fruit ripening processes. That is why we set out to investigate whether the genes *SAMS* and *SAMDC* play a part in the fruit ripening process of strawberry.

Also, both ethylene and the polyamines play a key role in biotic and abiotic stress responses of plants and in excess amount have a positive effect on the salt tolerance of plants. However, several earlier studies found that ethylene and polyamine metabolism show antagonistic responses to biotic and abiotic stresses. Therefore, the overproduction of SAM synthase, which is the common molecule of the two metabolisms and produces the SAM, and the overproduction of SAM decarboxylase, which is the precursor molecule of the polyamine metabolism and synthesizes the decarboxylated SAM, will help us build a more comprehensive picture of the role the two metabolisms play in the various physiological processes. Furthermore, it will allow us to investigate how the 'SAM flux' and the 'SAM deficiency' influence the polyamine/ethylene ratio. A further aim of our present study was to carry out comparative investigations under control and long-term salt stressed conditions between the *Nicotiana benthamiana* lines overexpressing *Fragaria vesca* SAM synthase (*FvSAMS*) and SAM decarboxylase (*FvSAMDC*) isolated from non-climacteric *Fragaria vesca* L. cv. Rügen strawberry and the wild type. Studies carried out so far have separately investigated the effects of overexpressing the enzymes SAMS and SAMDC on the abiotic stress tolerance of plants and showed that the overexpression of both *SAMS* and *SAMDC* increased the tolerance of plants against different abiotic stresses. However, studying the two enzymes in one experimental system, which has not been done before, allows us to relate quantitatively the physiological parameters tested to one another, which help us build a more comprehensive picture of the role the two enzymes play in salt stress related responses. We are

going to investigate the effect of different ethylene/polyamine ratios on the development, senescence and salt tolerance of plants. The transgenic and wild type lines are going to be tested both under control and stress conditions being exposed to 10 mM NaCl stress. We measured the quantity of ethylene and polyamines, chlorophyll *a, b* and the lignin content, electrolyte leakage, the relative quantities of H_2O_2 és O_2^-, the growth vigour, stem length, the biomass product, proline content as well as the responses of transgenic leaf discs to 300 mM NaCl stress. In addition, the relative expression rates of genes involved in ethylene and polyamine metabolism, S-nicotine and L-lignin metabolism, as well as in SAM recycling were measured (*SAM-synthase, SAM-decarboxylase, ACC synthase, spermidine synthase, spermine synthase, S-adenosyl-homocystein hydrolase, putrescine N-methyltransferase, Cinnamyl-alcohol dehydrogenase*). These genes play a part in metabolisms, in which SAM serves as a substrate.

It was also our further aim to determine the subcellular localization of the enzymes *FvSAMS::sGFP* and *FvSAMDC::sGFP*, as well as changes in their subcellular compartmentalization in response to salt stress since the binary vector pGWB405 to be used in our investigations contained the constitutive CaMV35S promoter and the inserts (*FvSAMS, FvSAMDC*) were suitable for the C-terminal fusion with *sGFP*. In earlier studies SAMS was described as an enzyme with cytoplasmic localization in plants but in most recent studies its nuclear and cytoplasmic localizations were also detected in rats. In contrast, the enzyme SAMDC was found to show chloroplast, mitochondrial and cytosolic activities in plants and cytoplasmic localization in animals.

Finally, we aimed to predict the possible amino acid residues playing a part in the post-translational modification of enzymes FvSAMS and FvSAMDC by means of bioinformatic analysis.

MATERIAL AND METHODS

Plant material, vector constructs and bioinformatic analysis

For the identification of sequences, the NCBI database was used. The primers were designed based on the available whole genome sequence of *Fragaria vesca* L. (Shulaev et al., 2011) and tested both on genomic and cDNA of *Fragaria x ananassa* Duch. cv. Asia. The main ORF of *FvSAMS* is 1182 bp (XM_004288294.2), while the *FvSAMDC* is 1080 bp long (XM_011464655.1). The leaf tissue of *Fragaria vesca* L. cv. Rügen was used for the isolation of RNA and cDNA was synthesized by reverse transcription according to the method described below. The cDNA sequences of *FvSAMS* and *FvSAMDC* were amplified with the primer pairs designed for the main ORFs and the CaMV35S promoter was amplified with specific primer pairs. NetNES 1.1 (La Cour et al., 2004), TargetP 1.1 (Emanuelsson et al., 2007), cNLS Mapper (Kosugi et al., 2009), GPS-SNO 1.0 (Xue et al., 2010), Nucleolar localization sequence Detector (NoD) (Scott et al., 2011) and PHOSIDA (Gnad et al., 2011) applications were used for bioinformatic analysis of the sequences. The cDNA fragments were ligated into pGWB405 (AB294429.1) and the CaMV35S was ligated into pGWB604 (AB543113.1) binary vector according to manufacturer's protocol (Invitrogen™ Gateway®, ThermoFisher Scientific, Waltham, USA). The pGWB405 contained a constitutive CaMV35S promoter and the *sGFP* reporter gene while the pGWB604 carried only the *sGFP* (**Figure 1**). The *FvSPDS::sGFP* construct used for microscopic analysis was built by us using the pGWB405 vector. The insert codes for the spermidine synthase gene isolated from *Fragaria vesca* L. cv. Rügen. *FvSPDS::sGFP* fusion protein showed only cytoplasmic localization and was used as a cytoplasmic control.

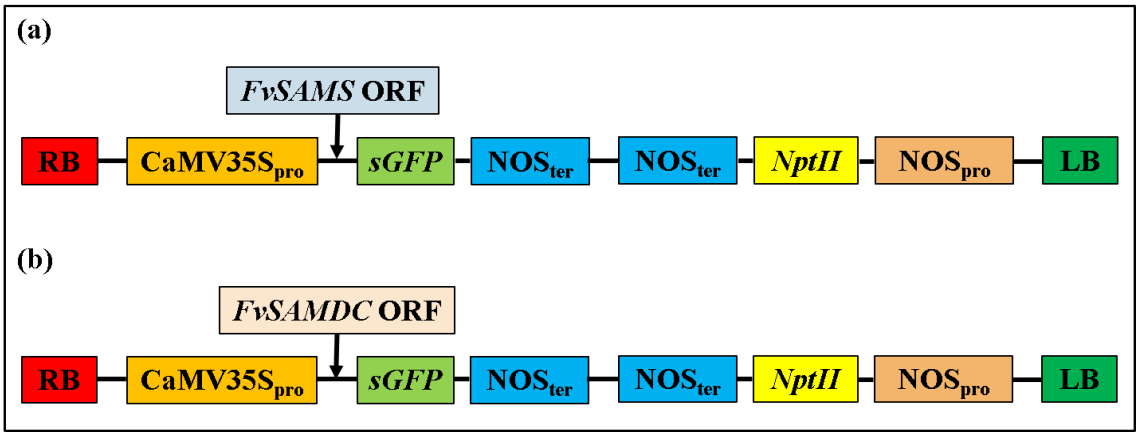

Figure 1. The *pGWB405::FvSAMS* (**a**) and the *pGWB405::FvSAMDC* (**b**) binary vector constructs. Abbreviations: RB- Right border; $CaMV35S_{pro}$ – Cauliflower mosaic virus 35S promoter; *sGFP* – Synthetic green fluorescent protein gene; NOS_{ter} – Nopalin synthase terminator; *NptII* – Neomycin phosphotransferase II; NOS_{pro} – Nopalin synthase promoter; LB – Left border.

DNA, RNA isolation and cDNA synthesis

The isolation of genomic DNA, plasmid DNA and total RNA was carried out by the CTAB method, PureYield™ Plasmid Miniprep System Kit (Promega, Madison, USA) and the Total RNA Mini Kit (Plant) (Geneaid®, New Taipei City, Taiwan), respectively, according to the manufacturer's protocols. cDNA was synthetized from total RNA by using Oligo $(dT)_{18}$ primer according to the protocol of the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, USA), which was used later for the RT qPCR analysis.

***Agrobacterium* mediated plant transformation and transient expression assay**

The *Agrobacterium tumefaciens* GV3101 strain was used for transformation of the *Nicotiana benthamiana* plants based on the method described by Clemente (2006). The *A. tumefaciens* GV3101 strain contained the *pGWB405::FvSAMS*, the *pGWB405::FvSAMDC* and the *pGWB604::CaMV35S* vectors. The same constructions were used for the *in vivo* infiltration of the 35-day-old *Nicotiana benthamiana* plants based on the method described by Li (2011).

Plant growth conditions

The pre-selection of the 2 transgenic T_1 lines from each construct was carried out on MS medium (supplemented with 75 mg/ml kanamycin) while the wild type *Nicotiana benthamiana* seeds were germinated on MS medium without antibiotics. On day 14, the seedlings were either transferred onto half strength MS medium without antibiotics or onto the same medium supplemented with 0.58 g/l (10 mM) NaCl, 3.5 g/l (60 mM) NaCl or 17.54 g/l (300 mM) NaCl. The plants and the explants were kept in 16 h light and 8 h dark periods at 23ºC. Samples were collected 85 days after sowing.

Chlorophyll *a, b* and relative chlorophyll content measurements

Chlorophyll *a* and *b* as well as the total chlorophyll contents were measured according to the method published by Porra et al. (1989). The chlorophyll *a* (Ca) and chlorophyll *b* (Cb), as well as the total chlorophyll (Ct=Ca+Cb) contents were determined based on the absorbance measured at 645 nm (Abs.645) and at 663 nm (Abs.663) by using NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, USA) and calculated according to the formulas: $Ca=0.0127\cdot(Abs.663)-0.00269\cdot(Abs.645)$; $Cb=0.0229\cdot(Abs.645)-0.00468\cdot(Abs.663)$. ImageJ 1.51j8 software was used for determining the relative chlorophyll content of leaf discs (Schneider et al., 2012).

Histochemical detection of H_2O_2 and O_2^-

The histochemical visualization of H_2O_2 and O_2^- was carried out according to the method described by Kumar et al. (2014). The chlorophyll-free leaves were fixed in 60% (v/v) glycerol. Photos were taken with Fuji FinePix S6500fd camera.

Determination of H_2O_2 content

The H_2O_2 content was determined according to Velikova et al. (2000). The absorbance of the samples was measured at 390 nm by using the WPA Biotech Photometer 1101 (Cambridge, UK). For plotting the standard curve dilutions of 30% (w/w) H_2O_2 (Sigma-Aldrich, Saint Louis, USA) were used.

Electrolyte leakage measurement

The electrolyte leakage measurement was carried out according to the method described by Rizhsky et al. (2002). The conductivity of samples was measured by using Radelkis Conductometer Type OK-104 (Budapest, Hungary).

Determination of proline content

The semi-quantitative determination of proline content was performed by the isatin paper assay of Ábrahám et al. (2010) protocol. L-proline (Sigma-Aldrich, Saint Louis, USA) solutions were used for plotting the standard curve. Photographs were taken with Fuji FinePix S6500fd camera and evaluated using ImageJ 1.51j8 software (Schneider et al., 2012).

Lignin measurement

The lignin content of the shoots was determined by the acetyl bromide method described by Moreira-Vilar et al. (2014). The absorbance of the samples was measured at 280 nm with WPA Biotech Photometer 1101 (Cambridge, UK). The standard curve was plotted using alkali lignin (Sigma-Aldrich, Saint Louis, USA).

Ethylene analysis

Ethylene release of the leaf discs was measured according to the method described by Langebartels et al. (1991). We put 5 leaf discs of 9 mm diameter on filter paper soaked in 1 ml 50 mM MES/NaOH (pH 5.6) and 2% (w/v) sucrose solution. We placed the scrolled filter papers into glass tubes covered by silica septum then incubated at 25°C for 1 hour. 1 ml of gas was injected into the Shimadzu GC-14A gas chromatograph (Kyoto, Japan) with the retention time of ethylene being 0.553 sec.

Polyamine analysis

Polyamines analysis was carried out from leaves as described by Németh et al. (2002). 200 mg plant sample was ground in liquid nitrogen, extracted with 2x1 ml 0.2 M ice cold perchloric acid, put on ice for 20 min, then centrifuged at 4°C for 20 min (10000g). In the case of free PA fraction, a dansyl-chloride derivative was prepared from 100 µl supernatant (Smith and Davies, 1985). Dansylated polyamines were measured in a WATERS W 2690 (Milford, USA) HPLC instrument using acetonitrile (Sigma-Aldrich, Saint Louis, USA) carrier.

qPCR analysis

Primers were designed for the sequences of the genes to be examined. The reactions were performed using Corbett RG-6000 real time PCR equipment (Qiagen, Hilden, Germany). The reaction mixture contained (in 20 µl final volume): 10 µl 2xABsolute qPCR SYBR Green Mix (ThermoFisher Scientific, Waltham, USA), 1.75 µl/primer (70 nM), 1 µl cDNA (diluted 1/50). For the determination of relative expression levels, the Comparative Quantification method was used supplied as part of Rotor-Gene Q Series Software 2.3.1. (Warton et al., 2004; McCurdy et al., 2008). *Nicotiana benthamiana* GAPDH and *Fragaria x ananassa* Duch. cv. Asia actin genes were used as an endogenous control.

Microscopic analysis

The detection of *FvSAMS::sGFP*, the *FvSAMDC::sGFP*, the *FvSPDS::sGFP* and the *35S$_{pro}$:sGFP* fusion proteins was performed by Leica TCS SP8 confocal laser scanning microscope and Leica/Leitz DMRB 301-371.010 fluorescence stereo microscope (Leica, Wetzlar, Germany). The leaves were examined without fixation with 1:1 87% (v/v) glycerol:bidistilled water being used for covering. For labelling the endoplasmic reticulum CellLight^{Tm} ER-RFP, BacMam 2.0 reagent was used (ThermoFisher Scientific, Waltham, USA). In contrast to the manufacturer's protocol with regard to the cell suspension, the CellLight^{Tm} ER-RFP was reinfiltrated into the living tissue (100-time dilution) followed by incubation at room temperature for 16 hours before the fixation of the tissue [4% (w/v) paraformaldehyde, 0.1% (v/v) Tween 20, 0.1% (v/v) Triton X-100, 0.15 M NaCl, 10 mM sodium phosphate buffer pH 7.5]. The LAS AF Lite 3.3.10134.0 software was applied for the processing of the pictures. ImageJ 1.51j8 software was used for determining the relative fluorescence of sGFP (Schneider et al., 2012).

Statistical analysis

The results are the mean values of at least 3 measurements and were statistically evaluated using the standard deviation and ANOVA methods.

RESULTS

The expression patterns of *Fragaria x ananassa* Duch. cv. 'Asia' S-adenosyl-L-methionine synthase (*FaSAMS)* and decarboxylase (*FaSAMDC)* during strawberry fruit ripening

The biosynthesis of ethylene proceeds through the following steps: S-adenosyl-L-methionine (SAM)→ 1-aminococyclopropane-1-carboxylic acid (ACC) →ethylene catalyzed by the SAM synthase (SAMS), catalyzed by 1-aminococyclopropane-1-carboxylate synthase (ACS) and by 1-aminococyclopropane-1-carboxylate oxidase (ACO) enzymes, respectively.

The polyamines are produced by binding an aminopropyl group from decarboxylated SAM (synthetized by SAMDC) to putrescine by the spermidine synthase (SPDS) and to spermidine by spermine synthase (SPMS) enzymes.

The common precursor of ethylene and polyamine metabolism is SAM and synthetized by *SAMS* genes. The *FaSAMS* shows its highest activity in the green and its lowest activity in the pink phase. The decarboxylated SAM is synthetized by the the *SAMDC* genes. These enzymes detour the flux from ethylene to polyamines, therefore SAMDCs are the key elements in balancing between these two products. The expression level of *FaSAMDC* increases gradually during the process of fruit ripening (**Figure 2**).

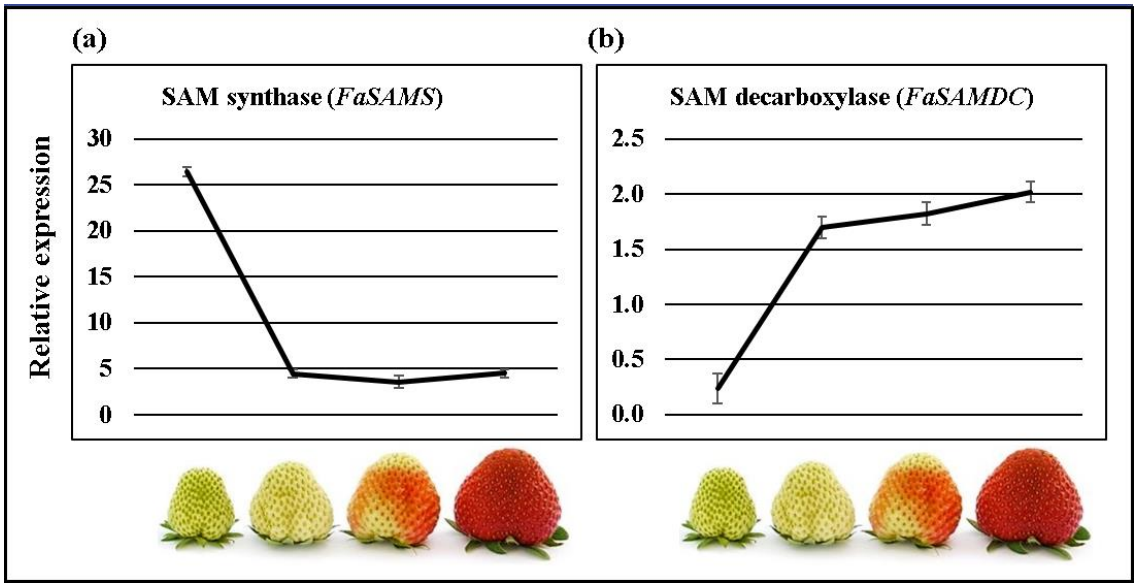

Figure 2. Relative expression levels of *FaSAMS* (**a**) and *FaSAMDC* (**b**) in four different ripening stages of *Fragaria x ananassa* Duch. cv. 'Asia'. Data are the means of four replicates (±SD). Error bars represent significant differences at $P< 0.001$.

The effects of NaCl stress on the physiological parameters of plants

The main ORF of *FvSAMS* codes for 394 amino acids and shows 95% similarity with AtSAMS1 (NP_171751.1), 93% with AtSAMS2 (NP_192094.1) and 90% with AtSAMS3 (NP_181225.1). The main ORF of FvSAMDC corresponds to 360 amino acids and its similarity

is 64% to AtSAMDC1 (NP_001154585.1), 66% to AtSAMDC2 (NP_197099.1), 68% to AtSAMDC3 (NP_001189972.1) and 43% to AtSAMDC4 (NP_197394.1). The FvSAMS-22 and FvSAMS-25 as well as the FvSAMDC-73 and FvSAMDC-76 transgenic lines are referred to hereinafter as FvSAMS and FvSAMDC lines.

The biomass of the plant is a good indication of its response to stress. The transgenic FvSAMS and FvSAMDC lines produced higher biomass (shoot and root) and increased shoot length than the non-transformed ones both in control and salt stress conditions.

We investigated the growing vigour and dry weight of the seedlings when exposed to 60 mM NaCl 30, 60 and 90 days after germination. We observed that both *FvSAMS* and *FvSAMDC* transgenics grew more intensively and produced higher biomass in MS medium containing 60 mM NaCl than the wild types (WTs).

Furthermore, we also examined the stress response of leaf discs of FvSAMS, FvSAMDC lines and WT *Nicotiana benthamiana* to short-term salt stress. We observed that the WT leaf explants died 48 hours after being placed on MS medium containing 300 mM NaCl. At the same time, the death of the leaf tissues of FvSAMS and FvSAMDC lines occurred later, 96 hours after salt exposure.

The chlorophyll content of plants demonstrates their physiological status, therefore we investigated the effect of long-term 10 mM NaCl stress on the total chlorophyll content of transgenic lines and wild type plants. The results of chlorophyll *a* and *b* content measurements show that the FvSAMS and FvSAMDC lines contained more chlorophyll than the WT ones both under stress-free and stressed conditions.

The accumulation of ROSs (Reactive Oxygen Species), similarly to that of H_2O_2 and O_2^- is in direct proportion with the stress levels of cells, therefore we investigated the H_2O_2 and O_2^- contents of tissues by means of histochemical staining as well as quantitative tests. The histochemical staining indicated that the presence of H_2O_2 could hardly be seen in the stress-free leaves of either the WT or FvSAMS and FvSAMDC lines. More intense coloration of the salt stressed WT leaves corresponds to higher concentrations of H_2O_2 than in the FvSAMS and FvSAMDC lines. Similarly, to H_2O_2 more intense coloration appeared in the WT leaves indicating that there is a higher amount of O_2^- in the non-transformed plants than in the FvSAMS and FvSAMDC lines. Furthermore, H_2O_2 measurements of salt treated leaves of FvSAMS and FvSAMDC lines showed low H_2O_2 contents compared to the WTs.

The measure of electrolyte leakage gives information about the status of the membranes. Therefore, we examined the electrolyte leakage of the leaf discs both under control and salt stress conditions. We detected lower rates of electrolyte leakage in both FvSAMS and FvSAMDC lines both under control and stress conditions than in the WTs.

We also measured proline content because the proline accumulation is also considered to be a good indicator of stress response. A slight increase in the proline content of FvSAMS lines could be observed in response to salt stress but not in the control conditions. In the non-stressed FvSAMDC lines slight proline surplus arose, which increased in response to the stress treatment.

Lignin is known to accumulate in between the primary and secondary cell wall to ensure the strength of cell wall during plant development. A firm cell wall provides the cells with increased protection against biotic and abiotic stresses. We also measured the lignin content of the stem under both control and salt stress conditions. Under the control conditions more lignin was found in the shoots of FvSAMS and FvSAMDC lines than in WTs. The salt stress caused a rise in the lignin content of the WT and FvSAMS lines, but a significant decrease was found in the FvSAMDC genotypes.

Since ethylene is implicated in numerous stress responses we investigated the effect of overproducing *FvSAMS* and *FvSAMDC* on the levels of ethylene both under control and salt stress conditions. In the FVSAMS lines, decreased ethylene levels were detected compared to WT both in response salt stress and in the control condition. In contrast, the FvSAMDC lines did not produce significantly higher levels of ethylene under control conditions than WTs, but the salt stress induced a substantial rise in their ethylene release.

We also investigated the effects of overproducing *FvSAMS* and *FvSAMDC* on the polyamine levels in addition to ethylene, therefore we examined the free polyamine content of the transgenic lines and WT plants both under control and salinity conditions. We detected significantly lower levels of putrescine (Put) in response to stress in the FvSAMS lines. However, the amount of Put was found to be significantly higher only in the FvSAMDC lines both under the control and stress conditions. In the FvSAMS lines significantly higher levels of spermidine (Spd) and spermine (Spm) were only detected in response to stress, while the amount of Spd and Spm was found to be significantly higher both under control and stress conditions in the FvSAMDC lines. In the FvSAMS lines significantly higher levels of total free polyamine content was only detected in response to stress, while the total free polyamine content was significantly higher in the FvSAMDC lines in response to both control and stress conditions.

Furthermore, we analyzed the expression of genes participating in ethylene (SAM synthase - *SAMS*; ACC synthase - *ACS*) and polyamine biosynthesis (SAM synthase - *SAMS*; SAM decarboxylase - *SAMDC*; spermidine synthase - *SPDS*; spermine synthase - *SPMS*), the recycling of SAM (S-adenosyl-L-homocysteine hydrolase - *SAHH*) and the production of two secondary metabolites such as lignin (Cinnamyl-alcohol dehydrogenase - *CAD*) and nicotine (Putrescine N-methyltransferase - *PMT*). The relative expression rate of *SAMS*, *SAMDC*, *SPMS*, *SAHH* and *CAD* was significantly higher in the FvSAMS and FvSAMDC lines than in the WTs both under control

and salt stress conditions. The transcription rate of *ACS* was found to be significantly lower in the FvSAMS lines under non-stressed condition, whilst in response to salt stress it decreased. At the same time, FvSAMDC lines displayed higher levels of *ACS* expression than in the WTs under stress condition. As for the transcription rate of *SPDS* in stress-free environment, a decrease could only be detected in FvSAMS lines, while it increased both in the FvSAMS and FvSAMDC lines in response to salt treatment. The relative expression rate of *PMT* was lower in the FvSAMS lines in the control environment and higher in the FvSAMDC lines than in the WTs. Significantly higher rate of *PMT* expression was measured in response to stress in both the FvSAMS and FvSAMDC lines. The expression levels of *CAD* were higher in the FvSAMDC lines than in the WTs and increased in both types of salt stressed transgenic lines.

The summary of the measurements is shown in **Table 1**.

Table 1. The summary of measurements.

	FvSAMDC	**FvSAMS**	**FvSAMDC**	**FvSAMS**
	Control condition (fold change)		**10 mM NaCl stress (fold change)**	
Biomass production/WT	~ 2.7 (↑)	~ 1.6 (↑)	~ 3.0 (↑)	~ 2.9 (↑)
Shoot length /WT	~ 1.7 (↑)	~ 1.5 (↑)	~ 1.8 (↑)	~ 1.7 (↑)
Electrolyte leakage/WT	~ 1.8 (↓)	~ 1.3 (↓)	~ 1.9 (↓)	~ 1.4 (↓)
Chlorophyll *a* and *b* content/WT	~ 1.6 (↑)	~ 1.3 (↑)	~ 1.9 (↑)	~ 1.7 (↑)
H_2O_2 concentration/WT	~ 1.4 (↓)	~ 1.2 (↓)	~ 1.8 (↓)	~ 1.3 (↓)
Proline content/WT	~ 1	~ 1.1 (↑)	~ 4.3 (↑)	~ 1.2 (↑)
Lignin content	~ 1.8 (↑)	~ 2.8 (↑)	~ 1.5 (↓)	~ 1.7 (↑)
Ethylene production/WT	~ 1.1 (↑)	~ 1.1 (↓)	~ 1.5 (↑)	~ 1.1 (↓)
Total free polyamine content				
Put/WT	~ 1.2 (↑)	~ 1.1 (↓)	~ 1.2 (↑)	~ 1.2 (↓)
Spd/WT	~ 1.2 (↑)	~ 1.1 (↑)	~ 1.9 (↑)	~ 1.3 (↑)
Spm/WT	~ 1.3 (↑)	~ 1.2 (↑)	~ 1.8 (↑)	~ 1.3 (↑)
Total free PA/WT	~ 1.2 (↑)	~ 1.1 (↑)	~ 1.7 (↑)	~ 1.2 (↑)
Relative expression measurement				
***SAMS*/WT**	~ 2.0 (↑)	~ 1.8 (↑)	~ 2.4 (↑)	~ 3.9 (↑)
***SAMDC*/WT**	~ 1.2 (↑)	~ 1.2 (↑)	~ 1.6 (↑)	~ 1.2 (↑)
***ACS*/WT**	~ 1.1 (↓)	~ 1.1 (↓)	~ 2.1 (↑)	~ 1.5 (↓)
***SPDS*/WT**	~ 1	~ 1.1 (↓)	~ 1.4 (↑)	~ 1.1 (↑)
***SPMS*/WT**	~ 1.3 (↑)	~ 1.2 (↑)	~ 1.5 (↑)	~ 1.2 (↑)
***SAHH*/WT**	~ 1.8 (↑)	~ 1.5 (↑)	~ 1.3 (↑)	~ 1.3 (↑)
***PMT*/WT**	~ 2.4 (↑)	~ 4.8 (↓)	~ 34.9 (↑)	~ 3.0 (↑)
***CAD*/WT**	~ 1.3 (↑)	~ 1.7 (↑)	~ 5.1 (↑)	~ 1.3 (↑)

It can be concluded that with the exception of the proline levels measured under control conditions and the lignin content under control and salt stress conditions, the overexpression of

FvSAMDC had more favourable effects on all the plant physiological parameters of the transgenic lines under both the control and salt stress conditions, than the overproduction of *FvSAMS*.

Subcellular localization of *FvSAMS::sGFP, FvSAMDC::sGFP* and bioninformatic analysis

We carried out transient expression investigations with the *FvSAMS::sGFP*, *FvSAMDC::sGFP*, *FvSPDS::sGFP* (*Fragaria vesca* L. cv. Rügen spermidine synthase) and *35S$_{pro}$:sGFP* constructs. Transient expression of *FvSAMS::sGFP*, *FvSAMDC::sGFP* and *35S$_{pro}$:sGFP* fusion proteins displayed nuclear and cytoplasmic localization, while *FvSPDS::sGFP* showed only cytoplasmic distribution.We noticed that each of the three fusion proteins (*FvSAMS::sGFP*, *FvSAMDC::sGFP* and *35S$_{pro}$:sGFP*) showed endoplasmic reticulum (ER) signals during their transient expression, therefore we used ER labelling with the application of CellLight^{TM} ER-RFP. The *FvSAMS::sGFP* and *FvSAMDC::sGFP* showed weaker ER signals than the *35S$_{pro}$:sGFP* construct. The fixation of the leaf tissue was carried out following the ER labelling, which resulted in the sGFP signal becoming less pronounced and therefore allowed us to observe the distinctive nuclear and nucleolar signals of *FvSAMS::sGFP*, which were detectable in the stable transformants as well. In the stable transformants the *FvSAMS::sGFP* did not show homogeneous cytoplasmic distribution, but it accumulated in the outermost regions of the cytoplasm near to the plasmalemma and plastid membrane. The *FvSAMS::sGFP* exhibited nuclear, nucleolar, cytoplasmic (near to the plasmalemma) and plastid membrane, while the *FvSAMDC::sGFP* showed nuclear and homogenous cytoplasmic localization (**Figure 2A, B, C, D**).

The distribution of *FvSAMS::sGFP* and *FvSAMDC::sGFP* fusion proteins in the stable transformants both under control and saline conditions is investigated (**Figure 3A**). We observed differences in the ratios of nuclear to cytoplasmic distribution under stress-free and salt stressed conditions (**Figure 3B**). The nuclear signal of *FvSAMS::sGFP* was more intense in the control environment, and the cytoplasmic expression became stronger in response to salt stress. In the case of *FvSAMDC::sGFP* the amount of fusion proteins increased substantially in response to salt stress both in the nucleus and the cytoplasm. At the same time it could be observed that the *FvSAMDC::sGFP* showed distinctive nuclear, cytoplasmic and nuclear-cytoplasmic localization concurrently. Furthermore, we also noticed that in the case of FvSAMS lines the abundance of the *FvSAMS::sGFP* fusion protein changed depending on the position of the leaves in the plant. In the leaves closest to the apex the sGFP protein could be detected in almost all of their cells while moving downwards the number of sGFP expressing cells decreased, finally leaving only the guard cells of the stomata giving off the sGFP signal. Therefore, we determined the amount of *sGFP* mRNA in the leaves from the top to 4th node and from the 5th to 7th nodes in the FvSAMS lines.

Despite the fact that the detectability of sGFP protein was higher in the upper leaves the amount of mRNA showed the opposite trend with the lower leaves containing ~2-5-times more mRNA than the upper ones. Similar phenomenon was not observed in the FvSAMDC lines. To further investigate the nuclear, nucleolar, cytoplasmic (near to the plasmalemma) and plastid membrane localization of *FvSAMS::sGFP* and the nuclear and cytoplasmic localization of *FvSAMDC::sGFP*, the amino acid of FvSAMS and FvSAMDC sequences were examined by means of various bioinformatic applications.

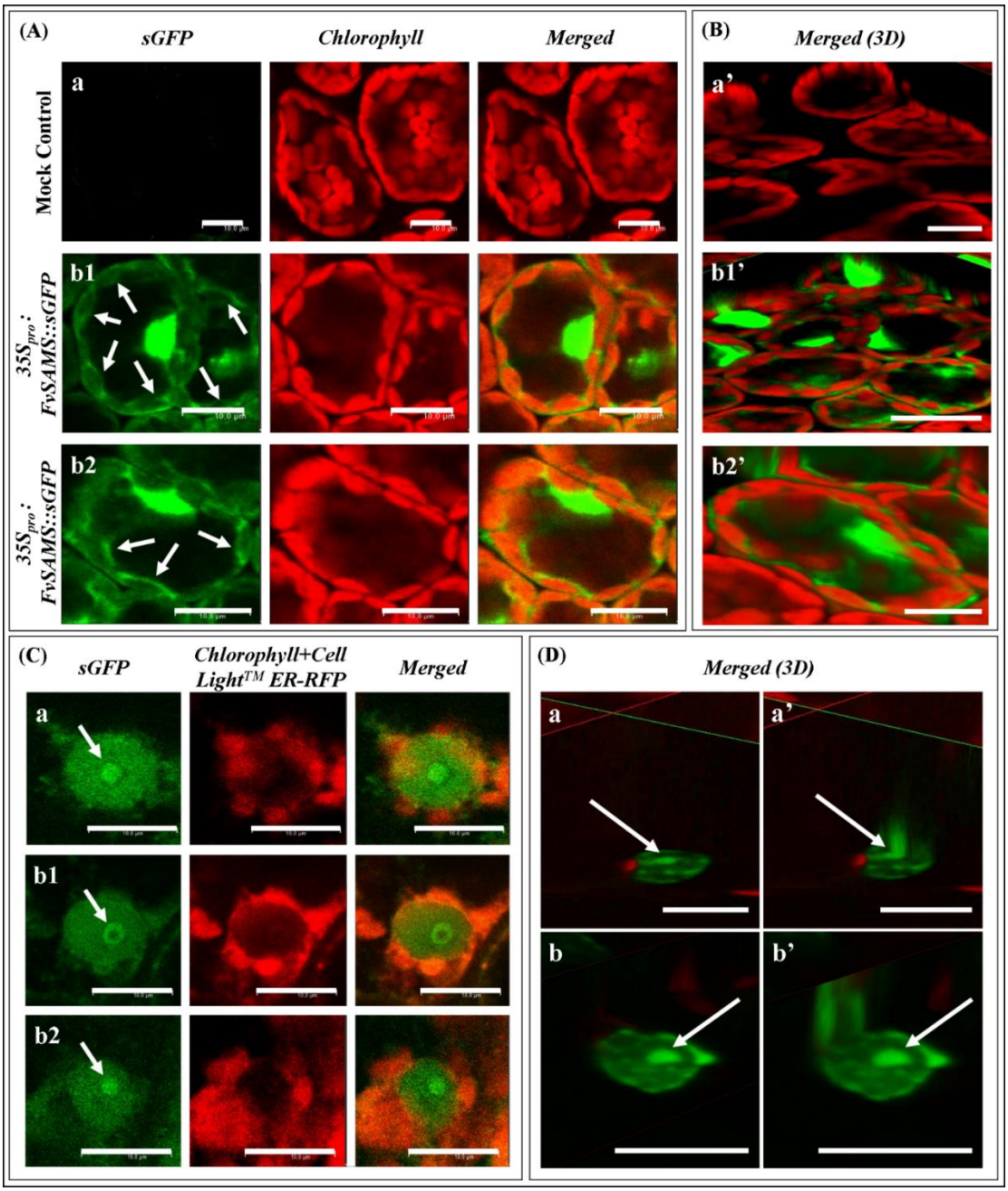

Figure 2. The cytoplasmic and plastid membrane localization of *FvSAMS::sGFP* (**b1, b2**) in palisade parenchyma cells. Bars indicate 10 µm (**A**). The cytoplasmic localization of *FvSAMS::sGFP* (**b1', b2'**) in palisade parenchyma cells in 3D. Bars indicate 20 µm (**B**). *FvSAMS::sGFP* signals in the cell nucleus and nucleolus of epidermal cells with transient

expression (**C)** (CellLight™ ER-RFP used only in these samples) and in the palisade parenchyma cells of the stable transformant plants in 3D (**D**). Arrows indicate plastid membrane and nucleolus localization of *FvSAMS::sGFP*.

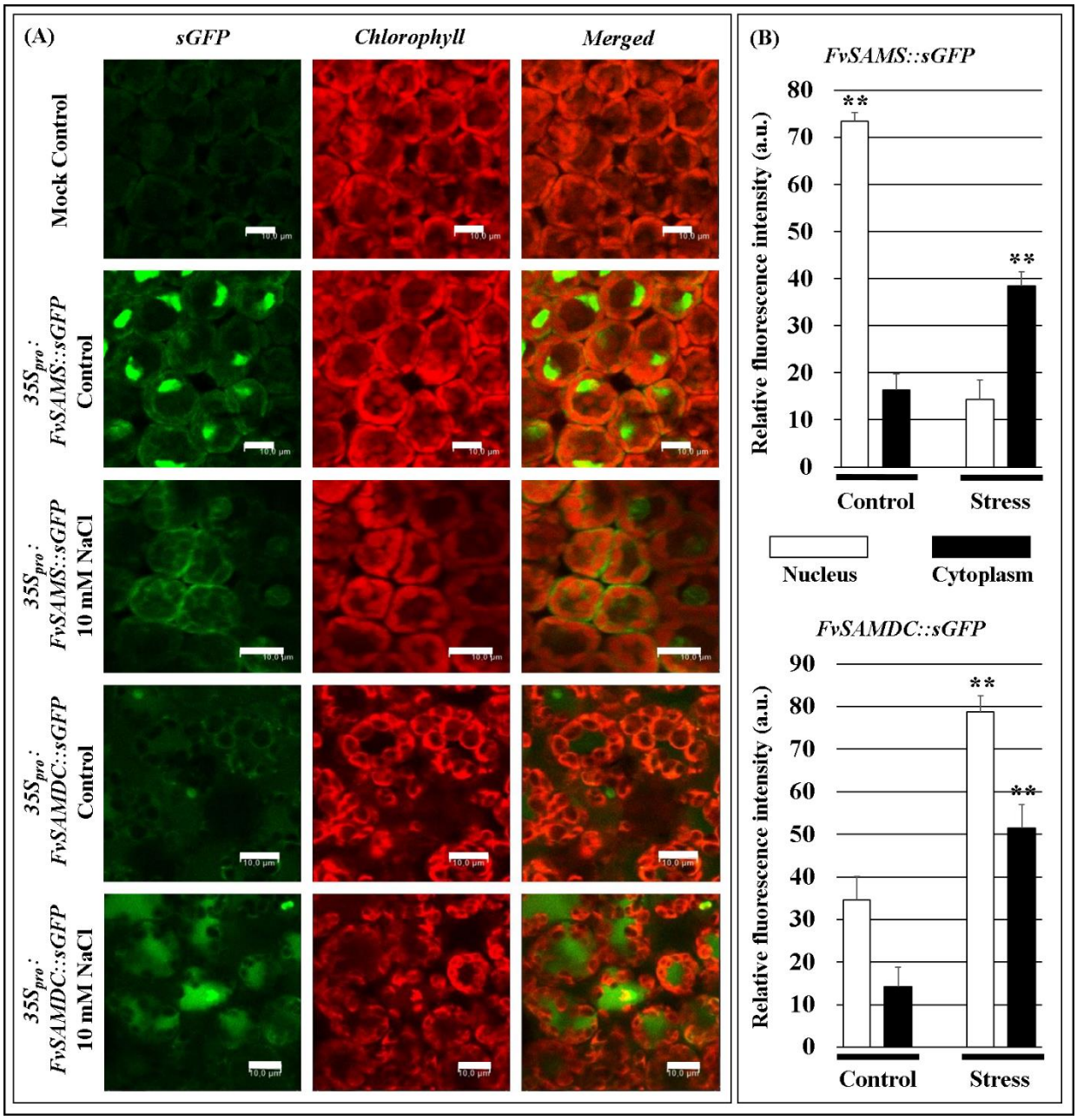

Figure 3. Expression pattern of *FvSAMS::sGFP* and *FvSAMDC::sGFP* in palisade parenchyma cells of stable transformants in control and salt stress conditions. Bars indicate 10 µm (**A**). Relative fluorescence intensity of *FvSAMS::sGFP* and *FvSAMDC::sGFP* fusion protein in the nucleus and cytoplasm under control and salinity conditions. Data are the means of ten replicates (SD±). Two asterisks represent significant differences at P<0.001 (**B**).

We searched these sequences for nuclear localization signals (NLSs), the nuclear export signals (NESs), the nucleaolar localization signals (NoLSs) and potential post-translational modification sites. **Figure 4A** shows the absence of a cysteine residue in the enzyme SAMS, which plays an important role in the inhibition of the SAMS by S-nitrosylation. Furthermore, **Figure 4B**

shows the recognition sequence involved in the inhibition of enzyme SAMS by Protein kinase C. Both FvSAMS and FvSAMDC contain putative bipartite nuclear localization signal and nuclear export signal sequences, but FvSAMS contained no predicted nucleolar localization signals. The predicted post-translational modification sites of FvSAMS include Ser-38 and Thr-112 for phosphorylation, Lys-335, 360 and 364 for acetylation, Cys-20 and 31 for S-nitrosylation, Lys-39 and 334 for SUMOylation. Potential sites of post-translational modifications based on Ser-25, 37, 39, 203, 204, 212, 316 and 317 for phosphorylation, Lys-199 for acetylation, Cys-312 for S-nitrosylation and Lys-334 for SUMOylation.

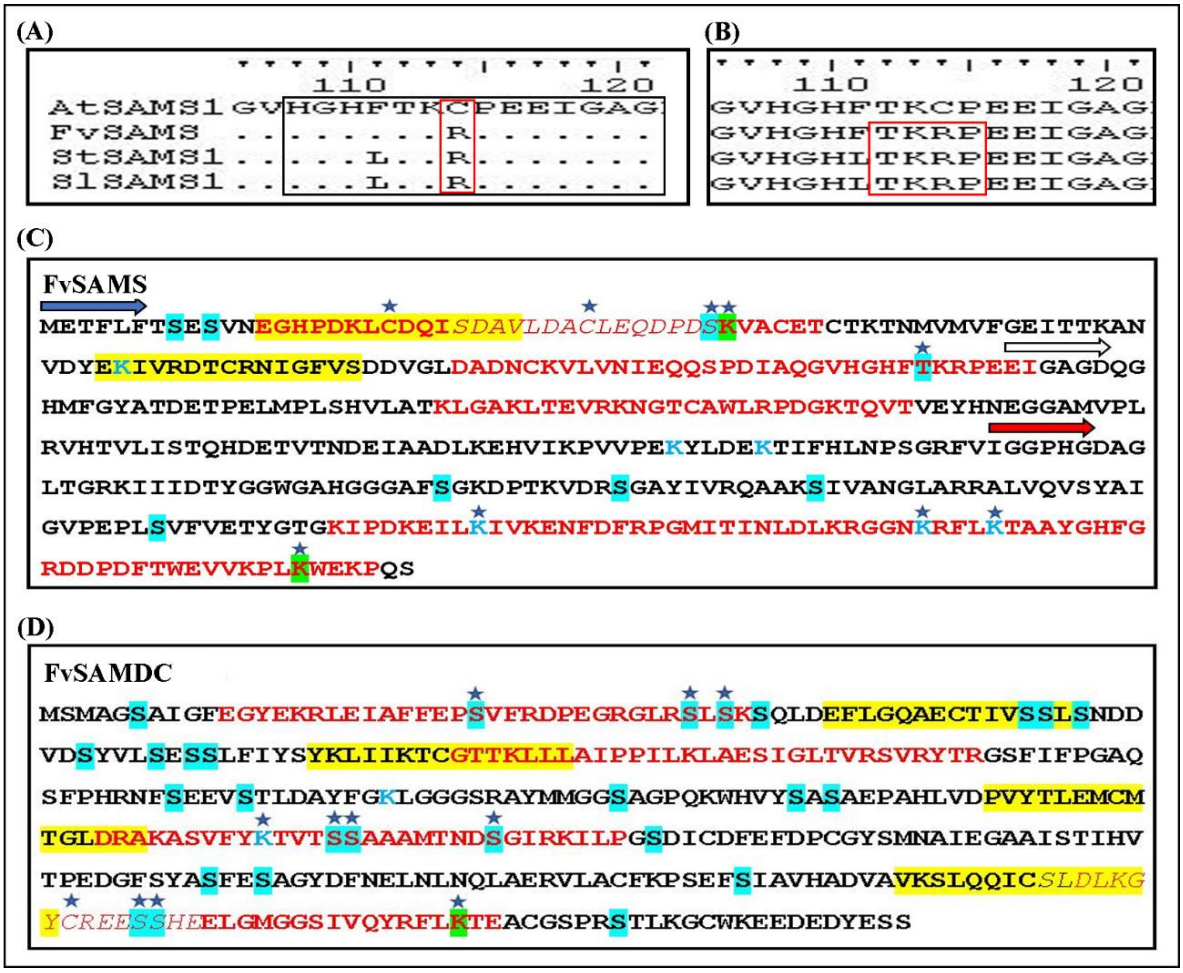

Figure 4. S-nitrosylation region of AtSAMS1 (NP_171751.1) proteins (black rectangular) and the lack of cystein (C) necessary for S-nitrosylation in FvSAMS (XP_004288342.1), SlSAMS1 (NP_001234425.1) and StSAMS1 (NP_001275609.1) (red rectangle). Dots mark amino acids identical to the sequence of AtSAMS1 (**A**). Predicted phosphorylation site of Thr-112 and the possible recognition sequence of Protein kinase C (red rectangle) (**B**). Bioinformatic analysis of the FvSAMS (**C**) and FvSAMDC (**D**) amino acid sequences. Color code: predicted bipartite NLS (red letters), predicted S-Nitrosylation Sites (highlighted in yellow, in areas of overlap not bold italics), predicted Phosphorylation Sites (highlighted in turquoise), predicted Acetylation Sites (blue letters), predicted SUMOylation (highlighted in green), overlap between predicted NLSs and the post-translational modification sites (star). In the case of FvSAMS the N-terminal (blue arrow), Central- (white arrow) and the C-terminal domains (red arrow) are indicated according to the UniProtKB - Q96551.

New Scientific Results

1. We have demonstrated that *FaSAMS* and *FaSAMDC* showed variable expression in *Fragaria x annana* Duch. cv. Asia strawberries during fruit ripening.
2. We proved that overproduction of *FvSAMS* and *FvSAMDC* increased the salt tolerance of transgenic *Nicotiana benthamiana* lines compared to the wild type.
3. Results of the measurements of FvSAMDC lines suggested that the levels of free polyamines, proline and ethylene content are positively correlated, whereas the amounts of free polyamines and lignin are negatively correlated as the plants respond to salt stress.
4. Based on one experimental system, we described first that that with the exception of the proline levels measured under control conditions and the lignin content under control and salt stress conditions, the overexpression of *FvSAMDC* had more favourable effects on all the plant physiological parameters of the transgenic lines under both the control and salt stress conditions, than the overproduction of *FvSAMS*.
5. In plants, we described first, that the *FvSAMS::sGFP* showed nuclear, nucleolar and cytoplasmic (near to the plasmalemma) and plastid membrane distribution, therefore SAM is assumed to be produced *in situ* for numerous biochemical reactions (e.g. ribosome biosynthesis, methylation of DNA, RNA, proteins in nucleus and plastid; lignin biosynthesis).
6. We described first the nucleus localization of *FvSAMDC::sGFP*.

CONCLUSIONS AND SUGGESTIONS

In summary, it can be concluded that *FaSAMS* and *FaSAMDC* showed altering expression in the strawberry fruit ripening and the overproduction of *FvSAMS* and *FvSAMDC* increased the salt tolerance of transgenic *Nicotiana benthamiana* plants in comparison with the wild type. Presumably, the overexpression of both the *FvSAMS* and *FvSAMDC* decreased the toxicity of Na^+ and increased the stability of membranes, as previous studies reported that polyamines with their positive charges are capable of blocking cation channels. It is assumed that the reduced quantities of H_2O_2 and O_2^- were indirectly caused by polyamines in *FvSAMS* and *FvSAMDC* overexpressing lines because earlier studies indicated that the enzymes of the antioxidant system were activated by polyamines. Measurements carried out in FvSAMDC lines suggested that the levels of free polyamines, proline content and the quantities of ethylene are positively correlated, whereas the levels of free polyamines and lignin content are negatively correlated as the plants respond to salt stress. Our measurements showed that under control conditions both 'SAM deficiency' and 'SAM flux' increased lignification in the transgenic lines. However, in response to salt stress 'SAM deficiency' substantially reduced, whereas 'SAM flux' substantially increased lignification, which could contribute to the enhanced tolerance of FvSAMS towards salt stress. In contrast to several earlier studies, we found that ethylene and polyamine biosynthesis had no antagonistic effect on one another in our experimental system. We assume that increased salt tolerance in FvSAMS lines was due to increased total polyamine and lignin contents, whereas in FvSAMDC lines salt tolerance was caused by substantially elevated levels of total polyamine and ethylene. This finding was further supported by the chlorophyll content of leaf discs exposed to 300 mM NaCl stress. In 24 hours after being exposed to stress the degree of chlorophyll degradation in FvSAMS lines was half of what was obtained in FvSAMDC, which was likely to be caused by the increased mechanical defence ability of FvSAMS lines due to their elevated lignin contents. These values evened out in 48, 72 and 96 hours following the treatment, which could be explained by the increased polyamine levels in FvSAMDC lines. The conclusion can be drawn that with the exception of the proline levels measured under control conditions and the lignin content under control and salt stress conditions, the overexpression of *FvSAMDC* had more favourable effects on all the plant physiological parameters of the transgenic lines under both the control and salt stress conditions, than overproduction of *FvSAMS*.

The localization of the *FvSAMS::sGFP* in the cytoplasm lining the plasmalemma and in the plasma membrane of the chloroplasts as well as its nuclear and nucleolar localization suggest that SAM is produced *in situ* for several biosynthetic processes. (e.g. methylation of genomic DNA, RNA and proteins, methylation of chloroplast DNA, RNA and RUBISCO enzyme,

biosynthesis of lignin and ribosome biosynthesis). It was observed that the quantity of detectable *FvSAMS::sGFP* decreased in the lower leaves, whereas the quantity of *FvSAMS* mRNA was ~2-5 times higher. Therefore, we investigated the amino acid residues involved in the post-translational modification of FvSAMS.

In an earlier study the enzyme SAMS was found to be inhibited by S-nitrosylation but the cysteine-114 required for the S-nitrosylation was absent in the amino acid sequence of FvSAMS, therefore we assumed that besides S-nitrosylation other translational or post-translational modifications are involved in the inhibition of enzyme SAMS. The phosphorylation of Thr-342 by Protein kinase C (PKC) discussed in other publications, decreased the enzyme activity of SAMS. The predicted threonine and serine that can be phosphorilated in FvSAMS are the Thr-112 and Ser-271, which are equivalent with the recognition sequences S/TXK/R and TXRX (TKRP and SGK for FvSAMS) preferred by PKC. Therefore, we assume that the phosphorilation of Thr-112 and Ser-271 can play a part in the inhibition of enzyme FvSAMS by PKC. In order to prove our hypothesis deletion vector construction with point mutations in the base sequence of Thr-112 and Ser-271 could be made to generate changes in these amino acids. This can be accomplished by designing specific primers to be used in a protoplast system in which transient expression and the induction of phosphorilation would make it possible to investigate whether these amino acids are involved in the post-translational inhibition of FvSAMS. The *FvSAMS::sGFP* fusion protein could only be detected in the guard cells of stomata in senescent leaves and cotyledons of seedlings, therefore it can be assumed that the enzyme SAMS plays an important role in the regulation of stomatal functions and thereby in cellular respiration, photosynthetic gas exchange and evaporation. Based on previous studies, it can be stated that the subcellular localization of the enzyme SAMS is determined by its C-terminal domain. According to bioinformatic analysis the subcellular localization of the enzyme FvSAMS can be determined by the acetylation of Lys-335, 360, 364 and SUMOylation of Lys-387. To provide experimental evidence deletion vector constructions similar to the ones used for the amino acid residues involved in inhibition of enzyme FvSAMS could be made to investigate whether these amino acid residues play a role in the subcellular localization of FvSAMS.

We noticed that *FvSAMDC::sGFP* showed distinctively nuclear, distinctively cytoplasmic and distinctively nuclear and cytoplasmic localizations concurrently. The fact that *FvSAMDC::sGFP* showed nuclear and cytoplasmic localizations separately and also simultaneously can be explained by the differential autocatalytic activation of the proenzyme SAMDC in the different cellular compartments as well as the unidirectional dislocation of the enzyme. However, we were not able to identify whether the direction of its movement is cytoplasm→nucleus or nucleus→cytoplasm. The dual localization of *FvSAMDC::sGFP* can be

further supported by an earlier study, in which the spermidine synthase was found to show nuclear and cytoplasmic localization while the spermine synthase only cytoplasmic localization. The same study also described that in the presence of exogenous spermidine synthase the cytoplasmic localization of spermine synthase shifted towards the nucleus, which suggests that the synthesis of spermidine and spermine is a complex process. Since dcSAM produced by SAMDC serves as a substrate for both spermidine synthase and spermine synthase providing the aminopropyl group, the dual localization of SAMDC can be explained by its inactive form after being synthesized, by its strict transcriptional and translational regulation and by its short half-life time due to the complexity of the process. This can be investigated in a protoplast system by relying on transient expression and supplying exogenous spermidine and spermine, which can explain the dual localization, inducibility and inhibition of FvSAMDC.

PUBLICATIONS

Thesis related scientific publication

Scientific article (English)

1. **László Kovács**, Ákos Mendel, Anna Szentgyörgyi, Sándor Fekete, Ferenc Söre, Katalin Posta and Erzsébet Kiss (2018). Comparative analysis of overexpressed *Fragaria vesca* S-adenosyl-L-methionine synthase (*FvSAMS*) and decarboxylase (*FvSAMDC*) during salt stress in transgenic *Nicotiana benthamiana*. **Frontiers in Plant Science**, (submitted). **IF: 3.678**
2. Ákos Mendel, **László Kovács**, Anna Szentgyörgyi, Sándor Fekete, Katalin Posta and Erzsébet Kiss (2018). Expression patterns of ethylene and polyamines biosynthetic genes during fruit ripening in strawberry. **Turkish Journal of Biology,** (submitted). **IF: 0.651**
3. Polgári D., Kalapos B., Tisza V., **Kovács L.**, Kerti B., Heszky L., Kiss E. 2010. *In silico* analysis of a putative *Spiral* gene related to strawberry ripening. **Acta Agronomica Hungarica**, 58 (3): 267-272 ISSN: 0238-0161.
4. Tisza V., **Kovács L.**, Balogh A., Heszky L., Kiss E. 2010. Characterization of FaSPT, a Spatula gene encoding a bHLH transcriptional factor from the non-climacteric strawberry fruit. **Plant Physiology and Biochemistry**, 48: 822-826 ISSN: 0981-9426. **IF: 2.485**
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Scientific article (Hungarian)

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8. **Kovács L.**, Mendel Á., Tóth Sz., Szentgyörgyi A., Kiss E. and Toldi O. 2015. Increased SAM pool and PA/Ethylene ratios in transgenic *Nicotiana benthamiana* plants resulted in

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10. Mendel Á., **Kovács L.**, Hidvégi N., Kiss E. 2013. Isolation and characterization of promoters of genes involved in polyamine metabolism in strawberry. Hungarian Molecular Life Sciences 2013: programme & book of abstracts. 287 p. ISBN: 978-615-5270-02-4.
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17. Mendel Á., **Kovács L.**, Tóth Sz., Szentgyörgyi A., Kiss E. 2014. *Fragaria vesca* S-adenozilmetionin szintáz gén (SAM-Sy) funkcionális jellemzése. XX. Növénynemesítési Tudományos Napok: Magyar Tudományos Akadémia Székháza, Budapest, 2014: Összefoglalók. p. 299-303.
18. Mendel Á., **Kovács L.**, Hídvégi N., Kiss E. 2013. A szamóca poliamin metabolizmusában működő gének promótereinek izolálása és jellemzése. XIX. Növénynemesítési Tudományos Nap. Összefoglalók. p. 118. ISBN 978-963-9639-50-8
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22. **Kovács L**., Koncz T., Tisza V., Kerti B., Szőke A., Kiss E., Heszky L. 2009. Szamóca érésében működő ACC-szintáz gén promóterének bioinformatikai jellemzése. Fiatal agrárkutatók az élhető Földért. ISBN 963993502-6. p. 49.
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Book, booklet, editing

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26. Alzohairy A. M., Gyulai G., Amin I. I., **Kovács L.**, Elazma M. H., Elsawy H., Youssef K., Elhamamsy A. R., Ibrahim H. M. M. and Bahieldin A. PLANT GENETICS BIOTECHNOLOGY and FORESTRY (Ed. Gyulai G.), 2016. Chapter 4. DNA methylation - Epigenetics of gene expression through DNA methylation in Human and plant genomes. ISBN: 978-963-269-580-8.
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