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

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Abstract

We investigated the effect of overexpressing *Fragaria vesca* L. cv. Rügen S-adenosyl-l-methionine synthase (*FvSAMS*) and decarboxylase (*FvSAMDC*) genes on control and salt stressed *Nicotiana benthamiana* Domin plants. According to previous studies the overproduction of both proteins enhances the abiotic stress tolerance of plants, but the two enzymes have not yet been studied in one experimental system. We found that the transgenic plants subjected to long-term salt stress displayed higher levels of tolerance than the wild type (WT). In contrast to several earlier studies no antagonistic effect between ethylene and polyamine biosynthesis was observed in our experimental system. Overexpression of *FvSAMDC* had higher impact on the plant physiological parameters both in control and salt stress conditions, than that of *FvSAMS*. Based on the data measured in the *FvSAMDC* lines there appears to be a positive correlation between the free polyamine levels and the proline content as well as the amount of ethylene, while there is a negative correlation between the free polyamine levels and the lignin content in the plants exposed to salt stress. The transformation vectors contained the CaMV35S promoter, the coding sequence of *FvSAMS* and *FvSAMDC* fused with synthetic green fluorescent protein (sGFP). We detected the subcellular localization of both enzymes and examined the possible stress induced changes in their distribution. In the case of *FvSAMS::sGFP* nuclear, nucleolar, cytoplasmic (near to the plasmalemma), plastid membrane, whereas in *FvSAMDC::sGFP* nuclear and homogenous cytoplasmic localization was detected. Therefore, SAM is assumed to be produced in situ for numerous biochemical reactions.

Keywords Ethylene · Polyamine · Lignin · Fusion protein · Subcellular localization

Introduction

Different abiotic stresses lead to severe economic losses each year (Mahajan and Tuteja 2005). High salt levels cause ion toxicity (mainly Na⁺), hyperosmotic stress, and secondary stresses (i.e., oxidative damage) (Zhu 2002; Chinnusamy et al. 2006). Both ethylene and polyamines (putrescine, spermidine and spermine) play an important regulatory role in the biotic and abiotic stress responses of plants (Müller and Munné-Bosch 2015; Romero et al. 2018) and in larger quantities they have a positive effect on the salt tolerance of plants (Jang et al. 2012; Peng et al. 2014; Shen et al. 2014). The antioxidant enzyme activity is growing on the effect of adequate concentration of both ethylene and polyamine, by this way they increase the homeostasis of reactive oxygen species (ROS) and regulate the balance of Na/K, enhancing the adaptation of plants to the salinity environment (Saha et al. 2015; Tao et al. 2015). S-adenosyl-l-methionine (SAM) is a common precursor of polyamine and ethylene biosynthesis. The biosynthesis of ethylene proceeds through the following steps: SAM → 1-aminocyclopropane-1-carboxylic acid (ACC) → ethylene catalyzed by ACC synthase (ACS) and ACC oxidase (ACO) enzymes. At the same time the SAM decarbosylase (SAMDC) converts SAM to decarboxylated SAM, which acts as an aminopropyl group donor in the synthesis of polyamines. Numerous earlier studies reported on the antagonistic effect of ethylene and...
polyamine metabolism in response to various biotic and abiotic stresses (Li et al. 2004; Nambeesan et al. 2012; Yu et al. 2016). Therefore, we also set out to study how the ‘SAM flux’ and ‘SAM deficiency’ influenced the ratio of ethylene and polyamine in response to salt stress. S-adenosyl-l-methionine synthase produces S-adenosyl-l-methionine from l-methionine and ATP. SAM is the second most frequently used enzyme substrate after ATP (Cantoni 1975) and can serve as a methyl group source for amino, ribosyl and aminopropyl groups (Fontecave et al. 2004). Furthermore, SAM is needed for the methylation of DNA, RNA, proteins, lipids, lignin, pectin, volatiles, flavonoids, nicotianamine and biotin (Roeder et al. 2009; Gong et al. 2014). Since DNA methylation causes genes to be shut down, SAM is definitely considered to be a gene expression regulator. According to estimates 95% of SAM is used for methylation and only 3–5% is decarboxylated (Merari and Clarkson 2004). SAM is not only the precursor of ethylene and polyamines, but the secondary metabolite lignin, too. Lignin is one of the main metabolic consumers of SAM. In mto3 Arabidopsis thaliana genotype a point mutation of the SAM3 gene, causing a single amino acid change in the ATP binding domain, is responsible for the mto3 phenotype. There is no modification either in SAM3 expression pattern or in the amount of SAMS protein produced but there is a decrease in SAMS enzyme activity and in the total amount of SAM and lignin content compared to the wild type (WT), providing evidence for the impact of SAM3 on lignin biosynthesis (Shen et al. 2002). Several former studies reported an increase in the amount of SAMS mRNA in response to salt stress in rose periwinkle, wheat and ginseng (Schröder et al. 1997; Pulla et al. 2009; Kamal et al. 2012). The overexpression of SAMS positively influenced the abiotic stress tolerance of tomato, tobacco and A. thaliana (Qi et al. 2010; Gong et al. 2014; Guo et al. 2014; Kim et al. 2015; Ma et al. 2017).

SAMDC forms a small gene family in A. thaliana—the 4 members of which show high similarity to each other (Franc-eschetti et al. 2001)—and is a strictly regulated enzyme (Hu et al. 2005). The in vivo production of the enzyme can be both transcriptionally and translationally regulated with the latter being directly influenced by polyamines (Pegg 1986; Kusano and Suzuki 2015). SAMDC participates in developmental processes, cell differentiation and it is synthesized as a proenzyme, which undergoes an autocatalytic cleavage. SAMDC functions as a dimer in mammals containing an alpha and a beta chain. In numerous higher organisms, its autocatalysis and decarboxylation are stimulated by putrescine, but in plants SAMDC is highly active in the absence of putrescine as well (Bennett et al. 2002). SAM decarboxylase plays a pivotal role in regulating spermidine and spermine biosynthesis in numerous plant species, since the decarboxylated SAM (dcSAM) produced by the SAMDC acts as a substrate for both spermidine and spermine synthase (Kusano et al. 2008). Embryonic lethality was observed in the double mutant of bud2 and samdc1, underlying the fundamental role of SAMDC in plant embryogenesis (Ge et al. 2006). The up- and down-regulation of the SAMDC showed that the ethylene and polyamine biosynthesis has antagonistic impact on each other in A. thaliana (Hu et al. 2006). Furthermore, the overexpression of carnation SAMDC resulted in elevated salt stress tolerance in transgenic tobacco plants (Wi et al. 2006) and the NaCl stress increased the amount of MdSAMDC1 and AtSAMDC1 mRNA in apple and A. thaliana (Urano et al. 2003; Liu et al. 2008). The silencing of SAMDC increased salt sensitivity in tobacco, at the same time dcSAM deficiency had a positive effect on the biomass production and stem length of transgenic plants (Mellidou et al. 2016).

Our goal was to elucidate the functions of these two enzymes (FvSAMS and FvSAMDC) both under control and salinity stress conditions, since they have not been investigated simultaneously in one experimental system. Earlier studies investigated the effect of overexpressing SAMS or SAMDC during abiotic stresses in Nicotiana tabacum L., Solanum lycopersicum L. and A. thaliana (Boerjan et al. 1994; Roy and Wu 2002; Waie and Rajam 2003; Wi et al. 2006, 2014; Cheng et al. 2009; Peremarti et al. 2009; Qi et al. 2010; Gong et al. 2014; Kim et al. 2015; Ma et al. 2017). Studying concomitant activity of the two enzymes in the same experimental system allows the plant physiological parameters investigated to be related to one another quantitatively, making it possible to build a clearer picture with regard to the role of the two enzymes in salt stress response. In addition, we planned to study the subcellular localization of FvSAMS::xGFP, FvSAMDC::xGFP and the possible changes in response to 10 mM NaCl stress. The SAMS is considered an enzyme with cytosolic localization in plants (Schröder et al. 1997; Ravanel et al. 1998; Hanson and Roje 2001), whereas it was found to show nucleocytoplasmic distribution in mammals (Reytor et al. 2009). Furthermore, in earlier studies SAMDC activity was found in the chloroplasts, mitochondria and cytosol of plants (Yamanoha and Cohen 1985; Torrigiani et al. 1986), while only cytoplasmic localization was detected in animals (Gritli-Linde et al. 1995).

Material and methods

Plant material, vector constructs and bioinformatic analysis

For the identification of sequences, the NCBI database was used. The main ORF of FvSAMS (XP_004288342.1) codes for 394 amino acids and shows 95% similarity with AtSAMS1 (NP_171751.1), 93% with AtSAMS2
(NP_192094.1) and 90% with AtSAM3 (NP_181225.1). The main ORF of FvSAMDC (XP_011462957.1) 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 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 (Table S2). 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 synthetic green fluorescent protein (sGFP) reporter gene while the pGWB604 carried only the sGFP. 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. was used for the in vivo infiltration of the 35-day-old N. benthamiana plants based on the method described by Li (2011).

Plant growth conditions

The seeds of two transgenic T1 lines from each construct (FvSAMS-22, FvSAMS-25 and FvSAMDC-73, FvSAMDC-76) were germinated on MS medium supplemented with 75 μg/ml kanamycin while the wild type N. benthamiana seeds on MS medium without antibiotics, respectively. Fourteen days after germination (14 DAG), 32 seedlings were germinated of each transgenic line and wild type were either transferred onto half strength MS medium without antibiotics or onto the same medium supplemented with 0.58 g/l (10 mM) NaCl. The plants and the explants were kept in 16 h light and 8 h dark periods at 23 °C. Samples were collected 85 days after sowing. We investigated the effect of 17.54 g/l (300 mM) NaCl on the survival of N. benthamiana leaf discs on same plant growth conditions than in the case of 10 mM NaCl stress. All plant physiology parameters were measured from N. benthamiana plant samples grown in control and 10 mM NaCl stress conditions. All photographs were taken with Fuji FinePix S6500fd camera.

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 (Abs.663) − 0.00269 (Abs.645); Cb = 0.0229 (Abs.645) − 0.00468 (Abs.663). ImageJ 1.51j8 software was used for determining the relative chlorophyll content of leaf discs (Schneider et al. 2012). Leaves of wild type N. benthamiana were used for calibration between 0 and 100% chlorophyll content (chlorophyll-free and control chlorophyll samples). We calculated the relative chlorophyll content from the measured values of 12 leaf discs.

Histochemical detection of H2O2 and O2−

The histochemical visualization of H2O2 and O2− was carried out according to the method described by Kumar et al. (2014). The chlorophyll-free leaves were fixed in 60% (v/v) glycerol.
**Determination of H$_2$O$_2$ content**

The H$_2$O$_2$ 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$_2$O$_2$ (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 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 five 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 h. 1 ml of gas was injected into the Shimadzu GC-14A gas chromatograph (Kyoto, Japan) with the retention time of ethylene being 0.553 s.

**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 2 × 1 ml 0.2 M ice cold perchloric acid, put on ice for 20 min, then centrifuged at 4 °C for 20 min (10,000×g). In the case of free PA (polyamine) 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 main parameters of which are shown in Table S2. 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 2×ABsolute 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). N. benthamiana glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was 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™ 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™ ER-RFP was reinfiltrated into the living tissue (100-time dilution) followed by incubation at room temperature for 16 h 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 three measurements and were statistically evaluated using the standard deviation and ANOVA methods.
Results

Short-term salt stress and relative chlorophyll content

We examined the stress response of leaf discs of FvSAMS, FvSAMDC and WT N. benthamiana to short-term salt stress (Fig. 1a). We observed that the WT leaf explants died 48 h 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 h after salt exposure. The relative chlorophyll content of the leaf discs shown in Fig. 1a was quantified and presented in Fig. 1b. Chlorophyll content measured in four sampling times (24, 48, 72 and 96 h after treatment) also supports that there is a difference in the rate of chlorophyll degradation between the leaf discs of WT and transgenic FvSAMS, FvSAMDC lines in response to 300 mM NaCl stress. In the WT leaf explants the chlorophyll content decreased to ~15% as early as 48 h after the treatment, while the average value of FvSAMS and FvSAMDC transgenic leaf discs approached this level only in the 96th h.

Fig. 1 Effect of 300 mM NaCl on the survival of Nicotiana benthamiana leaf discs. Young leaf discs (from the top to 4th node) were used 50 days after germination. Bars indicate 1 cm (a). Relative chlorophyll content of 300 mM NaCl stressed leaf discs, the control chlorophyll and chlorophyll-free samples were used for calibration (b)
Growth vigour, biomass production and chlorophyll content

The biomass of the plant is a good indication of its response to stress. We observed that both FvSAMS and FvSAMDC lines produced higher shoot biomass and shoot length than the non-transformed ones both in control and 10 mM NaCl stress conditions (Fig. 2a–c).

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 FvSAMS and FvSAMDC lines and wild type plants. The results of chlorophyll a and b content measurements in Fig. 2d shows that the FvSAMS and FvSAMDC lines contained more chlorophyll than the WT ones both under stress-free and stressed conditions.

Fig. 2 Visible effect of overexpression of FvSAMS and FvSAMDC on shoot and root biomass production in control (1–20) and 10 mM NaCl stress conditions (21–40). The numbers from 1 to 4 and 21 to 24 indicate the wild type, while 5 to 20 and 25 to 40 mark different transgenic lines. The same number refers to the same plant. Bars indicate 1 cm. Photographs were taken 85 days after germination (a). Dry weight of shoots (b). Results of shoot length measurements (c). Data are the means of six replicates (SD ±). Results of chlorophyll a and b content measurements (d). Data are the means of six (b) twelve (c) and three (d) replicates (SD ±), respectively. Three asterisks represent significant differences at P < 0.001 and one asterisk represents significant differences at P < 0.05.
H$_2$O$_2$, O$_2^-$ content and electrolyte leakage

The accumulation of ROSs (Reactive Oxygen Species), similarly to that of H$_2$O$_2$ and O$_2^-$ is in direct proportion with the stress levels of cells, therefore we investigated the H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ than in the FvSAMS and FvSAMDC lines (Fig. 3a, left panel). Similarly, to H$_2$O$_2$ 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 (Fig. 3a, right panel). Furthermore, salt treated leaves of FvSAMS and FvSAMDC lines showed low H$_2$O$_2$ contents compared to the WTs (Fig. 3b).

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 (Fig. 3c). We detected lower rates of electrolyte leakage in both FvSAMS and FvSAMDC lines both under control and stress conditions than in the WTs.

Proline and lignin content

Proline accumulation is also considered to be a good indicator of stress response. There was no change of proline content in control condition. Salt stress increased (4.3 fold) the proline accumulation only in FvSAMDC lines (Fig. 4a).

Lignin is known to accumulate 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 (Fig. 4b). 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.

Fig. 3 Histochemical detection of H$_2$O$_2$ (left) and O$_2^-$ (right) in Nicotiana benthamiana leaves. Bars indicate 1 cm (a). H$_2$O$_2$ content of the leaves (b). Results of electrolyte leakage measurements (c). Data are the means of three replicates (SD ±). One asterisk represents significant differences at P < 0.05.
Ethylene and polyamine content

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 stress conditions. In the FVSAMS lines there was no significant change in ethylene production either in control or salt stress conditions. The FvSAMDC lines did not produce significantly higher levels of ethylene under control conditions, but the salt stress induced a substantial rise in the ethylene production in the FvSAMDC transgenic plants than in WTs (Fig. 4c).

In addition to ethylene, we examined the free polyamine content of the transgenic FvSAMS and FvSAMDC lines and WT plants both under control and salinity conditions (Fig. 5). 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.
Relative expression measurement

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) (Fig. 6). The relative expression rate of SAMS, SAMDC and SAHH 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 lower in the FvSAMS lines under non-stressed condition and it was also in response to salt stress as well. At the same time, FvSAMDC lines displayed higher levels of ACS expression than in the WTs under stress condition. The transcription rate of SPDS did not show significant difference between the WTs and the FvSAMS, FvSAMDC 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 almost all transgenic lines in control condition and all transgenics under salt stress than in the WTs.

The summary of the measurements in control and 10 mM NaCl stress conditions is shown in Table S1.

Subcellular localization of FvSAMS::sGFP and FvSAMDC::sGFP

We carried out transient expression investigations with the FvSAMS::sGFP, FvSAMDC::sGFP, FvSPDS::sGFP (F. vesca L. cv. Rügen spermidine synthase) and 35Spro:sGFP constructs. Transient expression of FvSAMS::sGFP, FvSAMDC::sGFP and 35Spro:sGFP fusion proteins displayed nuclear and cytoplasmic localization, while FvSPDS::sGFP showed only cytoplasmic distribution in epidermal cells (Fig. 7). We noticed that each of the three fusion proteins (FvSAMS::sGFP, FvSAMDC::sGFP and 35Spro:sGFP) showed endoplasmic reticulum (ER) signals during their transient expression (Fig. S1), therefore we used ER labelling with the application of CellLight™ ER-RFP (Fig. S2). The FvSAMS::sGFP and FvSAMDC::sGFP showed weaker ER signals than the 35Spro: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 (Fig. 8a, b). In the stable transformants, the FvSAMS::sGFP did not show homogeneous cytoplasmic distribution, but it accumulated in the outermost regions of the cytoplasm near the plasmalemma and plastid membrane in palisade parenchyma cells as it is visible in Fig. 8c and d. The distribution...
of \textit{FvSAMS::sGFP} and \textit{FvSAMDC::sGFP} fusion proteins in the stable transformants both under control and saline conditions is illustrated in Fig. 9a. We observed differences in the ratios of nuclear to cytoplasmic distribution under stress-free and stressed conditions. The nuclear signal of \textit{FvSAMS::sGFP} was more intense in the control environment, and the cytoplasmic expression became stronger in response to salt stress (Fig. 9b, upper part). In the case of \textit{FvSAMDC::sGFP} the amount of fusion proteins increased substantially in response to salt stress both in the nucleus and the cytoplasm (Fig. 9b, lower part). At the same time it could be observed that the \textit{FvSAMDC::sGFP} showed distinctive nuclear, cytoplasmic and nuclear-cytoplasmic localization concurrently (Fig. 10a). 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 (Fig. 10b, ci, ii). 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 (Fig. 10d). Similar phenomenon was not observed in the FvSAMDC lines.

Bioinformatic analysis

To further investigate the nuclear, nucleolar and cytoplasmic (near to the plasmalemma), plastid membrane localization of FvSAMS::sGFP and the nuclear, homogenous cytoplasmic localization of FvSAMDC::sGFP, the amino acid of FvSAMS and FvSAMDC sequences were examined by means of various bioinformatic applications. We searched for nuclear localization signals (NLSs), the nuclear export signals (NESs), the nucleolar localization signals (NoLSs) and potential post-translational modification sites in these sequences. Figure 11a 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, Fig. 11b 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 of FvSAMDC 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 (Fig. 11c, d).

Discussion

In the present study we performed a comparative investigation in an attempt to find out how the overexpression of FvSAMS and FvSAMDC affects the various physiological parameters of the plants in response to salt stress. We found that the overproduction of FvSAMS and FvSAMDC decreased chlorophyll degradation in 300 mM NaCl stress (Fig. 1) and increased the biomass in response to 10 mM NaCl stress (Fig. 2), in agreement with earlier findings by others (Roy and Wu 2002; Wi et al. 2006; Qi et al. 2010; Gong et al. 2014; Kim et al. 2015; Ma et al. 2017). However, we found significant differences in the above mentioned parameters even under control conditions compared to the WT plants.

The chloroplasts are the main sites of the production of ROS, including superoxide anions, hydrogen peroxide, hydroxyl radical and singlet oxygen (Mignolet-Spruyt et al. 2016). ROS homeostasis is altered in response to stress. When present at high concentrations, ROS can damage the
cell and at low concentrations they act as signals in recognizing stress and triggering a response. Excessive accumulation of ROS causes oxidative stress, which leads to lipid peroxidation and membrane structure damage, leading eventually to cell death (Biswas and Mano 2015). Increased membrane stability accounts for significantly lower rates of electrolyte leakage and H$_2$O$_2$, O$_2^−$ in both FvSAMS and FvSAM lines both under control and stress conditions than

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**Fig. 8** FvSAMS::sGFP signals in the cell nucleus and nucleolus of epidermal cells with transient expression (a) and in the palisade parenchyma cells of the stable transformant plants in 3D (b). The cytoplasmic (near to the plasmalemma) and plastid membrane localization of FvSAMS::sGFP in palisade parenchyma cells (c) and these sGFP signals in 3D (d). The iv−iv’ and v−v’ refer to the same nucleus. Bars indicate 10 µm (a, c, d) and 20 µm (b). Arrows indicate plastid membrane (a) and nucleolus (c, d) localization of FvSAMS::sGFP.
in the WTs (Fig. 3). Lower electrolyte loss was measured in SlSAMS, MfSAMS1 overproducing tomato and tobacco plants in salt stress conditions (Gong et al. 2014; Guo et al. 2014) and in heat-stressed tomato plants overexpressing yeast SAMDC (Cheng et al. 2009) than in the wild types. Significantly lower levels of ROS accumulation could be detected in SISAMS and BvM14-SAMS2 overproducing tomato, A. thaliana (Gong et al. 2014; Ma et al. 2017) and in CaSAMDC overexpressing A. thaliana plants (Wi et al. 2014). Several previous publications reported that the overproduction of SAMS and SAMDC increased the activity of the ROS scavenging enzymes through increased polyamine biosynthesis (Wi et al. 2006, 2014; Gong et al. 2014; Guo et al. 2014; Ma et al. 2017). There are two biochemical pathways by which polyamines can exert their control over the homeostasis of ROS. One of the pathways leads to the inhibition of the auto-oxidation of metals, which decreases the electron supply required for the generation of ROS (Shi et al. 2010). The other pathway directly increases the activity of enzymes in the antioxidant system (Shi et al. 2010; Mostofa et al. 2014), which presumably leads to the reduced quantity of H$_2$O$_2$ and O$_2^-$ in the transgenic FvSAMS and FvSAMDC lines.

Besides the ROS, the amount of accumulating proline in the cell is also a good indicator of the level of stress the plants are exposed to. The elevated proline levels have numerous positive impacts on the abiotic stress tolerance of plants, with proline accumulation being directly proportional

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**Fig. 9** Subcellular localization 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 (a. u.: arbitrary unit). Data are the means of ten replicates (SD ±). Three asterisks represent significant differences at P < 0.001 (b).
to the levels of polyamines, in spite of the fact that they share a common precursor (glutamic acid) (Simon-Sarkadi et al. 2006; Wen et al. 2011; Cvikrová et al. 2012). Higher levels of total polyamine measured in our FvSAMS-expressing lines showed no positive correlations with the proline content detected. In contrast to our results, higher proline levels were found in *A. thaliana* plants overproducing *BvM14-SAMS2* in response to salt stress than in WT plants. The overexpression of FvSAMDC increased the levels of proline in response to salt stress (Fig. 4a), which is in agreement with the higher levels of total polyamine measured in FvSAMDC lines.

Since lignin is one of the main metabolic consumers of SAM, we investigated how ‘SAM flux’ and ‘SAM deficiency’ influence the lignin content of plants under control conditions and in response to salt stress. Lignin is deposited between the primary and secondary cell walls during plant development and thereby increases the strength of the stem,
which, in turn, improves the tolerance of plants against biotic and abiotic stresses. Accelerated lignification was observed in the roots of water-deficient sorghum, salt stressed soybean and salt stressed maize plants (Azaizeh and Steudle 1991; Cruz et al. 1992; Neves et al. 2010). Sánchez-Aguayo et al. (2004) concluded that the elevated activity of SAM in salt stressed tomato plants is commensurate with the increased amount of lignin deposited in vascular tissues. According to our result, both ‘SAM flux’ and ‘SAM deficiency’ increased the rate of lignification in the transgenic FvSAMS and FvSAMDC lines under control conditions. However, in response to salt stress ‘SAM deficiency’ substantially reduced, while ‘SAM flux’ substantially increased lignification, which could have directly increased the tolerance of FvSAMS lines against salt stress (Fig. 4b).

Since SAM is a precursor molecule of ethylene biosynthesis, we also investigated how the overexpression of FvSAMS and FvSAMDC influenced the amount of ethylene under control and stress conditions. Although the overproduction of FvSAMS had no influence on the release of ethylene, the overexpression of FvSAMDC upregulated ethylene production under stress conditions (Fig. 4c). In previous studies, down-regulated tobacco SAMDC activity did not cause any changes in ethylene production (Torrigiani et al. 2005), while the overexpression of AtSAMDC in A. thaliana plants reduced the amount of ethylene (Hu et al. 2006). Furthermore, neither the overexpression of FvSAMS, nor that of MfSAMS1 in tobacco plants resulted in any significant changes in the amount of ethylene produced (Guo et al. 2014), similarly to our results. However, the overexpression of SAMS increased the level of ethylene production in tomato and A. thaliana plants (Gong et al. 2014; Kim et al. 2015).

Polyamines are known to play an important role in osmotic regulations, membrane stability, and the removal of free radicals under abiotic stresses (Liu et al. 2007; Romero et al. 2018). In accordance with earlier studies, the overexpression of both FvSAMS and FvSAMDC was found to increase the total polyamine levels in response to stress (Roy and Wu 2002; Waie and Rajam 2003; Wi et al. 2006, 2014; Cheng et al. 2009; Peremarti et al. 2009; Gong et al. 2014; Ma et al. 2017). However, the overproduction of the FvSAMDC increased the total polyamine levels more...
substantially, while the “SAM surplus” caused a lesser degree of accumulation in the FvSAMS lines (Fig. 5). It follows therefore that in contrast to several earlier studies (Li et al. 2004; Nambeesan et al. 2012; Yu et al. 2016), no antagonistic effect between ethylene and polyamine biosynthesis was observed in our experimental system.

Based on the data measured in the FvSAMDC lines there appears to be a positive correlation between the free PA levels and the proline content as well as the amount of ethylene, while there is a negative correlation between the free PA levels and the lignin content when the plants are exposed to salt stress (Figs. 4c, 5).

The overexpression of FvSAMS and FvSAMDC increased the relative expression rates of genes SAMS, SAMDC, SPDS, SPMS, SAHH, PMT and CAD under stress condition (Fig. 6). However, the expression rates of ACS decreased in the FvSAMS but increased in the FvSAMDC lines under stress conditions, which was also supported by the ethylene production (Figs. 4c, 6). The relatively high levels of expression of CAD in response to stress in the FvSAMDC lines showed no positive correlation with lignin contents (Figs. 4b, 6), according to our assumption the lower lignin content measured in the FvSAMDC lines can induce the higher CAD expression in the case of salt stress in order to increase the lignin production. In earlier reports the overexpression of SAMS increased the relative expression rates of the genes SAMS, SAMDC, ACS, SPDS and SPMS in tomato, Medicago sativa and A. thaliana (Gong et al. 2014; Guo et al. 2014; Kim et al. 2015) and the overproduction of SAMDC increased the relative expression levels of SAMDC and decreased those of ACS in tobacco and A. thaliana (Wi et al. 2006, 2014).

In our opinion the prolonged survival and increased salt tolerance of the FvSAMDC lines can be attributed to the higher level of polyamines, while in the FvSAMS lines elevated lignin and polyamines contents together were among the contributing factors.

We investigated the subcellular localization of the proteins encoded by FvSAMS::sGFP and FvSAMDC::sGFP. The FvSAMS::sGFP exhibited nuclear, nucleolar and cytoplasmic (near to the plasmalemma), plastid membrane localization in stable transformants (Fig. 8c). Previous studies reported that the SAMS is considered to be an enzyme with cytosolic localization in plants (Schroeder et al. 1997; Ravelan et al. 1998; Hanson and Roje 2001), whereas in mammals it was found to show nucleocytoplasmic distribution (Reytor et al. 2009). Reytor et al. (2009) reported that the nuclear accumulation of MAT (methionine adenosyltransferase = SAMS in plant) showed positive correlations with the trimethylation of the histone H3K27. FvSAMDC::sGFP showed weak nuclear and homogeneous cytoplasmic distribution (Fig. 9a). In former studies SAMDC activities were found in the chloroplasts, mitochondria and cytosol of plants (Yamanoha and Cohen 1985; Torrigiani et al. 1986), while only cytoplasmic localization was detected in animals (Gritli-Linde et al. 1995). Since we detected no chloroplast or mitochondrial sGFP signals, the amino acid sequence of FvSAMDC was examined bioinformatically. According to application, prediction of the probability of FvSAMDC having chloroplast, mitochondrial and other localization was found to be 7%, 9% and 80%, respectively.

In the case of both constructs, endoplasmic reticulum subcellular localization was observed during transient expression, but no similar signals were detected in the FvSAMS and FvSAMDC stable transformants (Figs. S1, S2). We assumed therefore that these signals could be interpreted as a response to the physical and biotic stresses caused by the infiltration procedure. This phenomenon cannot be adequately explained by our current data and requires further investigations, but both biotic and abiotic stresses can cause the accumulation of unfolded proteins, which interact with the specific sensor proteins of the ER membrane. The response to the unfolded proteins (UPR—unfolded protein response) is a conserved eukaryotic stress reaction, and it was the unfolded protein ER-associated degradation (ERAD) that we presumably observed (Walter and Ron 2011).

In our experiments the FvSAMS::sGFP protein was localized to the nucleolus, an observation which has not been reported previously (Fig. 8a, b). Reytor et al. (2009) found no nucleolar localization of the MAT proteins. With the exception of FC (Fibrillar Center) shown in Fig. 8a ii, the FvSAMS::sGFP is localized either in DFC (Dense Fibrillar Component) or in GC (Granular Component) or in both nucleolar components, which take part in the biogenesis of ribosomes. DFC contains the protein fibrillarin (Sirri et al. 2008), which plays a role in the processing of rRNA. Furthermore, an MTase region (Ado-Met-dependent methyltransferase) was identified in the N. benthamiana Fibrillarin2 protein (NbFib2), which contained SAM binding motives and localized to the nucleolus (Zheng et al. 2016). Therefore, the nucleolar localization of FvSAMS::sGFP suggests that it plays a role in ribosome synthesis. The fact that the FvSAMS sequences contain no nucleolar localization signals makes it probable that there is an intermediate interaction required for the nucleolar localization of FvSAMS::sGFP proteins, but this phenomenon requires further investigations. Previous studies reported that SAM is delivered into plastids by specific SAM transporters (Ferro et al. 2002; Palmieri et al. 2006), where SAM is required to methylation of DNA, RNA and proteins (Montasser-Khousari et al. 1978; Block et al. 2002). According to our results the FvSAMS::sGFP showed nuclear, nucleolar and cytoplasmic (near to the plasmalemma), 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).
Our results showed that in stable transformants the subcellular localization of the \textit{FvSAMS}::sGFP protein was displaced in the direction of the cytoplasm (near to the plasmalemma and plastid membrane) from the nucleus in response to stress, while the quantity of \textit{FvSAMDC}::sGFP protein showed a parallel increase in both cellular compartments (nucleus, cytoplasm) (Fig. 9b). Several nuclear proteins carry both NLS and NES signals, which control their transport between the cell nucleus and the cytoplasm. The presence of putative bipartite nuclear localization and nuclear export signals in both \textit{FvSAMS} and \textit{FvSAMDC} is in agreement with the nuclear and cytoplasmic localization of both enzymes, though the fictionality of these signals remain to be demonstrated experimentally.

We could randomly detect \textit{FvSAMDC}::sGFP fusion proteins only in a few cases from several dozens of preparations, which can be due to the short half life time of the SAMDC (Pegg 1986), but it could be observed that the \textit{FvSAMDC}::sGFP showed distinctive nuclear, cytoplasmic and nuclear-cytoplasmic localization concurrently in the stable transformants (Fig. 10a). The fact that it showed nuclear and cytoplasmic localization separately and also simultaneously can be explained by the differential autocatalytic activation of the proenzyme SAMDC in the different cellular compartments as well as by 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 the \textit{FvSAMDC}::sGFP is also supported by an earlier study (Belda-Palazón et al. 2012) in which it was described that the spermidine synthase showed nuclear and cytoplasmic localization, whereas the spermine synthase had only cytoplasmic localization. Belda-Palazón et al. (2012) also observed that in the presence of exogenous spermidine synthase, the cytoplasmic localization of spermine synthase showed a displacement towards the nucleus, which suggests that the synthesis of spermidine and spermine is a complex process. Since SAMDC produces dcSAM, which serves as a substrate for both the spermidine and spermine synthases by providing them with aminopropyl group, the synthesis of SAMDC in an inactive form, its strict transcriptional and translational regulation as well as its short half-life can account for its dual localization due to the complexity of the process.

We also observed that the amount of detectable \textit{FvSAMS}::sGFP protein showed a decreasing tendency from the younger leaves towards the older ones but higher \textit{FvSAMS} mRNA rates were measured in the older leaves in spite of the lower levels of detectable \textit{FvSAMS}::sGFP protein. Finally, \textit{FvSAMS}::sGFP fusion proteins were only detected in the guard cells of older leaves (Fig. 10b–d), therefore we suppose that the SAMS enzyme has an important role to play in the regulation of the stomatal functions, such as cellular respiration, photosynthetic gas exchange and evaporation. Similar pattern was obtained in tobacco plants overexpressing \textit{ArSAMS1} by Boerjan et al. (1994), namely that \textit{ArSAMS1} mRNA accumulation grew from top to bottom but the activity of AtSAMS1 enzyme showed a reverse correlation to the amount of mRNA. Later it was proven that AtSAMS1 is inhibited by nitric oxide (NO). Structural analysis of AtSAMS1 showed that the Cys-114 is flanked with amino acids promoting S-nitrosylation. We found that the amino acid sequence of SAMS contained arginine (R) at the site of Cys-114 (Fig. 11a). The inhibitory effect of S-Nitrosoglutathione (GSNO) drastically decreased when Cys-114 was replaced by arginine (Lindermayr et al. 2006). Earlier studies focused on the reduction in the activity of enzyme SAMS by S-nitrosylation (Avila et al. 1997; Lindermayr et al. 2006), while other publications discussed the phosphorylation of Thr-342 by Protein kinase C (PKC), which decreased the enzyme activity of SAMS (Pajares et al. 1994) as well as the predicted phosphorylation of Thr, Ser, Thr-112 and Ser-271 in \textit{FvSAMS} (Fig. 11b), which is equivalent to the S/TXK/R (Woodgett et al. 1986) and TXRX (Pajares et al. 1994) recognition sequences preferred by PKC (in the case of \textit{FvSAMS} the TKRP and SGK). Therefore, it is possible that we assume that the phosphorylation of Thr-112 and Ser-271 play a role in the inhibition of enzyme \textit{FvSAMS} by PKC, but we currently do not have evidence for this possibility.

According to Reytor et al. (2009) the subcellular localization of the enzyme SAMS is determined by the structure of its C-terminal domain, which makes the involvement of Lys-335, 360, 364 acetylation and Lys-387 SUMOylation in the regulation of subcellular localization of \textit{FvSAMDC} probable (Fig. 11c). The results of earlier studies support this hypothesis since the SUMOylation of NLS of the human enzyme SAE resulted in its nuclear accumulation (Truong et al. 2012) and the acetylation of the NLS of the human RhoA GEF Net1A increased its cytoplasmic accumulation (Song et al. 2015). We have no data available with regard to the post-translational modifications determining the subcellular localization of the enzyme \textit{FvSAMDC} but based on predicted data the phosphorylation, acetylation, S-nitrosylation and SUMOylation of the NLSs can play a role in the process (Fig. 11d), since numerous published data provided evidence supporting the role of NLS phosphorylation, acetylation, S-nitrosylation, SUMOylation in the subcellular localization of enzymes (Harreman et al. 2004; Hara et al. 2005; Truong et al. 2012; Song et al. 2015).

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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