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Ectopic expression of a fruit phytoene synthase from *Citrus paradisi* Macf. promotes abiotic stress tolerance in transgenic tobacco

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Abstract Abscisic acid (ABA) is an important regulator of plant responses to environmental stresses and an absolute requirement for stress tolerance. Recently, a third phytoene synthase (*PSY3*) gene paralog was identified in monocots and demonstrated to play a specialized role in stress-induced ABA formation, thus suggesting that the first committed step in carotenogenesis is a key limiting step in ABA biosynthesis. To examine whether the ectopic expression of *PSY*, other than *PSY3*, would similarly affect ABA level and stress tolerance, we have produced transgenic tobacco containing a fruit-specific *PSY* (*CpPSY*) of grapefruit (*Citrus paradisi* Macf.). The transgenic plants contained a single- or double-locus insertion and expressed *CpPSY* at varying transcript levels. In comparison with the wild-type plants, the *CpPSY* expressing transgenic plants showed a significant increase on root length and shoot biomass under PEG-, NaCl- and mannitol-induced osmotic stress. The enhanced stress tolerance of transgenic plants was correlated with the increased endogenous ABA level and expression of stress-responsive genes, which in turn was correlated with the *CpPSY* copy number and expression level in different transgenic lines. Collectively, these results provide further evidence that PSY is a key enzyme regulating ABA biosynthesis and that the altered expression of other *PSY*s in transgenic plants may provide a similar function to that of the monocot's *PSY3* in ABA biosynthesis and stress tolerance. The results also pave the way for further use of *CpPSY*, as well as other *PSY*s, as potential candidate genes for engineering tolerance to drought and salt stress in crop plants.

Keywords Grapefruit · Drought tolerance · Abiotic stress · Abscisic acid · Carotenoids

Abbreviations

ABA	Abscisic acid
cDNA	Complementary DNA
GUS	β-glucuronidase
NaCl	Sodium chloride
nptII	Neomycin phosphotransferase II
PEG	Polyethylene glycol
PSY	Phytoene synthase
qPCR	Quantitative real-time RT-PCR
WT	Wild-type

Introduction

Abiotic stresses such as drought, salinity and extreme temperatures are the primary causes of crop productivity losses [1]. In perennial tree crops like citrus, severe water

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stress during the flowering, fruit-growth and fruit-maturity phases reduces both the number of fruits per tree and the size of fruits, resulting in up to a 21 % decrease in yield productivity [2]. As sessile organisms, plants have evolved various biochemical and physiological mechanisms to respond and adapt to these stresses and thus acquire stress tolerance, many of which are regulated by the phytohormone abscisic acid (ABA) [3]. ABA acts through a complex signaling network that changes, within minutes, the activity of various signaling molecules and ion channels, hence leading to stomatal closure, and also induces, within hours, the expression of several stress-related genes in order to maintain the osmotic homeostasis [4].

ABA biosynthesis-related genes [5–7] and their regulatory mechanisms [8] have been identified in higher plants over the past few years. The first committed and rate-limiting step in ABA biosynthesis is the oxidative cleavage of 9-cis-epoxycarotenoid [40-carbons (C40)] by 9-cis-epoxycarotenoid dioxygenases (NCEDs) to yield xanthoxin [15-carbons (C15)]. Xanthoxin is then oxidized to form abscisic aldehyde, which is then further oxidized to ABA [6, 9]. Overexpression of NCEDs in *Arabidopsis* [10, 11], tobacco [12–14] and bent grass [15] resulted in increased osmotic stress tolerance in transgenic plants.

Recently, a third member (*PSY3*) of the phytoene synthase gene family, which is known to encode the first committed and rate-limiting enzyme in the formation of carotenoids [16], was identified in monocots and implicated to play a specialized role in controlling ABA biosynthesis in response to abiotic stress [5, 7]. In contrast with *PSY1*and *PSY2*, which are required for endosperm carotenoid accumulation [17] and photomorphogenesis in photosynthetic tissues [18], *PSY3* was associated with root carotenogenesis and necessary for drought and salt stress-induced production of ABA [5, 7]. These findings provided evidence for a functional specialization of duplicated *PSY* genes in monocots, in which the family members show distinct tissue specificities and functions. Yet, in dicot species such as *Arabidopsis* [19] and tobacco [20], PSY is encoded respectively by single- or two-copy genes that control carotenogenesis in both photosynthetic and chromoplast-containing tissues. No *PSY* genes devoted to regulation of root carotenogenesis and ABA formation have been identified in dicots.

Once *PSY* subfunctionalization has been demonstrated in monocots, with one paralog (*PSY3*) exhibiting specificity of expression in roots to control ABA formation in response to abiotic stress, we have tested the hypothesis if the ectopic expression of *PSY*s, other than *PSY3*, in transgenic plants would likewise provide a similar function. In this regard, we have produced transgenic tobacco plants constitutively expressing a fruit *PSY* gene (*CpPSY*) isolated from grapefruit (*Citrus paradisi* Macf.). *CpPSY* expression was recently demonstrated to be upregulated during fruit development in albedo and juice vesicles of white- and red-fleshed grapefruits and correlated with the pattern of carotenoid accumulation in each variety [21]. Our results suggest that the ectopically expressed *CpPSY* is able to perform a similar function to that of the monocot's *PSY3* in transgenic plants. *CpPSY* can thus be considered as a potential candidate gene for engineering tolerance to drought and salt stress in crops.

Materials and methods

Plant material and *Agrobacterium* transformation

Preparation of leaf-disk explants of wild-type (WT) tobacco (*Nicotiana tabacum* cv. Havana), *Agrobacterium* transformation, shoot regeneration and rooting of the shoots were performed as described in da Silva et al. [22]. All independently transformed plants (T_0 generation) were transferred to soil and grown in standardized greenhouse conditions to generate seeds for further analyses.

Agrobacterium strain and plasmid

The disarmed *Agrobacterium tumefaciens* strain EHA105 [23] harboring the binary plasmid pCAMBIA 2301/CitPSY was employed in all experiments of transformation [electronic supplementary material (ESM) Fig. S1]. This plasmid contains the full-length cDNA sequence of a phytoene synthase (*CpPSY*) gene isolated from fruits of *C. paradisi* cv. Flame, under the control of the 35S promoter from Cauliflower mosaic virus (CaMV 35S), and the neomycin phosphotransferase II (*nptII*) selectable marker gene and the beta-glucuronidase (GUS) *uidA* scorable marker gene driven by the CaMV 35S promoter [24].

Histochemical assay of GUS activity

The expression of GUS in tissues of transformed plants was detected using the histochemical assay described by Jefferson et al. [25].

DNA extraction and PCR analysis

Total DNA from the leaves of T_0 transgenic plants was extracted using the CTAB method. The primer sequences used for amplification of the fragment of *nptII* gene were 5′-ATGGGGATTGAACAAGATGGATTG-3′ and 5′-TCAGAAGAACTCGTCAAGAAGGC-3′, generating an 800-bp product; those used for amplification of the fragment of the *CpPSY* gene were 5′-ATAGGGCATCGGCCTTGGATTGTG-3′ and 5′-CACTCACCATTTTCTCTGGTGCTC-3′,

generating a 1,300-bp product. PCR was carried out in 25 µl volumes containing 0.2 mM of each dNTP, 0.25 µM of each oligonucleotide primer, 0.25 U Taq polymerase (Promega, Madison, USA), and 50 ng of sample DNA. The reactions were amplified in a thermal cycler (Techne, Cambridge, UK) with an initial denaturing at 94 °C for 2 min; 35 cycles of 94 °C for 1 min, 58 °C (*nptII*) or 62 °C (*CpPSY*) for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 10 min. Amplified DNA fragments were electrophoresed on 1.5 % agarose gel, stained with ethidium bromide (0.5 µg ml^{-1}) and visualized under UV light.

Southern blot analysis

Total DNA was extracted from the leaves of T_0 transgenic plants, as previously described, and a Southern blot was prepared by digesting 80 µg genomic DNA with *Bam*HI (37 °C for 16 h), separating the DNA fragments on 0.8 % agarose gel and transferring them to a positively charged Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech, New Jersey, USA). The membrane was hybridized using a DNA probe conjugated with alkaline phosphatase for *nptII*, which was generated by amplifying an 800-bp DNA fragment from the *nptII* gene of pCAMBIA 2301, using the pair of primers and reaction conditions as described above. Labeling, hybridization and washing were performed according to the manufacturer's protocol supplied with the AlkPhos Direct labeling and detection system with CDP-star (Amersham Biosciences, Buckinghamshire, UK). The signals were detected by exposure of the probed membrane to Hyperfilm ECL film (Amersham Biosciences, Buckinghamshire, UK), at room temperature, using appropriate exposure time.

Quantitative real-time RT-PCR (qPCR) analysis

Total RNA was isolated from leaves of antibiotic-resistant T_1 transgenic plants with the RNAqueous® kit (Applied Biosystems, Foster City, USA), following the manufacturer's instructions. RNA was sequentially treated with DNase I (100 U g^{-1} total RNA) (FPLC pure, Amersham Pharmacia Biotech, Piscataway, USA). Purity and concentration of the isolated RNA samples were checked on 1 % (w/v) agarose gels and using a spectrophotometer. Reverse transcription (RT) was carried out using the RETROscript kit (Ambion, Austin, USA). The abundance of *CpPSY* mRNA in the transgenic plants was quantified using the ABI Prism 7500 and Sequence Detection System Software, version 1.6.3 (Applied Biosystems, Foster City, USA). All qPCR procedures, including tests, validations and experiments were carried out following the recommendations of Applied Biosystems. Actin was amplified along with the target gene as an endogenous control to normalize expression among different samples. The control primers were 5′-CATCCCTCAGCACCTTCC-3′ and 5′-CCAACCTTAGCACTTCTCC-3′. The primers for *CpPSY* were 5′-CCCGGACTGCTGTGTTTAAT-3′ and 5′-CAGCAGTGCTAGCAACCATACATG-3′. Primers for the stress-responsive genes 9-cis-epoxycarotenoid dioxygenase 1 (*NCED1*), catalase (*CAT*), glutathione S-transferase (*GST*), group 5 late embryogenesis abundant (*LEA5*) protein and group 2 LEAs (*ERD10C* and *ERD10D*) were as described in Huang et al. [26]. Reactions were performed in triplicate containing 100 ng of cDNA, 0.4 µl of each primer (5 pmol), 10 µl Power SYBR Green Master Mix (Applied Biosystems, Foster City, USA) and sterile Milli-Q water for a final volume of 25 µl. The amplification reactions were performed under the following conditions: (1) pretreatment at 50 °C for 2 min, (2) activation of Taq DNA polymerase at 95 °C for 10 min, (3) denaturation at 95 °C for 15 s, (4) annealing at 60 °C for 30 s, (5) extension at 60 °C for 1 min. The steps 3–5 were repeated for 30 cycles. In order to verify that only a single PCR product was generated for the amplified transcript, the multicomponent data for each sample was subsequently analyzed using the Dissociation Curve 1.0 Software (Applied Biosystems, Foster City, USA). Gene expression was quantified using the comparative methods Ct: $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ [27], with data obtained from a pool of at least three biological replicates that were individually validated.

Segregation analysis

Resistance to kanamycin in the T_1 progeny produced by self-pollination was tested by culturing sterilized seeds on Murashige and Skoog (MS) medium containing 100 mg l^{-1} kanamycin (Sigma, St. Louis, USA). The frequencies of the antibiotic-resistant and -susceptible T_1 seeds were assessed two weeks after germination.

Stress treatments

Fifteen 2-week-old WT and antibiotic-resistant T_1 tobacco seedlings were transferred to MS medium containing 25 % polyethylene glycol 6000 (PEG-6000) (Merck, Darmstadt, Germany), 150 mM NaCl (Merck, Darmstadt, Germany) or 200 mM mannitol (Vetec, Rio de Janeiro, Brazil). Seedlings were transferred to MS agar plates only in the control treatment. Root length and shoot biomass of individual seedlings were evaluated 25 days after the treatments.

ABA measurement

ABA concentration was determined in the mature (fully expanded) leaves of antibiotic-resistant T_1 transgenic plants according to Santa-Catarina et al. [28]. Samples (1 g

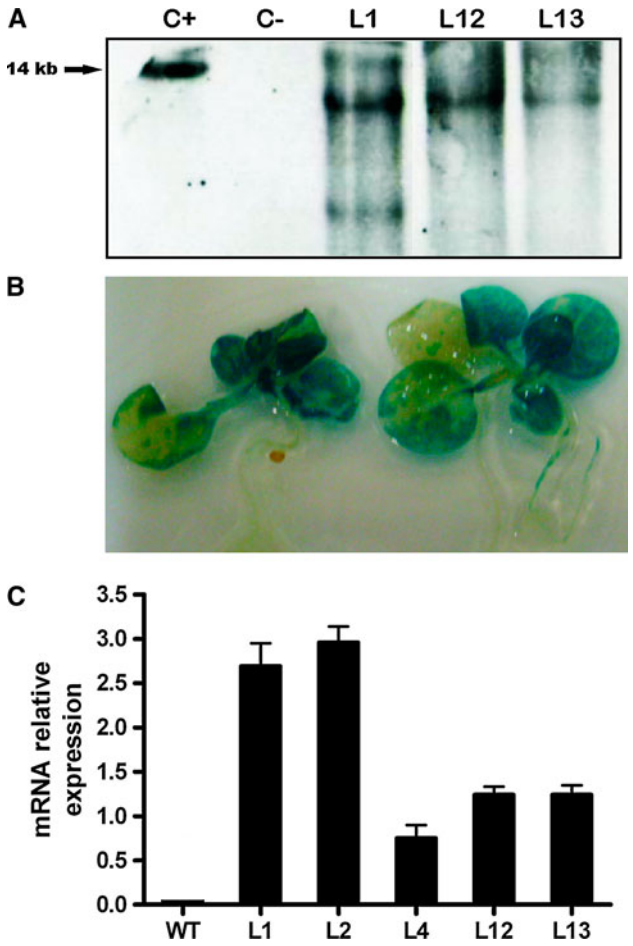


Fig. 1 Analysis of *CpPSY* integration and expression in transgenic tobacco plants. **a** Southern blot analysis of transgenic plants. DNA was digested with *Bam*HI, run in 0.8 % agarose gel, transferred to a nylon membrane and probed with alkaline phosphatase-conjugated fragment containing a region of the *nptII* gene. Lanes: *C* + pCAMBIA 2301/CitPSY plasmid, *C*- WT tobacco, *L1–L13* independent T_0 transgenic lines. **b** GUS activity detected in tissues of kanamycin-resistant T_1 tobacco seedlings. **c** *CpPSY* expression in transgenic tobacco plants. The transcript levels were measured in leaves of T_1 transgenic plants through qPCR, and standard errors were calculated from three biological replicates in which actin transcripts were used as internal controls. The relative mRNA expression in the transgenic plants as related to the L4 is indicated. *WT* WT tobacco, *L1–L13* independent T_1 transgenic lines

of dry tissue) were ground in 5 ml of 80 % (v/v) ethanol and 1 % (w/v) polyvinylpyrrolidone-40 (PVP-40) (Sigma, St. Louis, USA). [^{3}H]ABA was added as an internal standard. After 90 min of incubation, samples were centrifuged for 15 min at 20,000×*g* at 4 °C. Supernatants were concentrated in a speedVac at 45 °C, upon reaching 20 % of the initial volume (1 ml). The volumes were adjusted with Milli-Q water to 3 ml, and pH was adjusted to 2.5 using HCl (1 N). Samples were partitioned twice with ethyl ether. The organic phases containing ABA were completely dried in speed vac at 45 °C, dissolved in 300 µl of

Fig. 2 Stress tolerance assay of *CpPSY* expressing transgenic tobacco plants. **a** Seedling growth of *CpPSY* expressing transgenic tobacco in response to different stress treatments. WT and kanamycin-resistant T_1 tobacco seeds were germinated on MS agar plates and then transferred to a new MS agar plate supplemented with 25 % PEG-6000, 150 mM NaCl or 200 mM mannitol for 25 days. Seedlings were transferred to MS agar plates only in the control treatment. *WT* WT tobacco, *L1–L13* independent T_1 transgenic lines. **b** Analysis of root length (*left*) and shoot biomass (*right*) of *CpPSY* expressing transgenic tobacco plants under different stress treatments. WT and kanamycin-resistant T_1 tobacco seeds were treated with 25 % PEG-6000 (*top panel*), 150 mM NaCl (*middle panel*) or 200 mM mannitol (*bottom panel*), as described in Materials and Methods. Data are mean ± standard deviation of fifteen biological replicates. *, ** Significantly different from stressed WT at $P \leq 0.05$ or $P \leq 0.01$, respectively, according to Student's *t* test

100 % methanol and stored at -70 °C until analysis. Aliquots of stored extracts were analyzed by HPLC, using a 5-µm C18 reverse-phase column (Shimadzu Shin-pack CLC ODS, Shimadzu, Kyoto, Japan). The gradient was created by adding increasing volumes of methanol to 10 % methanol plus 0.5 % acetic acid in water. The methanol gradient was programmed as follows: 20 % over the first 15 min; 20–45 % between 15 and 22 min; 45–54 % between 22 and 33 min; 54–100 % between 33 and 34 min; and 100 % in 50 min, at a 1 ml min^{-1} flow rate at 40 °C. ABA concentration was determined using a UV–VIS detector at 254 nm. Fractions containing ABA were collected and analysed in the Packard Tri-Carb liquid scintillation counter to estimate losses.

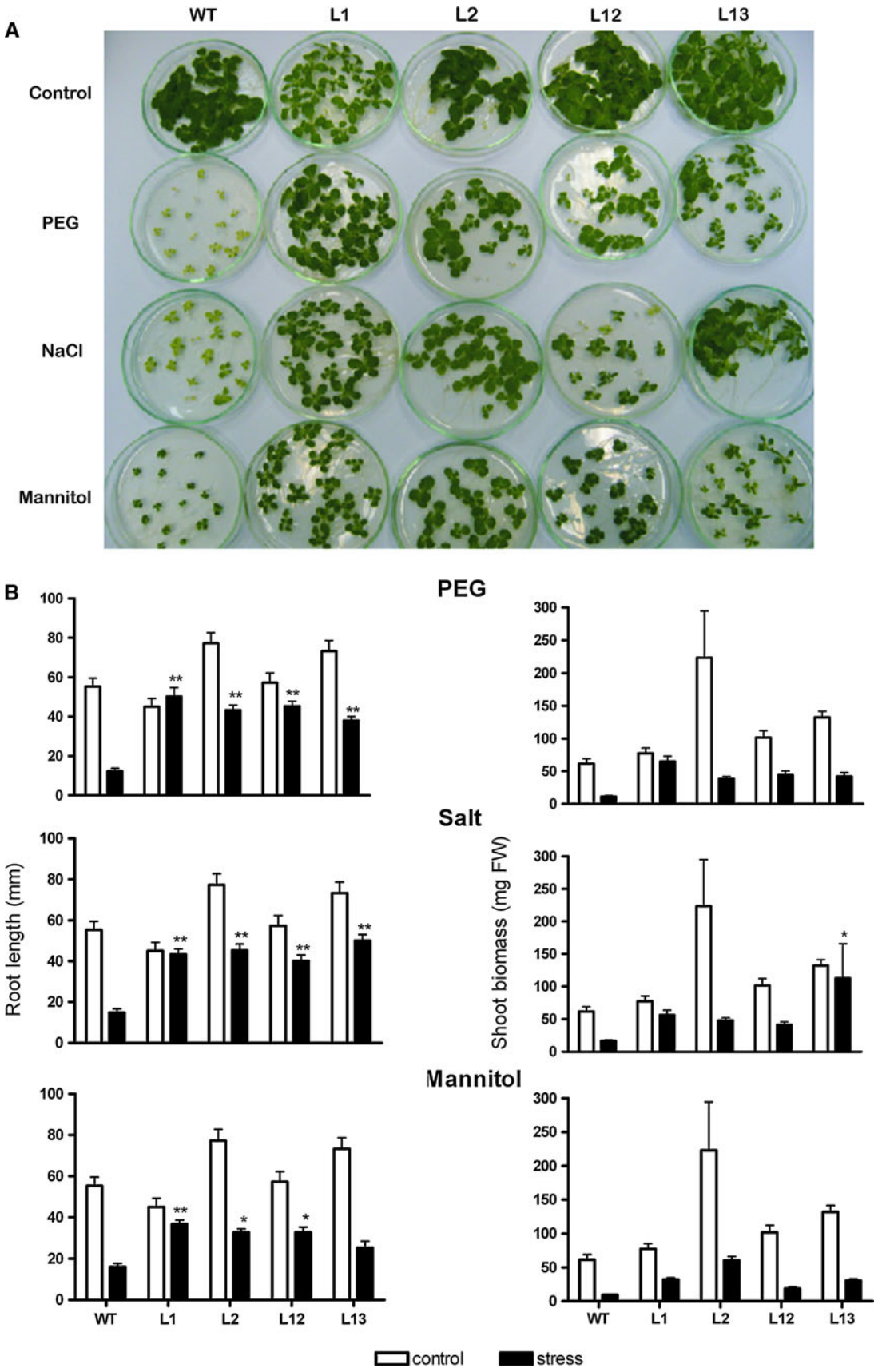
Statistical analysis

Statistical analysis was performed with the software BIOESTAT (Universidade Federal do Pará, Brazil), which tested the experiments as a completely randomized design. Segregation patterns were analyzed using the Chi-square test (χ 2) against the expected Mendelian ratios of 3:1 or 15:1 for single- or double-locus insertion, respectively. Statistical differences between WT and transgenic lines were assessed based on the analysis of variance (ANOVA) and means were separated by Student's *t* test, with a critical value of $P \leq 0.05$.

Results

Integration and expression of *CpPSY* in transgenic tobacco

Tobacco leaf disc explants were transformed with the plasmid pCAMBIA 2301/CitPSY, which contains a *PSY* cDNA isolated from fruits of *C. paradisi* (*CpPSY*) under control of the CaMV 35S promoter [24]. Fifteen out of a set of twenty regenerated tobacco plants randomly selected on kanamycin-


A
WT
L1
L2
L12
L13
Control
PEG
NaCl
Mannitol
B
PEG
Salt
Mannitol
Root length (mm)
Shoot biomass (mg FW)
WT
L1
L2
L12
L13
control
stress

containing medium, representing distinct transformation events, were confirmed to be GUS-positive (GUS +) by histochemical GUS assay (data not shown). Seven GUS + plants were screened for the presence of transgenes by PCR, using pairs of primers specifically designed to amplify a 800-bp fragment of the *nptII* gene and a 1,300-bp fragment of the *CpPSY* cDNA. Single bands of the expected sizes for *nptII* and *CpPSY* were obtained in all transgenic plants evaluated (ESM Fig. S1). Integration of T-DNA into genome of the transgenic plants was confirmed by Southern hybridization using a specific probe for the *nptII* gene. While the transgenic line L1 displayed two hybridization signals, only one was observed for L12 and L13 (Fig. 1a). The remaining transgenic lines analyzed have likewise exhibited only one hybridization signal (data not shown). Assuming that each hybridization signal corresponds to at least one gene copy, the copy number of transgenes integrated into the genome of transgenic plants varied from 1 to 2. That was further confirmed by segregation analysis of the *nptII* gene in the T_1 generation, based on the observed frequencies of kanamycin-resistant and -susceptible T_1 seeds (Table 1). The T_1 tobacco seedlings that survived to the antibiotic showed a complete GUS staining, thus confirming the transgenic nature of kanamycin-resistant seeds (Fig. 1b). T_1 transgenic plants were grown in a greenhouse and exhibited a normal phenotype and were fertile.

CpPSY expression was analyzed in the leaves of T_1 transgenic plants by qPCR. All the analyzed plants have expressed *CpPSY*, although differences in the transcript levels were observed among them (Fig. 1c). The transgenic lines L1 and L2 showed higher levels of *CpPSY* transcripts, whereas L12 and L13 presented an intermediate *CpPSY* transcript level. L4 had the lowest transcript level of *CpPSY*.

Transgenic tobacco expressing *CpPSY* shows enhanced tolerance to osmotic stress

Based on the results obtained in the analyses of *CpPSY* integration and expression, four transgenic lines (T_1 generation) were selected for the osmotic-stress tolerance assay. As can be seen in Fig. 2a, the transgenic lines showed increased tolerance to the different stresses as compared with the WT tobacco. The latter showed a ~5× decrease in root length and shoot biomass when subjected to different stress treatments (Fig. 2b). Such a stress effect was less noticeable in the transgenic lines, in which most of the stress treatments have caused a decrease in root length and shoot biomass as little as 2× as compared with the non-stress control treatment. A direct comparison between stressed WT and transgenic tobaccos has demonstrated that the latter showed a significantly higher root length than the former in all stress treatments (Fig. 2b), with the only exception of L13 in the mannitol treatment. The stressed transgenic lines have also showed a generally higher shoot biomass than the stressed WT tobacco, although in most cases these differences were not statistically significant (Fig. 2b). Collectively, these results show that the constitutive expression of *CpPSY* in transgenic plants is able to enhance their tolerance to stress.

CpPSY expressing tobacco plants show increased levels of endogenous ABA

In order to investigate whether the enhanced stress tolerance of the *CpPSY* expressing transgenic plants correlates with increased endogenous ABA levels, ABA concentration in leaves of T_1 transgenic plants was measured by HPLC and compared with that of WT tobacco leaves. Indeed, all the transgenic plants evaluated showed a remarkable increase in ABA content as compared with WT tobacco (Fig. 3). That increase was proportional to the *CpPSY* copy number and the expression level in transgenic lines. For example, the two-copy and high expression L1 contained ~10× more ABA than WT tobacco, whereas the one-copy and high expression L2 contained ~5× more ABA than WT tobacco (Fig. 3). The one-copy and intermediate expression L12 had ~3× more ABA than WT tobacco plants. The one-copy and intermediate expression L13, however, had only a 1.1× increase in ABA content,

Table 1 Segregation analysis in T_1 progeny of the transgenic tobacco plants transformed with pCAMBIA 2301/CitPSY

Transgenic line	Copy number of *nptII* in T_0 generation determined by Southern blot	Number of T_1 seeds		Number of loci estimated from Chi-square value	Chi-square value (ratio)	*P* value
		Kanamycin resistant	Kanamycin sensitive			
L1	2	226	17	2	0.07 (15:1)	0.79
L2	1	238	77	1	0.07 (3:1)	0.79
L4	1	244	56	1	6.42 (3:1)	0.02
L5	1	258	82	1	0.14 (3:1)	0.71
L11	1	246	72	1	1.07 (3:1)	0.30
L12	1	277	102	1	0.69 (3:1)	0.41
L13	1	318	88	1	2.57 (3:1)	0.11

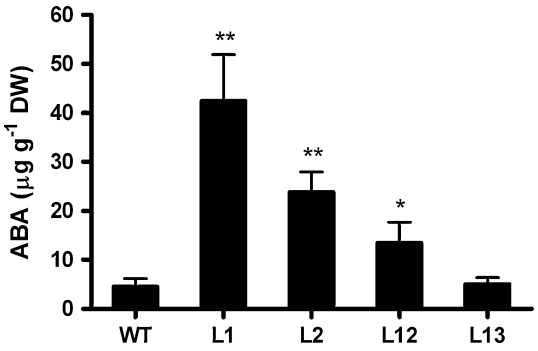


Fig. 3 HPLC analysis of ABA content in leaves of *CpPSY* expressing transgenic tobacco plants. Sixty-day-old plants of the WT and T_1 transgenic lines were used for ABA quantification. Data are mean ± standard deviation of two biological replicates. *, ** Significantly different from WT at $P \leq 0.05$ or $P \leq 0.01$, respectively, according to Student's *t* test

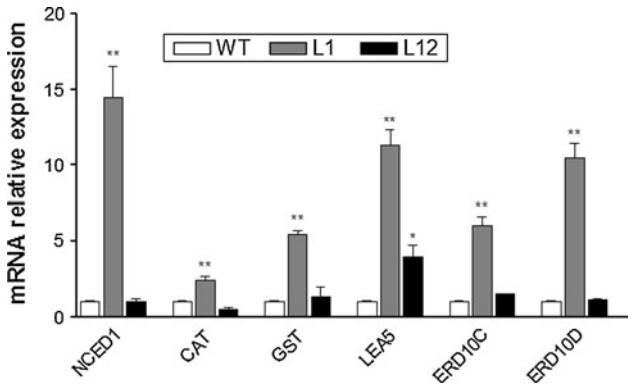


Fig. 4 Expression analysis of stress-responsive genes in transgenic tobacco plants. The transcript levels were measured in leaves of T_1 transgenic plants through qPCR, and standard errors were calculated from three biological replicates in which actin transcripts were used as internal controls. The relative mRNA expression in the transgenic plants as related to the WT tobacco is indicated. *WT* WT tobacco, *L1* and *L12* independent T_1 transgenic lines. *, ** Significantly different from WT at $P \leq 0.05$ or $P \leq 0.01$, respectively, according to Student's *t* test

probably caused by an altered post-transcriptional regulation. Taken together, these data indicate a consistent association among *CpPSY* copy number and expression, ABA content and stress tolerance.

Stress-related genes are upregulated in *CpPSY* expressing tobaccos

To analyse whether the increased ABA levels in the *CpPSY*-expressing transgenic plants altered expression of stress-related genes, the transcript abundance of six such genes was examined in leaves of T_1 transgenic (L1 and L12) and WT plants by qPCR. These genes encode enzymes for ABA biosynthesis (NCED1), ROS detoxification (CAT and GST), or stress-protective proteins (LEA5, ERD10C and ERD10D) [26]. As can be seen in Fig. 4, mRNA levels of all six genes significantly increased in L1 in comparison with the WT, whereas in L12 the increase in mRNA levels was significant for *LEA5*. These results demonstrate that *CpPSY* overexpression enhances the transcript levels of stress-responsive genes proportionally with the ABA levels produced by transgenic lines.

Discussion

The 9-cis-epoxycarotenoid cleavage reaction by NCEDs has long been known to be a rate-limiting step in ABA biosynthesis [29]. It was only recently recognized that the enzyme controlling the flux to carotenoid precursors, PSY, may also play a key regulatory role in ABA biosynthesis that is required for stress tolerance [5, 7]. To examine whether the ectopic expression of a *PSY* involved in fruit carotenoid accumulation [21] could similarly affect ABA production and stress tolerance, we have produced transgenic tobacco containing a fruit *PSY* from grapefruit (*CpPSY*). *CpPSY* integration was confirmed in seven transgenic plants, whereof six contained a single-locus insertion and one had a two-locus insertion. Analysis of *CpPSY* expression in leaves of the T_1 transgenic plants has revealed a variation in the transcript levels, even among the independent single-copy transformants. For example, the single-copy transgenic line L2 showed a level of *CpPSY* expression as high as that of the two-copy L1, whereas the single-copy L12 and L13 showed intermediate *CpPSY* expression levels between L2 and the single-copy L4. L4 showed the lowest *CpPSY* expression level. Such a transgene expression variability among the independent transformation events has been well documented in the literature and it is thought to reflect the influence of different sequences flanking the integration sites, a phenomenon known as "position effect" [30].

Transgenic tobacco plants expressing *CpPSY* showed significantly improved tolerance to osmotic stress induced by PEG, NaCl and mannitol as compared to WT plants. As a non-ionic and non-penetrating stress agent, high molecular weight (more than 4,000) PEG induces water stress in plants by decreasing the water potential of the nutrient solution without causing toxicity [31]. It simulates water deficit conditions similar to those observed in plants subjected to true drought conditions, in which water is withdrawn from both the cell wall and the protoplast, resulting in cytorrhysis, a process where both the cell wall and protoplast shrink [31]. Yet, the non-ionic and low molecular weight mannitol, which is also used to lower water potential, can penetrate the pores of the cell wall and causes plasmolysis, a loss of water and decrease in the protoplast volume while the volume of the cell wall

remains unchanged [31]. Salt stress also causes a low water potential stress at short-term, but an ionic imbalance and toxicity due to Na^+ excess at long-term [32]. Thus, our results indicate that the ectopic expression of *CpPSY* enhances the osmoregulation ability of plants.

WT tobacco displayed progressive chlorosis and a general growth inhibition in all osmotic stress treatments, whereas the *CpPSY* expressing transgenic plants had increased root length and shoot biomass. However, statistical difference for shoot biomass was not significant in most cases. The relative maintenance of root growth and inhibition of shoot growth is a well-established response of plants to low water potential [33] and it is the result of regulation of growth by increased ABA levels [34]. This could explain why the *CpPSY* expressing transgenic plants showed less noticeable differences in shoot biomass than in root length when subject to stress, as compared with WT plants. Besides, this could also explain why the phenotypes among the different transgenic lines, which produced distinct levels of endogenous ABA, were so different under control condition as shown in Fig. 2a.

The enhanced stress tolerance of the *CpPSY* expressing transgenic plants correlated with their upregulated endogenous ABA levels. ABA concentration was observed to increase in the transgenic lines as the *CpPSY* expression level and copy number increased. Thus, these results provide further evidence that the first committed step in carotenogenesis is a key limiting step in ABA biosynthesis and that the manipulation of ABA levels can be achieved by altering *PSY* expression. Besides, these data demonstrate that the altered expression of other *PSY*s in transgenic plants confers a similar function to that of *PSY3* in ABA biosynthesis and consequent stress tolerance. Actually, *PSY3* shows a conserved gene structure as compared to the monocot's *PSY1* and *PSY2*, as well as with *Arabidopsis* [5, 7] and *Citrus* [21] *PSY*s. What separates *PSY3* from members of the *PSY1* and *PSY2* groups is a distinct C-terminal motif, R(H/R)XS(S/T)LT, and an extended N terminus in the deduced protein sequences [5]. Probably the functional specialization of *PSY3* in stress-induced ABA formation is not caused by these discrete differences at the amino acid level, but rather by the distinct *cis*-regulatory elements observed in its promoter region [7]. In contrast with the light-responsive elements found in *PSY1* and *PSY2* promoters, the promoter of *PSY3* contains a combination of ABA-response element and coupling element [7] that is believed to play a crucial role in abiotic stress-dependent gene expression [35].

The increased ABA levels in *CpPSY*-expressing transgenic plants modulated transcriptional levels of stress-associated genes, which support our observations of stress tolerance in the transgenics. *NCED1* encodes a rate-controlling NCED enzyme involved in stress-induced de novo biosynthesis of ABA, particularly in leaf tissue [10, 36–39]. *CAT* and *GST* encode ROS-scavenging enzymes that are induced upon abiotic stresses in order to minimize the cellular damage caused by oxidative burst [40, 41]. *LEA5* and *ERD10C/D* encode group 5 and group 2 LEA proteins, respectively, which are presumed to function in dehydration tolerance by binding water, sequestration of ions, and stabilizing protein and membrane structures [42]. Induction of these target genes demonstrates some of the possible mechanisms mediating the observed stress tolerance in *CpPSY*-expressing transgenic lines.

In conclusion, endogenous ABA levels and expression of stress-responsive genes can be regulated by the ectopic expression of *CpPSY* in transgenic plants, hence resulting in enhanced stress tolerance. These results point the way towards the use of *PSY*s other than *PSY3* as potential candidate genes for engineering tolerance to drought and salt stress. Indeed, increased salt stress tolerance was recently reported in transgenic *Arabidopsis* overexpressing a *PSY* from euhalophyte *Salicornia europaea* [43]. The data obtained here support the use of *CpPSY* in genetic transformation of citrus rootstocks, currently in progress in our laboratory, as a strategy to increase drought and salt stress tolerance in this economically important fruit crop.

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