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ORIGINAL ARTICLE

Characterization of phytoene synthases from cassava and their involvement in abiotic stress-mediated responses

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Abstract Abiotic stress stimuli induce the increased synthesis of abscisic acid (ABA), which is generated through the cleavage of xanthophyll precursors. To cope with the increased xanthophyll demand, maize and rice contain a third stress-induced gene copy, coding for phytoene synthase (PSY), which catalyzes the first carotenoid-specific reaction in the pathway. To investigate whether this specific response extends beyond the Poaceae, cassava was analyzed, an important tropical crop known for its drought tolerance. We also found three *PSY* genes in cassava, one of which (MePSY3) forms a separate branch with the stress-specific Poaceae homologs. However, *MePSY3* transcripts were virtually absent in all tissues investigated and did not change upon abiotic stress treatment. In contrast, the two remaining *PSY* genes contributed differentially to carotenoid biosynthesis in leaves, roots, and flower organs and responded towards drought and salt-stress conditions. Detailed analyses of *PSY* and 9-*cis*-epoxycarotenoid cleavage dioxygenase (*MeNCED*) expression and resulting ABA levels revealed MePSY1 as the main stress-responsive paralog. In the presence of high carotenoid levels in leaves, MePSY1 appeared to support, but not to be rate-limiting for ABA formation; MeNCED represented the main driver. The inverse situation was found in roots where carotenoid levels are low. Moreover, ABA formation and the relative induction kinetics showed discrimination between drought and salt stress. Compared to rice as a drought-intolerant species, the drought response in cassava followed a different kinetic regime. The difference is thought to represent a component contributing to the large differences in the adaptation towards water supply.

Keywords ABA · Abiotic stress · Carotenoids · Cassava · 9-*cis*-Epoxycarotenoid cleavage dioxygenase (NCED) · Phytoene synthase

Abbreviations

ABA	Abscisic acid
NCED	9-*cis*-Epoxycarotenoid cleavage dioxygenase
PSY	Phytoene synthase

Introduction

In plants, carotenoids are involved in a variety of physiologically essential functions. In leaves, carotenoids are integrated in light-harvesting complexes and photosynthetic reaction centers to transfer energy and to mediate photoprotection (Demmig-Adams and Adams 1992, 2002; Niyogi 1999). Another proportion of carotenoids, however, is being metabolized into at least two classes of phytohormones, abscisic acid (ABA) and the strigolactones both emerging through the action of carotenoid-cleaving dioxygenases (CCDs; Bouvier et al. 2005; Auldridge et al. 2006). Strigolactones were shown to be involved in the inhibition of shoot branching and the establishment of mycorrhizal symbiosis (Dun et al. 2009) while ABA, well known for decades, regulates seed dormancy and mediates the response towards abiotic stress stimuli (Wilkinson and Davies 2002; Zhu 2002). The need for these

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phytohormones throughout the plant is probably reflected by the synthesis of carotenoids taking place at low rate in non-green tissues, such as in roots (Parry et al. 1992; Bouwmeester et al. 2007).

The enzyme phytoene synthase (PSY), which is the subject of the current work, catalyzes the first carotenoid-specific reaction condensing two molecules geranylgeranyl pyrophosphate to form the colorless carotene phytoene, which is converted into the red-colored lycopene through the action of two desaturases and two enzymes involved in isomerisation (Bartley et al. 1999; Park et al. 2002; Isaacson et al. 2002, 2004; Chen et al. 2010). Two cyclases introduce β- or ε-ionone rings at both ends of lycopene and define a branching point leading to either β-carotene (β-β-carotene) or α-carotene (β,ε-carotene). Hydroxylation reactions catalyzed by two P_{450} and two non-heme iron enzymes (Kim et al. 2009) and epoxydation reactions (Niyogi et al. 1998; Hieber et al. 2000) lead to the formation of lutein as the terminal derivative of the α-carotene derived branch of the pathway, while zeaxanthin, antheraxanthin and violaxanthin are formed within the β-branch. The latter reactions are reversible and represent the xanthophyll cycle which contributes to non-photochemical quenching of chlorophyll fluorescence (Szabó et al. 2005).

While the xanthophyll pattern is determined at the level of the cyclases (Ronen et al. 1999; Moehs et al. 2001) and the activity of oxygen introducing enzymes, the level/activity of PSY determines in most cases the quantity of carotenoids synthesized. *PSY* expression is light regulated, e.g. in seedlings (von Lintig et al. 1997; Welsch et al. 2000, 2003) but can also be developmentally regulated, for instance, during tomato fruit ripening (Fraser et al. 1994).

Corroborating rate-limitation, increased carotenoid levels have been achieved through overexpression of *PSY* alone in cases where a background of carotenoid formation existed, for instance in canola and *Arabidopsis* seeds (Shewmaker et al. 1999; Lindgren et al. 2003). This can be driven to levels where β-carotene crystals form, as in *Arabidopsis* cells and carrot roots (Maass et al. 2009). In other cases, additional enzymes may be needed, such as in Golden Rice where the co-expression of a bacterial desaturase (CrtI) was required (Ye et al. 2000; Paine et al. 2005; Schaub et al. 2005).

Substrates for ABA formation are the 9-*cis*-isomers of violaxanthin and neoxanthin. Their cleavage is catalyzed by a subclass of CCDs, 9-*cis*-epoxycarotenoid dioxygenases (NCEDs), yielding xanthoxin which leaves the plastids for conversion into ABA (Nambara and Marion-Poll 2005). In *Arabidopsis*, five NCEDs are involved in ABA biosynthesis. This was concluded from cleavage activity with 9-*cis*-epoxycarotenoids (NCED2, 2, 3, 6 and 9; Iuchi et al. 2001; Tan et al. 2003) or from enhanced ABA levels of an activation-tagged *Arabidopsis* line (NCED5; Fan et al. 2009). *AtNCED3* is predominantly induced by abiotic stress stimuli; accordingly, *AtNCED3* overexpressing *Arabidopsis* lines showed enhanced ABA levels and increased drought stress tolerance while *AtNCED3* antisense lines showed the opposite phenotype.

The increased requirement for epoxycarotenoid NCED substrates under stress conditions can have an impact on *PSY* expression. As revealed recently, maize and rice contain a third *PSY* gene copy, which is predominantly induced in roots upon drought and salt stress to support the biosynthesis of precursor xanthophylls (Li et al. 2008; Welsch et al. 2008).

The occurrence of this specialized *PSY* gene in rice and maize might suggest the development of a grass-specific mechanism to allow the high environmental adaptability of Poaceae. To question such potential exclusivity, we investigated cassava (*Manihot esculenta* Crantz) as a tropical, dicotyledonous species known for its marked drought tolerance. The results obtained suggest a wider occurrence of stress-mediated *PSY* regulation, however, with different contributions of individual PSY homologs.

Materials and methods

Plant material

Cassava (*Manihot esculenta* Crantz) landraces CM 3306-4 and MPER183 were obtained from the International Center for Tropical Agriculture (CIAT, Cali, Columbia). In vitro culture of landrace MPER183 plantlets were maintained in plastic boxes (diameter 68 mm, height 100 mm, Greiner Bio-One, Frickenhausen, Germany) containing propagation medium (4.4 g l^{-1} MS salts/KOH, pH = 5.7, 20 g l^{-1} saccharose, 100 mg l^{-1} myo-inositol, 1 mg l^{-1} thiamin-HCl, 0.04 mg l^{-1} 6-benzylaminopurine, 0.05 mg l^{-1} gibberellin A3, 0.02 mg l^{-1} 1-naphthaleneacetic acid, 0.25 mg l^{-1} $CuSO_4$, 4 mg l^{-1} $AgNO_3$, 4.5 g l^{-1} plant agar). The plants were grown under long day conditions with 16 h light (100 µmol photons m^{-2} s^{-1})/8 h dark at 26°C. Plants were propagated by transferring about 3 cm long explants to fresh medium every 10 weeks.

Rice plants (*Oryza sativa* var TP309, kindly provided by Paola Lucca, Swiss Federal Institute of Technology, Zurich, Switzerland) were grown in the greenhouse at 28°C (day) and 21°C (night) with 80% relative humidity.

Salt and drought stress treatment

Six- to eight-week-old in vitro grown cassava plants (landrace MPER183) were removed from the boxes, remaining agar was carefully removed and the plants were transferred into propagation medium (similar as described

above but without agar) containing 250 mM NaCl (salt stress) or 40% (w/v) PEG 6000 (drought stress) in propagation medium. The lid of the box was closed to maintain high humidity and the plants were incubated further with ongoing illumination for the times indicated. Plants used as a control were transferred into propagation medium only and incubated for identical times. After harvest, plants were separated into roots and leaves and used for RNA isolation as well as for the quantification of ABA. For stress treatments of detached cassava leaves, leaves were cut from in vitro cultivated plants and placed into NaCl or PEG 6000 containing propagation media as above, respectively, in closed plastic boxes and incubated for the times indicated. All experiments (stress treated samples and controls) were started 2 h after the end of the 8 h dark period.

Rice leaves were cut from adult rice plants and incubated in Yoshida solution (Yoshida et al. 1976) containing 40% (w/v) PEG 6000 for the times indicated; leaves were incubated in Yoshida solution only and used as the control. One measuring point represents the data of pooled tissues derived from three individual plants.

Cloning and identification of *PSY* genes

Cassava *PSY1* and *PSY2* were amplified by RT-PCR on total RNA from leaves of landrace CM 3306-4, using primer pairs *MePSY1sen* GAG AAC TGT CAG ATA GAT AGC CAA AA/*MePSY1asen* TAT CAT GGG TTT GCC AAA GG and *MePSY2sen* ATG ACT GTA GCA TTA CTA TGG GTT G/*MePSY2asen* AGC TTT TGT CAC AGG AGA TGA and cloned into the vector pCR2.1 (Invitrogen, Heidelberg, Germany). Genomic DNA was isolated as described (Arango et al. 2010). Complete genomic sequences for *MePSY1* and *MePSY2* were obtained with the GenomeWalker Universal Kit (Clontech, Heidelberg, Germany) according to the manufacturer's instructions. *MePSY3* was identified by BLAST analysis against the cassava genome assembly (version 1.1, available at http://www.phytozome.net/cassava.php). Scaffold positions of cassava *PSY* genes (translational start to stop) are as follows: *MePSY1*, scaffold01964:195640-197670 (locus name: cassava30274); *MePSY2*, scaffold02756:277111-279642 (locus name: cassava35536); *MePSY3*, scaffold01735:25274-27082 (locus name: cassava32745).

Bioinformatics/sequence analysis

Sequence alignments were performed with the Vector NTI advance 10.0.1 software (Invitrogen). Cassava contigs with homologies to *AtNCEDs* contained the following ESTs (GenBank Accession Numbers are given): contig 1: CK646917, CK652044, CK647474, FF381673, FF379872; contig 2: DB934068, DB930946, DB922665, DB925879, DB936486, CK643557, DV452473, DV448557. Exon/intron prediction for *MePSY3* was performed with FGENESH (http://mendel.cs.rhul.ac.uk/mendel.php?topic5fgen).

Real-Time RT-PCR assays

Total RNA was isolated using the plant RNA purification reagent (Invitrogen). RNA purification, DNAseI digestion and Real-Time RT-PCR assays were performed as described (Welsch et al. 2008). 6FAM-labeled TaqMan probes were designed for *MePSY1* and *MePSY2*; *NCED* transcripts were quantified using SYBR and for *MePSY3* both TaqMan as well as SYBR-based detection was used. The presence of single amplification products in SYBR assays was confirmed by melting curve analyses. Primer and probe sequences were as follows: *MePSY1,* forward, CCG ACG AGA CGG CCA TT; reverse, CAT AGG ATT AGG TAG TGG AAG CAA TTT A, TaqMan probe, CCT GCC GAG CTG GCT ACC ATG C; *MePSY2,* forward, GCA GCA TCA AGC ATA TCA AAG G, reverse, TGG GAA GCA AGG TTG GAA GA, MGB probe, ACG ACC TCG AAA CAT; *MePSY3*, forward, CAT TAG GGC CAT CCA CTA GTT CA, reverse, GAG GAG CGA CAG AAA GCC ATA, TaqMan probe, TGT TCT CCT GCA CCA GAC ATA AAT TGC CC; SYBR forward, GGC GCT TGA GCT TTG GTG TA, SYBR reverse, AAA GAG AGC TTA TGT CGG AAG GAC TA; *MeNCED,* forward, GAG GGA TTG CTT GCA GAT ACG, reverse, CAA ATA AAA AAC GAA CCC AGA AAA A. Primer functionality for *MePSY3* combinations was confirmed using genomic DNA.

Carotenoid extraction and quantification

Carotenoids were extracted from 5 mg lyophilized cassava leaves with acetone using α-tocopherolacetate as internal standard to correct for extraction errors and analyzed by HPLC as described (Welsch et al. 2008).

ABA extraction and quantitative analysis

ABA extraction from roots was performed as described (Welsch et al. 2008). For ABA extraction from leaves, 20 mg lyophilized leaf powder was spiked with 50 ng of internal standard ([6]-2-cis,4-trans-ABA-d6, Icon Isotopes, Summit, NJ, USA) and extracted with 1.2 ml acetone:H_2O:acetic acid (80:19:1, by vol.) for 2 min at 30 Hz in a vibration mill (MM301, Retsch, Haan, Germany). After centrifugation (15,000*g*, 2 min) the supernatant was transferred to a new tube. The extraction was repeated twice and the combined supernatants were evaporated

(Concentrator 5301, Eppendorf, Hamburg, Germany) to the aqueous phase. Then 100 µl 1% (v/v) MeOH was added and adjusted to a volume of 1 ml with 1% (v/v) acetic acid. Interfering organic substances were removed by extracting two times with 1 ml of hexane. The remaining aqueous phase was applied onto an SPE column (Oasis HLB 1 cc/30 mg; Waters, Eschborn, Germany) equilibrated with 1 ml MeOH, followed by 1 ml MeOH:H_2O:acetic acid (10:89:1, by vol.). The column was washed with 1 ml MeOH:H_2O:acetic acid (10:89:1, by vol.) and extracts were eluted with 1 ml MeOH:H_2O:acetic acid (80:19:1, by vol.), dried and redissolved in 200 µl 12 mM acetic acid in acetonitrile:H_2O (15:85, v/v). Then 10 µl was subjected to LC–MS analysis as described (Welsch et al. 2008). Internal standard-based quantification of ABA was done using the MS data and the quantification software available in the Xcalibur 2.0 software package. Retention times and MS^2 fragmentation patterns were used for identification with the help of authentic reference standards; trans-ABA was from OlChemIm (Olomouc, Czech Republic) and (±)-ABA was from Sigma (Deisenhofen, Germany). Phaseic acid (PA) and dihydroxy phaseic acid (DPA) were identified according to their retention times and MS^2 fragmentation patterns as described (Owen and Abrams 2009).

Results

PSY paralogs of cassava

In the absence of sequence information on cassava carotenoid biosynthetic genes, we used RT primers designed to conserved regions of *PSY* cDNAs to clone a cDNA fragment of 400 bp length from cassava leaf RNA (landrace CM 3306-4). Southern blot analysis using this fragment as a probe revealed two to three bands in most genomic DNA digests, thus suggesting the presence of at least two *PSY* genes (Fig. 1a). Using the primer set from above, two genomic fragments of different sizes were amplified by PCR, corresponding to two cassava *PSY* genes, *MePSY1* and *MePSY2* (see Fig. 1b for primer positions). We determined the genomic sequences of these *MePSYs* [Fig. 1b; sequences are deposited in GenBank with Accession No: *MePSY1*, GU111714 (genomic), GU111719 (mRNA), *MePSY2*, GU111715 (genomic), GU111720 (mRNA)]. However, the restriction patterns did not always match with the hybridization patterns observed upon Southern blot analysis.

A cassava genome assembly became available at that time of this research (http://www.phytozome.net/cassava.php, version 1.1). BLAST analyses using the cassava *PSY* sequences obtained revealed the presence of an additional

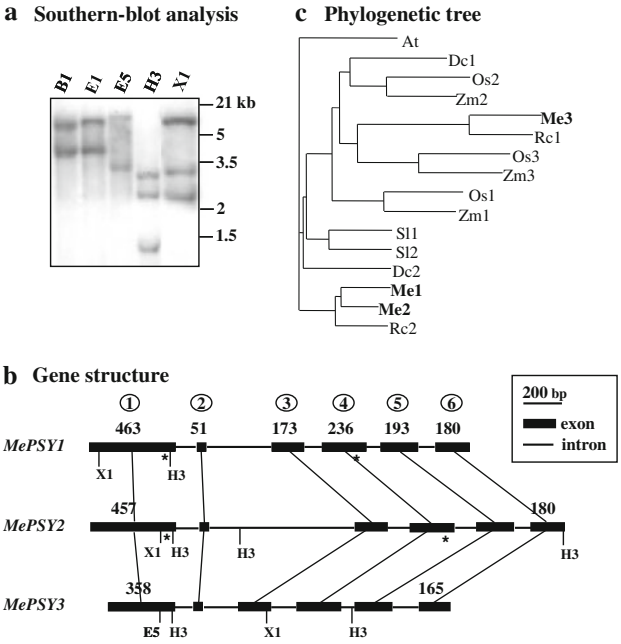


Fig. 1 Three *PSY* genes in cassava: Southern blot analysis, gene structures and phylogenetic analysis. **a** Southern blot analysis with cassava (landrace CM 3306-4). A PCR product from *MePSY1* of 400 bp was used as a probe. DNA sizes of lambda DNA, digested with *Eco*RI/*Hin*dIII are indicated on the *right*. *B1*, *Bam*HI; *E1*, *Eco*RI; *E5*, *Eco*RV; *H3*, *Hin*dIII; *X1*, *Xba*I. **b** Gene structures of cassava *PSY1, PSY2 and PSY3*. Exon/intron structures for *MePSY1* and *MePSY2* were deduced from comparisons of transcript with genomic sequences while those for *MePSY3* were predicted using FGENESH. The *numbers* above exons indicate exon sizes in base pairs; exons 2–5 had similar sizes in all *PSY* genes. Positions of restriction sites used in the Southern blot analysis are indicated; the position of the primer pair used to amplify genomic fragments of *MePSY1* and *MePSY2* is denoted with *asterisks*. **c** A phylogenetic tree of selected PSY amino acid sequences was calculated using the neighbor-joining algorithm (Saitou and Nei 1987). Abbreviations and GenBank accession numbers are as follows: *At A. thaliana*, NP_197225; *Dc D. carota*, 1: Q9SSU8, 2: ABB52068; *Os O. sativa*, 1: NP_001058647, 2: ABA99494, 3: ACI62767; *Zm Zea mays*, 1: AAR08445, 2: AAX13807, 3: ABD1761; *Rc R. communis*, 1: XP_002527067, 2: XP_002532975; *Sl S. lycopersicum*, 1: EF534740, 2: EF534738

third *PSY* gene (*MePSY3*; scaffold information for all *PSY* genes is given in "Materials and methods").

Exon/intron was predicted for *MePSY1* and *MePSY2* by comparing genomic sequences with transcript sequences (Fig. 1b). For *MePSY3*, exon/intron was predicted using the program FGENESH. All the three cassava genes showed an identical number of introns/exons with similar sizes, except for exon 1, which was only 358 bp in *MePSY3* while it was about 460 bp in *MePSY1* and *MePSY2*.

MePSY1 and MePSY2 shared 88% amino acid sequence identity, which is relatively high when compared to other PSY paralogs (see Supplemental Fig. S1 for amino acid alignment). In contrast, the amino acid identity between these two cassava PSYs and MePSY3 was only about 50%. Main sequence differences were found at the N-terminal

150 amino acids containing the transit peptide known for its low degree of sequence conservation (Bruce 2001). However, various regions of sequence divergence (compared to the consensus sequence determined for PSY proteins of other taxa) were also present in the remaining MePSY3 peptide sequence.

A phylogenetic analysis of the MePSY protein sequences identified revealed a branch harboring MePSY1 and MePSY2 together with one PSY homolog from *Ricinus communis*, representing a member of the Euphorbiaceae family, like cassava (Fig. 1c). In contrast, MePSY3, and a second PSY from *Ricinus*, formed a different branch together with specifically stress-induced *PSY3* homologs from the Poaceae. All three cassava PSYs exhibited enzymatic functionality in assays for recombinant protein activity (data not shown).

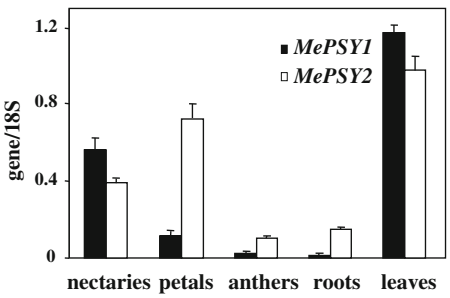
Expression of *PSY1* and *PSY2* in cassava tissues

For all the three cassava genes, TaqMan probes were designed and used to determine their transcript amounts in roots, leaves, and different flower organs by Real-Time RT-PCR (Fig. 2a). For *MePSY1* and *MePSY2*, leaves showed the highest transcript levels with very similar relative ratios for both *MePSYs*, suggesting an equal contribution to photosynthesis-related carotenoid formation. Roots and anthers showed much lower levels; however, *MePSY2* transcripts dominated quantitatively. A similar dominance of *MePSY2* over *MePSY1* was found in cassava petals although at much higher expression which is unexpected, since cassava petals are white. The nectaries of cassava contain carotenoids like in tobacco (Mann et al. 2000) and here, *MePSY1* and *MePSY2* appeared to contribute almost equally to carotenoid formation.

In contrast, transcripts of *MePSY3* were virtually absent in these tissues as concluded from their very late amplification in Real-Time RT-PCR analyses with all primer combinations used. A comparison of C_t values obtained for the three cassava *PSY*s is shown in Fig. 2b.

In summary, transcript levels of *MePSY2* dominate those of *MePSY1* in petals, anthers, and roots, suggesting a major role of MePSY2 in determining the basic carotenoid content in these tissues. However, the high carotenoid content in leaves and nectaries suggests the presence of additional biosynthetic capacity, provided by MePSY1. Evidently, *MePSY3* did not contribute to carotenoid formation in any of the tissues investigated.

a **Expression level**



b **Ct values**

Organ/Tissue	*MePSY1*	*MePSY2*	*MePSY3*	*MePSY3*-S
Nectaries	27.2 ± 0.4	27.0 ± 0.2	36.3 ± 0.5	39.5 ± 0.3
Petals	27.5 ± 0.3	25.0 ± 0.3	34.8 ± 0.8	34.6 ± 0.4
Anthers	30.7 ± 0.3	29.2 ± 0.4	35.9 ± 0.2	36.1 ± 0.9
Roots	30.5 ± 0.4	28.1 ± 0.2	36.7 ± 0.8	36.9 ± 0.3
Leaves	24.8 ± 0.6	25.2 ± 0.3	36.5 ± 0.7	36.0 ± 0.1

Fig. 2 Expression of cassava *PSY* genes in different tissues. **a** Transcript abundances of *MePSY1* and *MePSY2* in different cassava tissues. Tissues were harvested from field-grown cassava plants. RNA was isolated and used for Real-Time RT-PCR. Expression levels are expressed as relative to 18S rRNA levels of the corresponding sample. Data represent the mean ± SD of three independent biological replicates. **b** Cycle threshold (C_t) values ± SD of *MePSY1, MePSY2* and *MePSY3* determined by Real-Time RT-PCR analysis from cassava samples with each comparable 18S rRNA levels. TaqMan probes were used for all three *PSY* genes. For *MePSY3* a different primer combination was used in addition and C_t values were analyzed with SYBR green (*MePSY3*-S)

MePSY1 and *MePSY2*, but not *MePSY3* respond towards salt-stress treatment

For investigation of abiotic stress responses, cassava plants (landrace MPER183) were grown in vitro for 6 weeks and subjected to salt stress by transferring them to a 250 mM NaCl solution (in propagation medium). Roots and leaves were harvested at various times and analyzed by Real-Time RT-PCR; concomitantly ABA levels were determined by LC–MS.

MePSY3 transcripts did not respond to salt-stress treatment in roots and leaves, remaining at the detection limit although this PSY groups with stress-responsive homologs of the Poaceae. In contrast, *MePSY1* and *MePSY2* showed comparable transcript induction levels in roots and increased sixfold at the sixth hour of salt-stress application relative to the untreated controls (Fig. 3a). Untreated roots contained 0.5 nmol ABA per gram DW, which increased fourfold after 6 h, followed by a decline. Therefore, the kinetics of the onset of ABA formation followed the expression levels of both *MePSYs* in roots but was not correlated with the decline in ABA levels, which occurred after the sixth hour (see below).

In leaves, *MePSY1* showed a much weaker, ca. 1.5-fold induction relative to the untreated controls, while *MePSY2* did not respond (Fig. 3b). The ABA levels were at 1.5 nmol ABA per gram DW at the onset of salt stress and increased linearly to ca 7 nmol g^{-1} DW after 12 h

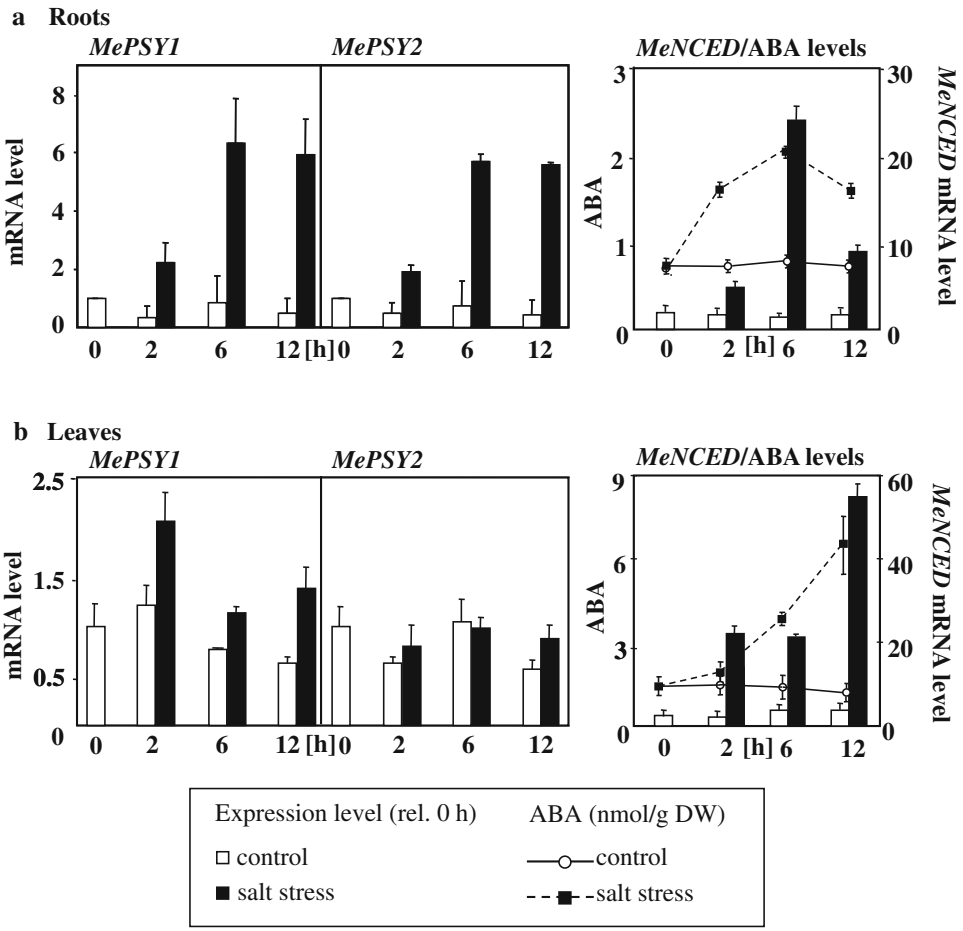


Fig. 3 *MePSY1, MePSY2* and *MeNCED* expression and ABA levels in cassava plants following salt stress treatment. Cassava plants grown in vitro on propagation medium were subjected to salt stress with 250 mM NaCl in propagation medium. Control plants were transferred into fresh propagation medium. Roots (**a**) and leaves (**b**) were harvested at the times given for ABA quantification and the determination of *MePSY1*, *MePSY2* and *MeNCED* transcript levels using Real-Time RT-PCR. Expression levels were normalized to 18S rRNA levels of the corresponding samples and expressed relative to levels detected in untreated roots or leaves, respectively (0 h). ABA contents were determined by LC–MS using internal standardization with d6-ABA. Data represent the mean ± SD of three independent biological replicates

treatment. Thus, while a strong induction of both *MePSY1* and *MePSY2* led to moderately increased ABA levels in roots, much weaker induction rates of *MePSY1* alone was sufficient to achieve much higher ABA levels in leaves. In an interpretation, the low carotenoid content in roots necessitated the transcriptional upregulation of *PSYs* to push the pathway, while this is not so much required in leaves with their high xanthophyll (ABA-precursor) content. In roots, both MePSY1 and MePSY2 are needed to achieve adequate levels of ABA-precursor carotenoids.

Cassava *PSY* induction upon salt stress correlates with kinetics of both *NCED* transcripts and ABA levels

NCEDs, catalyzing the cleavage of carotenoids are considered to be rate-limiting in ABA formation. We used *Arabidopsis NCED* sequence information of those paralogs known to respond towards abiotic stress to identify cassava ESTs (*NCED3*, AGI No. At3g14440; *NCED5*, AGI No. At1g30100; Iuchi et al. 2001). Blast searches and EST assembly revealed two contigs composed of cassava ESTs, one of which showed highest sequence identity to *AtNCED3* (contig 1: 75%, contig 2: 34%) comparing its deduced amino acid sequence with those of *Arabidopsis* CCDs (see Supplemental Fig. S2). Real-Time RT-PCR using RNA from salt-treated cassava samples confirmed that only transcripts corresponding to contig 1 (here named *MeNCED*) showed strong induction rates while there was no response with the contig 2 mRNA. As shown in Fig. 3, *MeNCED* induction rates correlated with the time course of ABA formation both in roots and leaves. *MeNCED* transcript levels also determined the drop of ABA levels in roots while *MePSY* transcript levels were still at maximum, corroborating the rate-limiting function of NCED at adequate activity of the carotenoid biosynthetic pathway.

Drought stress leads to a different response

Drought represents the abiotic stress that cassava is known to be able to deal with in the first place. Therefore, we extended our analysis to water-stress conditions and used an established protocol used to assess drought-susceptible and drought-tolerant cassava varieties (Sundaresan and Sudhakaran 1995). This method uses the widely used polyethylene glycol (PEG)-mediated reduction of water potential (e.g. Pilon-Smits et al. 1995). For this, in vitro

grown cassava plants were transferred to 40% (w/v) PEG 6000 in propagation medium and analyzed as above (Fig. 4).

Similar to salt stress, *MePSY3* transcripts also did not respond towards drought stress treatments. For *MePSY1* and *MePSY2*, PEG-induced drought stress led to much weaker *MePSY* induction rates in roots as compared to salt stress (Fig. 4a), but again with *MePSY1* showing a somewhat higher participation as compared to *MePSY2*. The ABA levels followed this time course, but at lower levels as expected, given that *PSY* expression is rate-limiting for ABA formation in roots. Again, *MeNCED* transcript levels correlated with ABA formation, but reached only about 50% of those detected under salt stress conditions.

In contrast to salt-stressed leaves, ABA levels in drought-stressed leaves changed pronouncedly but in a biphasic manner. A moderate rise in ABA levels, about threefold up to the sixth hour was followed by a burst of ABA formation thereafter. A large difference as compared to salt stress is that the *MeNCED* transcript level did not follow this kinetic at all, increasing 80-fold much earlier, already 2 h after drought stress application, and then gradually declining.

As expected from the salt stress data, concomitant upregulation of *MePSY* transcript levels was evidently not required, given the high carotenoid precursor availability in leaves.

Detached cassava leaves show pronounced response towards drought stress

The picture on tissue and stress-specific differences noted above might be blurred by the known involvement of root-to-shoot ABA signaling (Christmann et al. 2007; Jiang and Hartung 2008). Moreover, stress conditions applied to the roots of complete plants may not have led to the full establishment of a systemic stress situation. This led us to analyze the leaf response separately. For this, we subjected detached leaves from in vitro grown cassava plants to salt or drought stress by incubating them in propagation medium containing 250 mM NaCl or 40% (w/v) PEG 6000, respectively. Control leaf samples were incubated in the same medium without salt or PEG (Fig. 5a, b).

In essence, the stress-specific characteristics in the response kinetics of *MeNCED* transcript and ABA levels were maintained but at much higher levels. Like with "on

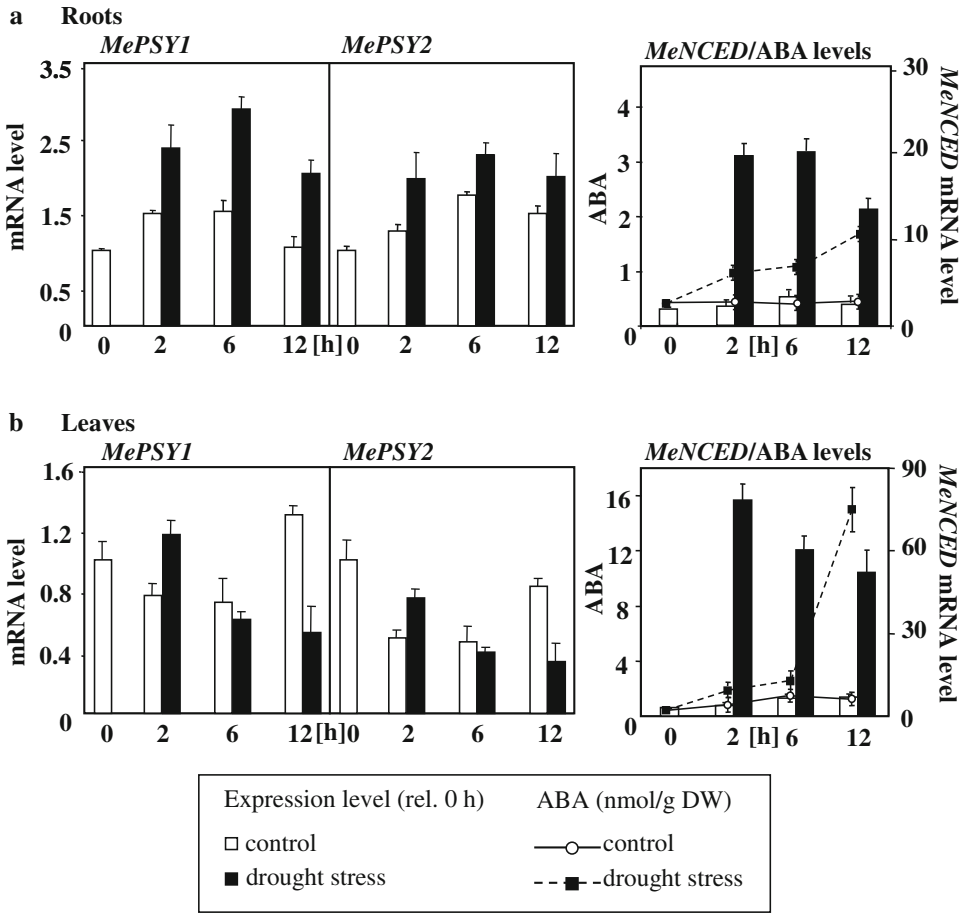


Fig. 4 *MePSY1, MePSY2* and *MeNCED* expression and ABA levels in cassava plants following drought stress treatment. Cassava plants grown in vitro on propagation medium were subjected to drought stress with 40% (w/v) PEG 6000 in propagation medium. Control plants were transferred into fresh propagation medium. Roots (**a**) and leaves (**b**) were harvested at the times given for ABA quantification and the determination of *MePSY1*, *MePSY2* and *MeNCED* transcript levels using Real-Time RT-PCR. Expression levels were normalized to 18S rRNA levels of the corresponding samples and expressed relative to untreated roots or leaves, respectively (0 h). ABA contents were determined by LC–MS using internal standardization with d6-ABA. Data represent the mean ± SD of three independent biological replicates

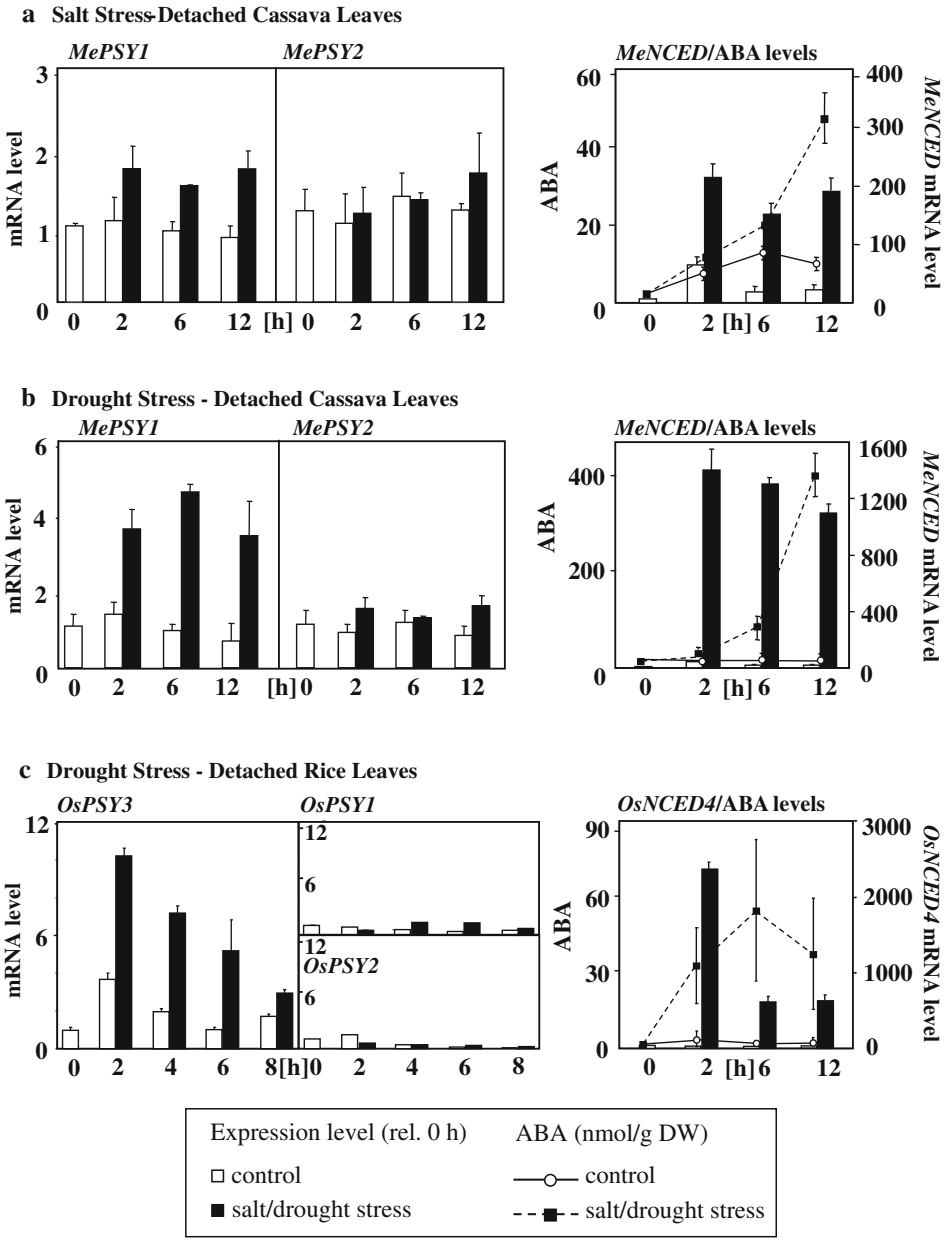


Fig. 5 *PSY, NCED* expression and ABA levels in detached cassava and rice leaves following salt and drought stress treatment. Leaves from cassava plants grown in vitro were detached and incubated in 250 mM NaCl (**a**) or 40% (w/v) PEG 6000 in propagation medium (**b**), for the times indicated. Control plants were transferred into fresh propagation medium and harvested at the same times. **c** Rice leaves were detached and subjected to drought stress by incubation in 40% (w/v) PEG 6000 in mineral solution; control plants were transferred into mineral solution only. ABA levels were quantified using LC–MS using d6-ABA as an internal standard and transcript levels were determined using Real-Time RT-PCR. Transcript levels were normalized to the 18S rRNA levels of the corresponding samples and expressed relative to those detected in the untreated sample (0 h). *Data* represent the mean ± SD of three independent biological replicates

plant" leaves, salt stress led to linearly increasing ABA levels reaching up to 50 nmol g^{-1} DW (compared to 6 nmol g^{-1} DW, see Fig. 3b), driven by an accordingly higher, namely 200-fold *MeNCED* induction (compared to ca. 60-fold "on plant"). Predominantly *MePSY1* levels contributed to ABA formation, which seems required at such elevated rates of ABA formation.

Drought conditions led to very high levels of ABA reaching 400 nmol g^{-1} DW after 12 h (compared to ca. 15 nmol g^{-1} DW, see Fig. 4b). This high ABA level was sustained to the sixteenth hour (not shown). Like with "on plant" leaves, ABA formation was biphasic increasing moderately relative to the burst of ABA formation after the sixth hour (compare Figs. 5b and 4b: mind the different ordinate scaling). This is preceded by a very large induction of *MeNCED* at the second hour followed by a shallow decrease, maintaining high levels. Evidently, such high demand for ABA-precursor carotenoids necessitates *MePSY* upregulation, even in leaves. Figure 5b corroborates the tendency shown in all previous experiments that MePSY1 is the drought responsive paralog.

Control samples showed an about tenfold increase in ABA formation, correlating with a slight induction of *MeNCED* (but not of *MePSY*) transcript levels which is most probably due to the known phenomenon of wounding-induced ABA (Reymond et al. 2000).

We wondered whether such massive ABA formation corresponding to about 6% of the total carotenoids

present in cassava leaves might affect leaf carotenoid amounts. HPLC analysis revealed that carotenoids remained unchanged until 6 h of drought stress, but were reduced after 12 and also 16 h (see Supplementary Table S1). This was mainly due to reduced violaxanthin and neoxanthin levels, but also of lutein, which is not an ABA-precursor. In contrast, detached leaves subjected to salt stress with their much lower *MeNCED* induction rates showed an unchanged carotenoid content and pattern. Because the very high ABA levels are accompanied by a notable drop in carotenoids, the most likely interpretation is that cassava possesses a yet unidentified NCED paralog that is drought-specific and does not respond to salt stress.

Response of detached rice leaves towards drought stress

The distinct response of cassava leaves towards drought might represent a cassava-specific environmental adaptation. Using the same protocol as above, we determined the response of detached leaves from a drought-sensitive species, rice (Fig. 5c). This induced ABA levels at the 6th h almost similar to those in detached cassava leaves (55 nmol g^{-1} DW vs. 80 nmol g^{-1} DW, respectively); however, this appeared as a peak which followed closely the induction kinetic of *OsNCED4*. Neither was a biphasic time course of ABA formation found nor were ABA levels sustained beyond the twelfth hour like in cassava leaves. Similarities were only revealed upon monitoring the expression pattern of the three rice *PSY* paralogs. Like in cassava leaves, the formation of large ABA levels was supported by *PSY* induction; however, this was restricted to the stress-responsive paralog *OsPSY3*, as expected (Welsch et al. 2008).

Two ABA breakdown products, PA and DPA, were investigated by LC–MS (see Supplementary Fig. S3). These were found to be correlated with the ABA levels in drought-stressed cassava and rice leaves; however, DPA was not detected in cassava.

Discussion

The *PSY* genes of cassava

Poaceae like maize and rice contain three gene copies coding for *PSY*, the enzyme that is thought to represent the rate-limiting step in the carotenoid biosynthetic pathway in most plant tissues. According to recent findings, one of these paralogs (*ZmPSY3* and *OsPSY3*) is specialized in supporting carotenogenesis under abiotic stress conditions to enable enhanced ABA formation in each of these species (Li et al. 2008; Welsch et al. 2008).

In cassava, a similar situation is given at first glance. Similar to Poaceae, three *PSY* genes are present, one of which does not contribute to carotenoid biosynthesis in the tissues analyzed, like leaves, flower organs and roots. However, in contrast to rice and maize, cassava *PSY3* did not respond at all towards salt or drought stress treatment. Stress-induced support of ABA synthesis does also occur in cassava, but is achieved by the induction of the other two *PSY* genes present, with a preferential involvement of *MePSY1* (see below).

The virtual absence of *MePSY3* transcripts raises questions on the functionality of the gene and gene product. The derived amino acid sequence of MePSY3 exhibits frequent deviations at highly conserved positions (see amino acid alignment in Supplemental Fig. S1). However, a gene product from the closely related species *Ricinus* (like cassava a member of the Euphorbiaceae) is about 80% identical to MePSY3 and shares similar deviations from the consensus sequence. Because subtle differences in the amino acid sequence of PSY can have profound effects (Paine et al. 2005), it is therefore necessary to define the function of MePSY3 in terms of its enzymatic properties and the conditions required for expression.

Under normal growth conditions the remaining two cassava *PSY* genes are almost equally expressed in leaves and at highest levels of all tissues examined suggesting a shared contribution to leaf carotenoid biosynthesis. High transcript levels of both *MePSYs* were also observed in the orange-colored nectaries containing carotenoids, like in tobacco (Mann et al. 2000). In contrast, MePSY2 appears to mainly control carotenoid biosynthesis in roots, anthers and petals. In the latter tissue, *MePSY2* transcript levels approached those in leaves although they are white. It remains to be determined whether carotenoid accumulation is not possible because of their rapid degradation, like in chrysanthemum petals where the knock-down of the carotenoid cleavage oxygenase *CCD4a* led to coloration (Ohmiya et al. 2006).

Cassava *PSY* expression under stress conditions

In roots of entire cassava plants, both *MePSY1* and *MePSY2* reacted towards salt stress almost equally with pronounced transcript upregulation (Fig. 3a). Both *MePSYs* were also found upregulated when roots were drought-stressed by PEG treatment (Fig. 4a). In contrast, only a marginal upregulation was met with "on plant" leaves under both stress conditions (Figs. 3b, 4b), maybe with some higher participation of *MePSY1*. This difference in magnitude of the response may be interpreted in terms of carotenoid precursor requirement, which is very high in roots, which are almost carotenoid-free and low in carotenoid-rich leaves. With respect to the relative contribution

of the two *PSY* genes one might speculate that in roots both *PSYs* are required to drive carotenogenesis to adequate levels, while with "on plant" leaves the system was not sufficiently challenged to the point where ABA-precursor carotenoids become limiting.

Applying PEG-mediated drought stress to detached leaves directly (Fig. 5a, b) drove the system into such a situation yielding ABA levels which accounted for a substantial fraction of the xanthophylls present in leaves (about 6% of the total carotenoid amount; see Supplementary Table S1). This clarified that in fact, MePSY1 was the primary driver in leaves without any notable contribution of MePSY2. Accordingly, salt stress, directly applied to detached leaves (Fig. 5a) defined more robustly than with "on plant" leaves (compare Fig. 3b) *MePSY1* as the drought and salt stress responsive *PSY* form. Thus, in cassava, *PSY1* is exclusively stress responsive with the exception of roots, where the reserve is called into action in the form of *PSY2* to cope with the high demand for ABA in carotenoid shortage. If correct, PSY can determine the rate of ABA formation in roots but not in leaves (see below).

In detached leaves of cassava as well as rice, drought stress response is restricted to only one of the *PSY* genes, namely *MePSY1* or *OsPSY3*, respectively. These findings call for the presence of a common feedback loop linking ABA levels or ABA-precursor xanthophyll levels to *PSY* expression rates. The nature of this mediator which necessarily needs to be able to leave the plastid is not known.

Relation of *PSY* induction to ABA formation: the role of NCED

NCED3, catalyzing the cleavage of carotenoids, has been found to be rate-limiting for ABA formation in *Arabidopsis* (Iuchi et al. 2001). A close *AtNCED3* homolog of cassava responded to abiotic stress conditions and was used to analyze its expression relative to *MePSYs* and to ABA levels.

When whole plants were used, the induction of *MeNCED* occurred simultaneously with both *MePSYs* upon salt and drought stress treatment in roots and correlated with the ABA levels formed (Figs. 3a, 4a). However, compared to leaves, the *MeNCED* levels attained were small. ABA formation thus appears as a concerted action of both *MePSY* genes and *MeNCED* in roots so that there is no clear "pacemaker". Only in salt-stressed roots, the downregulation of *MeNCED* at the twelfth hour (Fig. 3a), followed by decreased ABA levels indicates dominance at this point and rapid ABA turnover.

In leaves, regardless whether "on plant" or detached, it appears that MeNCED, not the upregulated MePSY1 is determining. This is judged by the tremendously higher induction rates of *MeNCED* in the range of 100-fold as compared to two- to fourfold for *MePSY1*. Moreover, ABA levels followed the *MeNCED* expression levels; however, the kinetic modalities were different. While under salt stress, *MeNCED* induction was accompanied by increased ABA levels in an almost co-linear fashion (Figs. 3b, 5a), this was not observed under drought conditions. Under drought stress, *MeNCED* transcript levels responded very promptly and pronouncedly (much faster and stronger than under salt stress) reaching highest levels (Figs. 4b, 5b). ABA levels rose biphasically to first intermediate (sixth hour) and then to very high levels both in detached as well as "on plant" leaves. High *MeNCED* transcript levels and ABA abundance were then maintained up to the sixteenth hour (data not shown). This is in marked difference to the situation found with detached rice leaves, where the response of *OsNCED4* and ABA appeared rapidly and almost synchronously but as a "peak".

It may be speculated that the inability of rice leaves to sustain the signal over longer periods of time may be a component of its low drought tolerance while cassava, known for its ability to withstand drought conditions, may have developed this sustained response. It is not clear how, in cassava leaves, the biphasic ABA response under drought stress is regulated. A possible downregulation of ABA catabolism with a reversed activity relative to MeNCED (i.e. high ABA catabolism during the initial response upon drought stress and low within later phase) is not involved since both PA and DPA, the two most widespread ABA catabolites, approximately followed the time course of ABA formation (see Supplementary Fig. S2).

Drought and salinity are thought to share common principles. While drought stress results in dehydration, salt stress lead to hyper-ionic conditions in addition; consequently salt stress affects more genes and more intensely (Chaves et al. 2009). However, our data indicate that at least at the level of ABA synthesis, cassava differentiates clearly between both types of stress by sustaining ABA formation at high levels over longer periods of time and with the support of a specialized *PSY* gene.

The need for enhanced carotenoid precursor supply to enhance ABA formation led to the development of stress-inducible *PSY* paralogs. The results presented suggest a wider occurrence of stress-responsive *PSY* genes beyond grasses. However, different *PSY* homologs were recruited for this purpose and provided with modalities of induction not necessarily relying on the closest homolog across taxa.

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