**PSY3, a New Member of the Phytoene Synthase Gene Family Conserved in the Poaceae and Regulator of Abiotic Stress-Induced Root Carotenogenesis**

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Abscisic acid (ABA) plays a vital role in mediating abiotic stress responses in plants. De novo ABA biosynthesis involves cleavage of carotenoid precursors by 9-cis-epoxycarotenoid dioxygenase (NCED), which is rate controlling in leaves and roots; however, additional bottlenecks in roots must be overcome, such as biosynthesis of upstream carotenoid precursors. Phytoene synthase (PSY) mediates the first committed step in carotenoid biosynthesis; with PSY3 described here, maize (Zea mays) and other members of the Poaceae have three paralogous genes, in contrast to only one in Arabidopsis thaliana. PSY gene duplication has led to subfunctionalization, with each paralog exhibiting differential gene expression. We showed that PSY3 encodes a functional enzyme for which maize transcript levels are regulated in response to abiotic stresses, drought, salt, and ABA. Drought-stressed roots showed elevated PSY3 transcripts and ABA, responses reversed by rehydration. By blocking root carotenoid biosynthesis with the maize y9 mutation, we demonstrated that PSY3 mRNA elevation correlates with carotenoid accumulation and that blocking carotenoid biosynthesis interferes with stress-induced ABA accumulation. In parallel, we observed elevated NCED transcripts and showed that, in contrast to dicots, root zeaxanthin epoxidase transcripts were unchanged. PSY3 was the only paralog for which transcripts were induced in roots and abiotic stress also affected leaf PSY2 transcript levels; PSY1 mRNA was not elevated in any tissues tested. Our results suggest that PSY3 expression influences root carotenogenesis and defines a potential bottleneck upstream of NCED; further examination of PSY3 in the grasses is of value for better understanding root-specific stress responses that impact plant yield.

Abiotic stresses, such as water deficit, salinity, and high or low temperatures, have profound negative effects on plant growth; such stresses are the primary causes of crop productivity losses (Bray et al., 2000). For example, during the flowering and silking stages of maize (Zea mays), 4 d of mild drought stress can cause up to a 50% decrease in yield productivity (Classen and Shaw, 1970). Plants have evolved various levels of adaptation in response to stress conditions, and best understood is mediation by the hormone abscisic acid (ABA; Xiong et al., 2002).

Given its important role in plant stress tolerance, regulation of ABA biosynthesis and accumulation is a focal point of research. In higher plants, ABA is derived from the 9-cis-epoxycarotenoids 9-cis-violaxanthin and 9-cis-neoxanthin; these C_{40} compounds are cleaved by 9-cis-epoxy-carotenoid dioxygenase (NCED) to form xanthoxin, a C_{15} intermediate, which is subsequently converted to ABA in two steps of oxidation (see Fig. 1; for review, see Nambara and Marion-Poll, 2005). ABA accumulation is a balance of biosynthesis and catabolism, the latter process primarily regulated by ABA 8′ hydroxylase (ABA8ox; Kushiro et al., 2004; Saito et al., 2004). ABA biosynthesis, as derived from de novo biosynthesis in a given tissue, may be potentially limited by the plastid enzymes involved in producing the carotenoid (epoxycarotenoid) precursors (Li et al., 2007; Matthews and Wurtzel, 2007; Quinlan et al., 2007) and/or the enzymes involved in conversion of specific epoxycarotenoids to ABA (Taylor et al., 2005; Fig. 1); factors affecting flux to ABA may vary according to tissue (photosynthetic versus nonphotosynthetic) and/or to particular species (and possibly plant families), as we demonstrate in this article for maize.

In leaves of dicot and monocot plants, including maize, de novo biosynthesis of ABA is induced by water stress (Sindhu and Walton, 1987). The cloning and characterization of maize Viviparous14, which encodes NCED1, led to identification of NCED as a rate-controlling enzyme in stress-induced de novo biosynthesis of ABA, particularly in leaf tissue (Schwartz et al., 1997; Tan et al., 1997). Similar results were obtained from characterization of other ortholo-
gous genes, *PnNCED1* of bean (*Phaseolus vulgaris*; Qin and Zeevaart, 1999), *VuNCED1* of cowpea (*Vigna unguiculata*; Iuchi et al., 2000), *LeNCED1* of tomato (*Solanum lycopersicum*; Thompson et al., 2000a), and *AtNCED3* of Arabidopsis (*Arabidopsis thaliana*; Iuchi et al., 2001). Stress-induced elevation of NCED transcript levels was observed to precede ABA accumulation (Qin and Zeevaart, 1999), and up- or down-regulation of the gene affected both ABA levels and drought sensitivity (Thompson et al., 2000b; Iuchi et al., 2001). Conversion of the downstream xanthoxin to ABA is not limiting as evidenced by the invariant levels of a requisite cytosolic enzyme activity in comparing normal and water-stressed leaves (Sindhu and Walton, 1987). Similarly, the upstream carotenoid levels are not thought to limit flux to ABA in leaves; leaf epoxycarotenoids are abundant (Parry et al., 1990), and transcripts for zeaxanthin epoxidase (*ZEP,* which converts zeaxanthin to violaxanthin, the precursor for epoxycarotenoids, were shown to be constant or deceased in leaves under drought stress (Audran et al., 1998; Iuchi et al., 2000; Thompson et al., 2000a).

Roots of maize and other plants also respond to osmotic or water stress through elevation of ABA, only some of which is due to increased translocation from other tissues (Rivier et al., 1983; Cornish and Zeevaart, 1985). However, the mechanism for increasing flux to ABA in nonphotosynthetic tissues, such as roots, contrasts with that operating in leaves. ABA epoxycarotenoid precursors are lower in roots as compared with leaves (Parry and Horgan, 1992). Although NCED is a limiting enzyme for ABA biosynthesis in roots, just as it is in leaves (Qin and Zeevaart, 1999; Thompson et al., 2000a), increased rates of carotenoid synthesis in roots may also be necessary for elevating flux to root ABA. Unlike the constant levels seen in leaves, drought stress-induced elevation of root ABA was associated with *ZEP* transcript level increases of 3- to 7-fold and 4-fold in roots of tobacco (*Nicotiana plumbaginifolia*) and tomato, respectively (Audran et al., 1998; Thompson et al., 2000a). Another indication that there is an additional rate-controlling step upstream of NCED that limits flux to ABA in roots is suggested by experiments where transgenic tomatoes were modified for constitutive overexpression of NCED1; transgenic plants showed greater accumulation of ABA in leaves and only a modest increase in root ABA, suggesting that in roots there might be other steps upstream of NCED that limited flux to ABA (Thompson et al., 2007). Therefore, induction of elevated root ABA must also require enhanced levels of NCED precursors to accommodate elevated NCED levels induced under drought stress, but absent in NCED-overexpressing transgenic plants. The observation that stress-induced accumulation of ABA was also associated with elevated transcripts for the nonheme diiron β-carotene hydroxylase (HYD), an enzyme catalyzing hydroxylation of β-carotene to zeaxanthin, suggests that this and possibly other components of the carotenoid biosynthetic pathway may represent putative upstream bottlenecks that must be released to elevate root ABA levels in response to abiotic stress.

To examine the nature of the root ABA bottleneck, we decided to look at maize, an important food crop worldwide, and model for translational genomics in the grass family (Poaceae; Lawrence and Walbot, 2007); insight from study of maize paralogs will be useful in predicting ortholog targets in related grasses, many of which also serve important agronomic roles worldwide. The carotenoid biosynthetic pathway in maize and other grasses is complex compared to Arabidopsis and other dicots; many of the enzymes in the grasses are encoded by small gene families for which much ongoing investigation is focused on elucidating specific roles in carotenogenesis (Wurtzel, 2004; Li et al., 2007; Matthews and Wurtzel, 2007).

The first enzyme in the plastid-localized carotenoid biosynthetic pathway, phytoene synthase (PSY), which is known to control flux to carotenoids in the seed (Gallagher et al., 2004), is nuclear encoded by a small gene family consisting of PSY1 and PSY2, which we had previously shown to exist throughout the Grasses (Gallagher et al., 2004). In the process of searching for orthologs of PSY1 and PSY2 in sorghum (*Sorghum bicolor,* a cultivated species in drought- and salt-stressed environments, we stumbled upon a new and unrelated PSY gene, PSY3. The sorghum *PSY3* gene led us to the rice (*Oryza sativa*) ortholog, from which synteny with maize provided a strategy to identify a syntenic chromosome region harboring the maize *PSY3* gene.

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**Figure 1.** Carotenoid and ABA biosynthetic pathways in higher plants. Left, Carotenoid precursors of carotenones to ABA. Enzymes are bold: LCYE, lycopene ε-cyclase; CYP97A, P450 carotene β-ring hydroxylase; VDE, violaxanthin de-epoxidase; ABA2, short-chain alcohol dehydrogenase; AA03, ABA oxidase.
PSY3 cDNAs that we identified in the public databases were primarily associated with abiotic stress, suggesting that the PSY3 gene might play a role in regulating carotenoid flux in response to stress. We therefore tested this possibility and showed that, indeed, PSY3 gene expression represented at least one bottleneck in controlling flux to carotenoid precursors that are required for elevating ABA in maize roots.

RESULTS

Isolation of the Third PSY Gene Paralog from Maize, Rice, and Sorghum

We previously showed that PSY was encoded by two paralogs, PSY1 and PSY2, in 12 species across eight subfamilies of the grasses (Poaceae; Gallagher et al., 2004) in comparison to a single PSY gene in Arabidopsis; we also demonstrated enzymatic functions for maize PSY1 and PSY2 and rice PSY2. While attempting to expand PSY studies in sorghum, which is an important cereal crop in Africa and other parts of the world, we discovered a novel PSY cDNA, which we termed PSY3. Sorghum PSY3 (GenBank accession no. BG464544) has a unique 3’ end distinguishing it from sorghum PSY1 and PSY2 homologs. To rule out the possibility that sorghum was unusual among the grasses in having a third gene, we used BLAST analysis to test whether a homolog even existed in rice because earlier genome data had not revealed any additional genes; we did find a rice PSY3 gene located on chromosome 9 (Gramene ID LOC_Os09g38320) and six rice ESTs in GenBank. For maize, we identified only one genomic contig (The Institute for Genomic Research ID AZM4_60808), which contained an incomplete maize PSY3 gene, but no maize ESTs were found. To clone the maize PSY3 gene, we exploited synteny between maize and rice and used flanking markers to identify the maize syntenic region in maize bin 7.03 near umc1865 (Fig. 2); bacterial artificial chromosome (BAC) clones in this region were screened by PCR to identify one containing the maize PSY3 gene, which was sequenced by primer walking (GenBank accession no. DQ372936; see details in “Materials and Methods”). To facilitate gene annotation and later functional analyses, we used reverse transcription (RT)-PCR to clone full-length PSY3 cDNAs for maize and rice (as described in “Materials and Methods”). The cDNA clones containing the sorghum PSY1 (GenBank accession no. CD234165) and PSY3 (GenBank accession no. BG46454) genes were requested from the Comparative Grass Genomics Center. Analysis of genomic and cDNA sequences revealed conserved gene structure between PSY3 as compared to PSY1 and PSY2 in the grass family, as well as with Arabidopsis PSY (Fig. 3). All PSY genes possess six exons and five introns, except sorghum PSY3, whose third and fourth exons are fused. All of these PSY genes have long first exons and a very short second exon. With the exception of the sorghum-fused PSY3 exons, the sizes of the second, third, fourth, and fifth exons of all three groups of PSY genes in the Poaceae and Arabidopsis are identical with sizes of 51, 173, 236, and 193 bp, respectively. In addition, all PSY1s have a small first intron (approximately 100 bp) and a large second intron (>600 bp), but all PSY2s have a large first intron (approximately 400 bp) and a small second intron (approximately 100 bp; Fig. 3).

The deduced protein sequences for all three PSY genes of maize, rice, and sorghum were determined and used for phylogenetic analysis (Fig. 4A). This analysis showed that all PSY3 proteins belong to a novel group, whereas the PSY1 group in monocot species in the grass family is most closely related to the
PSY in the dicot plant Arabidopsis. PSY3 proteins possess a distinct domain found at the carboxyl terminal, R(H/R)XS(S/T)LT, a motif that separates these proteins from the members of the PSY1 group with the SLRNXQ(T/K) motif and PSY2 with the ARAAVAS(S/P) motif (Fig. 4B); H/R are charged amino acids, and S/T are polar but uncharged amino acids. All PSY3 proteins possess transit peptides for chloroplast targeting as predicted by the ChloroP 1.1 server (Emanuelsson et al., 1999). Maize PSY3 was predicted to be 47.3 kD (426 residues), having a 51-residue transit peptide and processed to a 41.9-kD (375 residues) mature plastid protein; rice PSY3 was predicted as 49.3 kD (444 residues) and having a 54-residue transit peptide and processed to a 43.5-kD (390 residues) mature plastid protein. Sorghum PSY3 was predicted as 48.9 kD (441 residues) with a 57-residue transit peptide and processed to a 42.7-kD (384 residues) mature protein. When comparing the processed and plastid-localized PSY proteins, the 41.9-kD maize PSY3 is expected to be larger by 2 kD at the N terminus as compared to maize PSY1 (39.8 kD) and PSY2 (39.4 kD). The unconserved first exon of the PSY genes encodes the PSY transit peptide and the N-terminal 2-kD extension in the case of PSY3 proteins; the last gene exon, which also lacks conservation among PSY genes, encodes the distinguishing C-terminal PSY motif (Fig. 4B).

PSY3 Encodes a Functional PSY

We used heterologous functional complementation to verify whether the novel PSY3 proteins were functional (Gallagher et al., 2003, 2004; Matthews et al., 2003; Quinlan et al., 2007). Maize PSY3 and sorghum PSY1 and PSY3 cDNAs were subcloned into the pET23 expression vector (Novagen) and transformed into Escherichia coli harboring pACCAR25ΔcrtB, which carries a bacterial carotenoid gene cluster missing the bacterial PSY gene crtB (Misawa et al., 1990). Cells produce the pathway end product, zeaxanthin diglucoside, only when a functional PSY enzyme is present; a peak corresponding to this end product is seen in an HPLC chromatogram of extracted carotenoids from positive control cells containing the entire carotenoid cluster, including bacterial PSY (pACCAR25; Fig. 5A), but not when the empty vector only is cotransformed with the PSY deletion construct, pACCAR25ΔcrtB (Fig. 5B). HPLC analysis of carotenoid extracts from transformants containing either maize PSY3 or sorghum PSY1 and PSY3 showed a peak corresponding to zeaxanthin diglucoside (Fig. 5, C–E), which matches in retention time and spectrum that seen for the positive control (Fig. 5A). These results indicate that maize PSY3 and sorghum PSY1 and PSY3 all encode functional enzymes.

Maize PSY3 Is Mainly Expressed in Root and Embryo Tissue

To assess the role of maize PSY3, its tissue specificity was investigated with quantitative RT-PCR using endosperm and leaf tissues, where carotenoids generally accumulate to visible levels, and root and embryos, where carotenoids are barely detectable (Fig. 6). In leaf
and endosperm tissue, maize PSY3 mRNA levels were 4- to 5-fold lower than those of PSY2 and 10- to 15-fold lower than those of PSY1, which is consistent with the semiquantitative data we previously reported (Gallagher et al., 2004). In contrast, maize PSY3 mRNAs represented the most prevalent PSY transcript in roots, being about 4-fold higher than that of PSY2 and 10-fold higher than that of PSY1 (Fig. 6). In embryo, transcript levels of maize PSY2 and PSY3 were 3- and 2-fold higher than that of maize PSY1, respectively. The observed abundance of PSY3 transcripts in tissues that accumulate little colored carotenoids suggests that PSY3 may have a unique role in maize instead of merely being a redundant copy.

Maize PSY2 and PSY3 mRNA Levels Up-Regulated by Drought

We used E-northern from the National Center for Biotechnology Information (NCBI) to reveal possible factors that might influence PSY transcript levels as indicated by the source of abundant ESTs. We identified only six PSY3 ESTs from rice, but none from maize; as described in “Materials and Methods,” five were related to the ABA pathway or associated with plants subjected to drought conditions. E-northern

data suggested that PSY3 in the grass family may be involved in drought stress or in regulation of ABA biosynthesis under abiotic stresses. To test these hypotheses, we subjected maize seedlings to various abiotic stresses and measured PSY3 transcript levels in comparison to transcript levels for PSY1 and PSY2.

To test for the effect of drought stress on PSY transcript levels, maize seedlings were subjected to drought conditions at the five-leaf stage. After 4 d of

Figure 4. PSY1, PSY2, and PSY3 protein sequence comparisons. A, Phylogenetic analysis of amino acid sequences. B, Conserved carboxyl termini of PSY proteins. GenBank accessions are in parentheses. Arabidopsis (AtPSY AAA32836), rice (OsPSY1, AAS18307; OsPSY2, AAK07735; OsPSY3, DQ356431), sorghum (SbPSY1, AY705389; SbPSY2, AW679367; SbPSY3, AY705390), maize (ZmPSY1, P49085; ZmPSY2, AAC91837; ZmPSY3, DQ356430). Amino acid sequences were aligned using ClustalW and a neighbor-joining tree was constructed with a 500-bootstrap replication support using MEGA3 software (Kumar et al., 2001). The carboxyl-terminal conserved domain of each PSY group was identified manually after alignment. Black highlighting indicates identical residues; gray denotes similar residues.

Figure 5. Functional complementation of PSY3 from maize and sorghum. E. coli cells were transformed with pACCAR25 (A); pACCAR25ΔcrtB + pET23a (empty vector; B); pACCAR25ΔcrtB + maize PSY3 (C); pACCAR25ΔcrtB + sorghum PSY1 (D); and pACCAR25ΔcrtB + sorghum PSY3 (E). Chromatograms show HPLC separation of extracted pigments; inset in A shows the spectral fine structure for the pathway end product, zeaxanthin diglucoside.
water deprivation, leaves began to wilt and ABA levels in leaf and root tissue began to increase (Fig. 7, A and B); in parallel, mRNA level increases were observed for both PSY2 and PSY3 in leaves (Fig. 7A) and only PSY3 in roots (Fig. 7B). Transcript levels of PSY2 and PSY3 reached their highest levels in seedlings subjected to continuous drought stress for 8 d, showing a 16- and 17-fold increase in leaves, respectively; in leaves, PSY2 encoded the most abundant (3.4-fold compared to PSY3) transcript at 8 d, the time point after which water was restored. In contrast to leaves, at 4 d, roots showed a 38-fold increase of PSY3 transcripts and, by 8 d, roots showed 50-fold induction of PSY3 transcript levels as compared to predrought levels. Upon rewatering at 8 d, both ABA and mRNA levels of PSY2 and PSY3 dropped to normal levels within 4 h in both tissues. The rapid disappearance of PSY2 and PSY3 transcripts is suggestive of tight control of mRNA stability and/or gene transcription rate. In contrast, maize PSY1 mRNA levels were only slightly altered by drought stress in both tissues.

Response of Maize PSY Transcript Levels to Salt and ABA Treatment

For plants, the responses to drought and salt are closely related and their mechanisms overlap (Zhu, 2002). Moreover, in Arabidopsis, many drought-inducible genes can also be induced by exogenous application of ABA (Seki et al., 2002). Therefore, salt and exogenous ABA treatments were also used to study PSY responses.

When maize inbred B73 seedlings were subjected to salt stress, PSY3 transcript levels were barely altered in leaves (Fig. 8A), but increased in roots within 30 min; within 2 h of salt stress, PSY3 transcripts peaked 6-fold, compared to untreated controls, and then dropped to lower levels by 5 h (Fig. 8B). In contrast, PSY2 transcripts only slightly increased in salt-stressed root tissue. In leaves, PSY1 transcript levels decreased rapidly within 30 min and then remained at a low level, whereas transcript levels of PSY2 and PSY3 did not show significant changes (Fig. 8A).

To test PSY responses to ABA treatment, maize B73 seedlings were subjected to 100 mM ABA. In leaves, PSY2 transcript levels increased about 8-fold within 15 min, then dropped down to normal levels within 4 h (Fig. 9A). In contrast, PSY1 mRNA levels steadily decreased upon ABA application, whereas PSY3 transcripts remained at a low level for the entire 4-h period. In roots, within 15 min of treatment, both PSY2 and PSY3 mRNA levels increased in response to ABA, with distinguishable temporal patterns; the PSY3 response was higher and peaked earlier at 15 min compared to 1 h for PSY2; PSY3 mRNA levels increased about 7-fold in 15 min and PSY2 mRNA levels increased 4.5-fold in 1 h, and then dropped after 2 h (Fig. 9B).

Up-Regulation of PSY3 Expression Correlates with the Increase of Carotenoid Flux under Salt Treatment

The up-regulation of maize PSY3 in roots in response to drought, salt, and exogenous ABA strongly
suggested that PSY3 plays a role in stress-induced carotenogenesis required for ABA and other apocarotenoids. Because roots do not ordinarily accumulate carotenoids, it is difficult to assess the correlation between increased PSY3 transcripts and increased carotenoid accumulation. However, it is possible to block the pathway using mutants that condition accumulation of pathway intermediates (Wurtzel, 2004; Matthews and Wurtzel, 2007). We therefore chose the maize y9 mutation, which blocks carotenoid biosynthesis in nonphotosynthetic tissues through interference with isomerization of \( \zeta \)-carotene by \( \zeta \)-carotene isomerase (Z-ISO; Li et al., 2007); homozygous mutants accumulate the Z-ISO substrate 9,15,9\( \zeta \)-carotene and earlier pathway intermediates (Fig. 1), which can be easily measured by HPLC. Therefore, in this set of experiments with y9, we were able to compare changes in gene expression for carotenoid enzymes with changes in carotenoid content and ABA. Homozygous maize y9 seedlings were salt treated, and carotenoid content, ABA, and PSY transcript levels in roots were measured and compared with ABA levels in normal B73 roots subjected to salt stress. In salt-treated y9 roots, we observed that the total carotene content, including phytoene, phytofluene, and \( \zeta \)-carotene isomers, began to increase within 1 h after salt treatment (Fig. 10A). The total carotene content peaked after 2 h to levels 1.7-fold higher than that of untreated controls. ABA levels peaked at 5 h both in B73 and in y9 roots; due to the block at Z-ISO, the increase in ABA concentration in the root tissue of the y9 mutant was 46% less than that of the normal B73 control (Fig. 10B). The 2-fold increase in ABA levels in y9 roots may be due to leaf ABA redistribution or incomplete blocking at the Z-ISO step. In comparison to the carotenoid increase seen at 1 h in y9 roots, PSY3 transcripts began elevating at 30 min and peaked by 2 h (Fig. 10C) as seen for PSY3 in inbred B73 roots (Fig. 8B), whereas no changes were seen for PSY1, PSY2, or PDS (phytoene desaturase; Fig. 10C). Therefore, the elevated carotenoid content appears to be preceded by elevation in PSY3 transcripts. A block in the pathway leads to reduced ABA, implying that elevated carotenoids are needed for stress-induced elevated ABA. These results demonstrate that salt-induced elevation of PSY3 mRNA level correlates with increased carotenoid flux in roots, which contributes precursors needed for conversion to ABA.
PSY3 Is a Key Regulator of Carotenoid Biosynthesis in Roots under Stress

We showed that PSY3 transcript levels are increased in response to drought, salt, and ABA treatment; elevated PSY3 transcripts were also shown to precede carotenoid and ABA accumulation in y9 roots. It has been previously shown in dicots that ZEP and HYD transcripts are elevated in roots subjected to drought stress (Audran et al., 1998; Thompson et al., 2007). Therefore, we questioned whether PSY3 was the only gene encoding a carotenoid pathway enzyme that is abiotic stress responsive or whether other genes, including ZEP and HYD, contribute to enhanced carotenoids required for NCED1 activity and subsequent ABA induction. In maize, ZEP is encoded by two copies; we found that neither showed significant variation in transcript level in response to drought stress (Fig. 11, A and B). maize HYD is encoded by a small gene family (R. Vallabhaneni and E.T. Wurtzel, unpublished data), only one of which was significantly induced under drought conditions. HYD transcript level increases were first observed at 6 d and peaked at 8 d following drought stress (Fig. 11). In comparison, PSY3 mRNAs increased 38-fold already at 4 d and by 8 d roots showed 50-fold induction of PSY3 transcript levels as compared to predrought levels (Fig. 7B); parallel to PSY3, NCED1 also increased 3.7-fold at 4 d (Fig. 11D). These observations suggest that modulation of HYD transcript levels is delayed relative to induction of PSY3 and NCED mRNA levels. We also tested PDS, ZDS (ζ-carotene desaturase), CrtISO (carotenoid isomerase), and LCYB (lycopene β-cyclase), none of which showed significant changes in response to drought stress (data not shown). Therefore, temporal control of stress-induced root carotenogenesis and ABA biosynthesis appears to be regulated first through induction of PSY3 and NCED1 transcript levels, followed by induction of HYD transcript levels, but not induction of ZEP as seen in dicots.

Reversal of drought stress in roots was observed within 2 h of rehydration, when PSY3 transcript levels decreased and ABA levels plummeted (Fig. 7B). One explanation for the rapid loss of ABA, besides loss of PSY3 transcripts, is that rehydration likely induced transcript levels of the ABA degradative enzyme ABA8ox; in Arabidopsis and bean, ABA8ox genes were up-regulated in response to drought stress and rehydration caused additional increases in transcript levels associated with reduced ABA (Kushiro et al., 2004; Yang and Zeevaart, 2006). To test whether reduced ABA was due to induction of the degradative enzyme ABA8ox, we measured corresponding maize transcripts. We identified maize orthologs of the recently identified rice gene (Saika et al., 2007) and examined two maize orthologs (ABA8ox1a and ABA8ox1b) that were most abundantly expressed in maize roots (R. Vallabhaneni and E.T. Wurtzel, unpublished data). Transcript levels of these root-abundant paralogs were measured in response to drought stress and rehydration; both genes showed increased transcript levels upon drought stress (Fig. 11, E and F). ABA8ox1a, having the most abundant root transcripts, showed a more rapid response to drought stress in comparison with ABA8ox1b; ABA8ox1a levels peaked at 4 d of drought stress compared to ABA8ox1b transcripts peaking at 6 d. ABA8ox1a, but not ABA8ox1b, also showed further elevation in response to rehydration within 2 h of watering at day 8 (Fig. 11E). These results explain the rapid loss of ABA upon watering and are consistent with previous reports of ABA8ox gene responses to drought stress in other plants (Kushiro et al., 2004; Yang and Zeevaart, 2006).

Figure 10. HPLC analysis of carotenoid content of maize y9 root tissue after salt treatment. Maize y9 mutant seedlings and normal B73 seedlings at the five-leaf stage were salt treated for 0, 0.25, 0.5, 1, 2, 5, 10, and 20 h. A, HPLC chromatogram (at Amax) showing carotenoid content of y9 mutant seedling roots (the mean of N = 5) subjected to salt stress for hours indicated. B, ABA content of roots from maize y9 mutant seedlings and normal B73 seedlings subjected to salt stress for hours indicated. C, Quantitative RT-PCR of salt-treated roots for analysis of genes listed. Transcript levels were normalized to levels of β-actin transcripts measured in the same sample and are shown relative to PSY1 transcript levels in root at 0 h. Values represent the mean of three RT-PCR replicates ± so from five pooled plants. Other genes are abbreviated as in Figure 6.
DISCUSSION

The role of carotenogenesis in plants is multifaceted, including functions in photosynthesis and photoprotection, precursors to ABA and to other apocarotenoids that function as signals in development, and in communication between plants and their biotic environment. Yet, in a species such as Arabidopsis, where a rate-controlling carotenoid biosynthetic enzyme, PSY, is encoded by a single-copy gene, responses leading to altered flux are limited to control of that single-copy gene. In contrast, we showed that maize and other members of the Poaceae have three paralogs; gene duplication has provided an opportunity for subfunctionalization, whereby gene family members vary in tissue specificity of expression and in responses to abiotic stress.

Database searching led to the fortuitous discovery of PSY3 in the grasses; syntenic comparisons between the published rice genome and the available maize physical map facilitated isolation of the maize gene. We showed that PSY3 is present in maize, sorghum, and rice, three species that span two subfamilies in the Poaceae. Enzymes encoded by each of the three paralogs were shown to be functional, as demonstrated in a commonly used E. coli platform. Each enzyme is also predicted to have an extended N terminus as compared to PSY1 and PSY2. The fact that PSY3 sequences across three species are more closely related than they are to PSY1 and PSY2 within a species suggests that gene family members may share paralog-specific roles in the plant either in terms of gene regulation or with regard to metabolon assembly and/or membrane-specific localization within different plastids.

We discovered PSY3 among sorghum EST sequences, but we could not find any evidence that the gene was expressed in maize, suggesting that specific tissues or conditions were needed to elicit expression, which later proved to be true. In maize endosperm and leaves, tissues high in carotenoids, low levels of PSY3 transcripts were observed. In contrast, the gene is expressed in minimally carotenogenic tissues; further analysis of roots revealed that the absence of accumulated carotenoids is likely due to carotenoid cleavage because carotenoid accumulation can be observed if further conversions are blocked by mutations affecting carotenoid biosynthetic enzymes.

The rationale for investigating stress-induced regulation was based on prevalence of rice PSY3 ESTs associated with abiotic stress. Plant responses to drought and salt show overlapping mechanisms (Zhu, 2002) and many drought-inducible genes can also be induced by exogenous application of ABA (Seki et al., 2002).
transcripts were those of accumulated carotenoids, and the most prevalent PSY sected at 20 d after pollination (DAP), contains few 2003; Havaux et al., 2007). Nonstressed embryo, dis- temperature stress tolerance, and photoprotection in roots, although the PSY3 transcripts in roots, containing PSY2, were 3.4-fold higher than that of PSY2 and PSY3 transcripts in roots, thus the observed pattern of rice ESTs associated with plant stress, suggesting that PSY3 responses seen in maize are not unique to this species.

In nonstressed leaves, the mRNA levels of maize PSY2 were 3.4-fold higher than that of PSY3, suggesting that PSY2 is the primary gene responding to drought stress in leaves. However, the response mechanism may not involve increased flux to ABA because leaf ABA biosynthesis is limited not by the carotenoid precursor pool, but by NCED-mediated conversion of the xanthophyll precursors to ABA (Parry et al., 1990; Marin et al., 1996; Audran et al., 1998; Thompson et al., 2000a). Instead, increased carotenoid flux in leaves may relate to other processes, such as photosynthesis, temperature stress tolerance, and photoprotection (Davison, 2002; Rossel et al., 2002; Woiwisch and Romer, 2003; Havaux et al., 2007). Nonstressed embryo, dis- sected at 20 d after pollination (DAP), contains few accumulated carotenoids, and the most prevalent PSY transcripts were those of PSY2 followed by PSY3; perhaps, in this tissue, which is lacking in photosyn- this ability of Arabidopsis thaliana to impact flux to carotenoids in maize was consistent with the observed pattern of rice ESTs associated with plant stress, suggesting that PSY3 responses seen in maize are not unique to this species.

In roots, transgenic overexpression of NCED previously suggested that there were other factors that were bottlenecks to ABA (Thompson et al., 2007). The results shown here suggest that overexpression of PSY3 in combination with NCED may overcome the bottleneck that was observed when NCED was over- expressed alone. By using the maize y9 mutation to block root carotenoid biosynthesis, we observed elevated root PSY3 mRNAs in parallel with carotenoid accumulation; the block in carotenoids also led to reduction in accumulated ABA. PSY3 and NCED shared a simi- lar temporal response to drought stress and were followed by elevation of ABA. PSY3 was the only gene in the carotenoid pathway that showed significant changes in transcript levels needed to control flux to carotenoids in roots; induction of elevated PSY3 transcripts was followed by moderate induction of HYD transcript levels, which might be interpreted as a response to the increased carotenoid flux mediated by elevation of PSY3 transcripts. By using the y9 mutant to block Z-ISO in the carotenoid pathway, we showed that the PSY3 transcript level correlated with increased root carotenoids that are produced upstream of HYD; at this time, we are unable to evaluate the effect of elevated HYD transcripts in driving flux further to xanthophylls. We rule out later steps, such as ZEP, because we did not observe any significant change in ZEP transcripts in roots when plants were subjected to drought stress. The lack of changes in maize root ZEP transcripts is in contrast to dicots, where stress did cause changes in ZEP mRNA levels in roots, but not in leaves (Audran et al., 1998; Thompson et al., 2000a).

Other factors that might additionally affect flux in roots may be attributed to the upstream nonmevalonate isopentenyl diphosphate biosynthetic pathway for which expression of certain enzymes has been shown to impact flux to carotenoids in maize (R. Vall Abraham and E.T. Wurtzel, unpublished data) and in other organisms (Matthews and Wurtzel, 2000).

**CONCLUSION**

In summary, PSY3 expression plays a role in controlling flux to carotenoids in roots in response to drought stress; changes in PSY3 transcripts were accompanied by induced levels of carotenoid intermediates, elevation of HYD and NCED transcripts, and followed by accumulation of ABA. PSY3, which exists in multiple species within two subfamilies of the Poaceae, is a new target to consider for enhancing tolerance to drought and salt stress. Stress tolerance is an important factor affecting plant yield that could contribute to increasing the food supply or to improved biofuel production from grass species of the Poaceae.

**MATERIALS AND METHODS**

**Plant Materials**

Maize (*Zea mays*) inbred line B73 and mutant y9 (X07C; Maize Genetics Cooperation Stock Center, University of Illinois) and rice (*Oryza sativa* 'indica') var. IR36 were propagated as follows. Maize B73 and y9 mutant were grown in a greenhouse with a photoperiod of 16 h supplemented with artificial lighting at 25°C with appropriate watering prior to drought, salt, or ABA treatment. Rice was grown under the same conditions and leaves were collected for cDNA isolation and gene cloning. The endosperm and embryo tissues of maize B73 were dissected at 20 DAP from field-grown plants and stored at −80°C until analysis.

Cloning of Maize PSY3

Maize PSY1 cDNA sequence (GenBank accession no. ZMUS2636) was used in BLAST analysis to identify a putative homolog from sorghum (*Sorghum bicolor*; GenBank accession no. BG46454; Alschul et al., 1997). Further se- quence comparisons revealed that this sorghum EST had a unique 3’ end, which distinguished it from PSY1 and PSY2, and it was therefore named PSY3. To search for PSY3 homologs in other grass species, we used the sorghum PSY3 cDNA (GenBank accession no. BG46454) in BLAST analysis, which led to six rice PSY3 ESTs (CF305089, CF312254, CF312253, CF307565,
AK108134, and AY078162), although none was found for maize. Among these, five ESTs (except AK108134) were drought induced or associated with ABA signaling; the first four ESTs originated from a cDNA library prepared from transgenic rice modified for overexpression of ABF3, an ABA-responsive element-binding factor, which belongs to a distinct subfamily of bZIP proteins (Choi et al., 2000). BLAST analysis (Altschul et al., 1997) with the sorghum PSY3 cDNA (BG46454) was then used to identify the rice PSY3 gene between loci Aa666377 and rz404 on chromosome 9 (GenBank AB005238). Next, we used synteny between rice and maize (http://www.tigr.org/tdb/ synten/) to identify the putative maize PSY3 locus; rice PSY3 flanking markers were used to mark the syntenic region, which putatively encompassed maize PSY3, in bin 7.03 between loci rz404 (ccp) and umc1865. This maize region was covered by BAC contig 309 developed by the Maize Agarone FPC Map project (http://www.genome.arizona.edu/tpc/maze). Maize B73 BAC clones within this contig were requested and validated via PCR; PCR primers were designed from maize genomic contig AZM4_60808, which contained a partial maize PSY3 genomic DNA sequence deduced by alignment with sorghum and rice PSY3 cDNAs. Maize PSY3 containing BAC clone b012105 was identified and both strands were sequenced by primer walking (DNA Sequencing Facility, Biotechnology Resource Center, Cornell University) and deposited into GenBank (DQ372936).

Sequence Analyses
cDNA sequences and corresponding protein sequences of PSYs of Arabidopsis (Arabidopsis thaliana), rice, sorghum, and maize were obtained from NCBI GenBank, some of which were deposited as a result of this work (DQ372936, DQ565431, AY705389, AY705390, DQ565430; Arabidopsis (AtPSY AA32626), rice (OsPSY1, AAS18307; OsPSY2, AA007373; OsPSY3, DQ565431), sorghum (SbPSY1, AY705389; SbPSY2, AY705390; SbPSY3, DQ565390; SbPSY4, DQ565391, DQ565392; ZmPSY1, P49085; ZmPSY2, AA019837; ZmPSY3, DQ565430). Amino acid sequences were aligned using ClustalW and a neighbor-joining tree was constructed with 500 bootstrap replication support using MEGA3 software (Kumar et al., 2001). The carboxyl-terminal conserved domain of each PSY group was identified manually after alignment. The genomic DNA sequences of PSYS of Arabidopsis, rice, sorghum, and maize were obtained from NCBI, GenomeNet, and PlantCDB for gene structure analysis: maize PSY1 (ZmPSY1; GenBank AB142344); rice PSY1 (OsPSY1; GenBank AP005750); sorghum PSY1 (SbPSY1; PlantCDB SbGSStuc11-12-04.5154.1); maize PSY2 (ZmPSY2; GenBank AB142344); rice PSY2 (OsPSY2; GenBank AL831803); sorghum PSY2 (SbPSY2; PlantCDB SbGSStuc11-12-04.1260.1); maize PSY3 (ZmPSY3; GenBank DQ372936; described in this article); rice PSY3 (OsPSY3; Gramene LOC_Os09g38320); sorghum PSY3 (SbPSY3; PlantCDB SbGSStuc11-12-04.766.1); Arabidopsis PSY3 (AtPSY; GenBank AB005238). The PSY gene structures were analyzed using Vector NTI Suite Version 9.0 (InforMax).

Plasmids
Sorghum PSY-containing ESTs, CD234165, AW679367, and BG46454, were requested and verified by further sequencing. Both CD234165 and BG46454 contained full-length sorghum PSY1 and PSY3 cDNAs, respectively. The maize PSY3 (sequence deposited as DQ564340) and rice PSY3 (sequence deposited as DQ56431) full-length cDNAs were amplified from cDNAs prepared from leaf tissue of the corresponding plant species using RT-PCR primers designed based on genomic DNA sequences of maize BAC clone b012105 and rice LOC_Os09g38320, respectively. The rice PSY3 (DQ56431) was subcloned into the pGEMT-vector (Promega) and renamed pGEMT- RY3 prior to sequencing of both strands and use in phylogenetic analysis. The maize PSY3 cDNA (DQ56430) was inserted into the pET23b (+) vector between EcoRI and XhoI, named pETb-PSY3. Sorghum PSY1 from CD234165 and PSY3 from BG46454 were inserted between the EcoRI and HindIII sites of the pET23a (+) vector, designated as pETa-SPSY1 and pETA- SPSY3, respectively.

Functional Complementation
To test the function of PSY gene products, a heterologous complementation assay was carried out as previously described (Gallagher et al., 2004). Briefly, Escherichia coli BL21 (DE3) cells (Novagen) were transformed with combinations of pACCAR253ΔNtB and the expression constructs pETb- MPSY3, pETA-SPSY1, pETA-SPSY3, or empty vector pET23b (+). Carotenoids were extracted (Gallagher et al., 2004), resuspended in methanol, and subjected to HPLC analysis (Quinlan et al., 2007); zeaxanthin diglucoside was identified as before (Gallagher et al., 2004) and in comparison with literature data (Misawa et al., 1990).

Quantitative Real-Time PCR
RNA isolation and cDNA synthesis were carried out as described (Gallagher et al., 2004). Real-time PCR was performed using iQ SYBR green supermix (Bio-Rad) with 10 ng synthesized cDNA. For primers and PCR conditions for test genes and the internal actin control, refer to Supplemental Table S1. Specificity of amplification was confirmed via melt curve analysis of final PCR products by ramping the temperature from 50°C to 90°C with fluorescence acquired after every 0.5°C increase. All quantifications were normalized to the signal of actin cDNA for the same sample. The fold change of transcript abundance of target genes was first calculated as 2^−ΔCt, where ΔCt is the number of PCR cycles required to reach the log phase of amplification for the target gene minus the same measure for actin. Transcript abundance of maize PSY1 was then adjusted to 100% and fold changes of transcripts from other genes from the same tissue were normalized via comparison with that of maize PSY1. Values represent the mean of three RT-PCR replicates ±SD from five pooled plants.

Stress Treatments
To carry out the drought stress experiment, maize B73 seedlings at the five-leaf stage (about 3 weeks) were deprived of water for 8 d and then rewatered. Leaves began wilting 4 d after withholding water. Therefore, leaves and roots were collected at 0, 4, 6, and 8 d after water was withheld and 2, 4, and 24 h after rewatering.

For high salt and ABA treatments, maize B73 seedlings were carefully removed from soil to avoid injury, rinsed with water, and then hydroponically grown in solutions that contained either 250 mM NaCl or 100 mM ABA [c(+) ABA; catalog A1049, Sigma]. For an effective concentration of 100 mM ABA, 200 mM of the emunctiome mixture was used. Leaves and roots were collected after 0, 0.25, 0.5, 1, 2, 4, 5, 10, and 20 h of salt treatment, and after 0, 0.25, 0.5, 1, 2, and 4 h of ABA treatment. All plant materials were stored at −80°C until analysis. For the salt treatment of y9 mutants, y9 seedlings were treated with 250 mM NaCl and roots were collected for HPLC analysis to measure carotenoid content, which was performed and quantified as previously described (Li et al., 2007). Values represent the mean of three RT-PCR replicates ±SD from five pooled plants.

ABA Measurement
ABA extraction was carried out according to the method described by Xiong et al. (2001). Briefly, 1 g of frozen tissue was ground with liquid nitrogen and suspended in 5 mL of extraction solution (80% methanol, 100 mg L−1 butylated hydroxytoluene, and 1.7 g L−1 NaHCO3). The suspension was stirred 48 h at 4°C for extraction and centrifuged at 3,000g for 30 min. The supernatant was transferred to a new tube and dried under vacuum. Samples were dissolved in 100 μL of methanol and 900 μL of Tri-buffered saline (50 mM Tris, 0.1 mM MgCl2, and 0.15 mM NaCl, pH 7.8) and ABA concentration determined using the Phytodetect ABA immunoassay kit (Idetect). Note that the monoclonal antibody is specific for ABA and does not bind to ABA-GE [2-cis-(s)-ABA-6-glucopyranosyl ester]. Values are reported as the mean of five samples ±SD.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AY705389, AY705390, DQ565430, DQ565431, and DQ372936.

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Table S1. Primers for gene isolation, BAC sequencing, and quantitative real-time PCR.

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