PSY3, a New Member of the Phytoene Synthase Gene Family Conserved in the Poaceae and Regulator of Abiotic Stress-Induced Root Carotenogenesis[1][W][OA]

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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-epoxycarotenoid 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-

[1] This work was supported by the National Institutes of Health (grant nos. S06–GM08225 and 1SC1GM081160–01), the Professional Staff Congress of The City University of New York, and New York State.

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www.plantphysiol.org/cgi/doi/10.1104/pp.107.111120

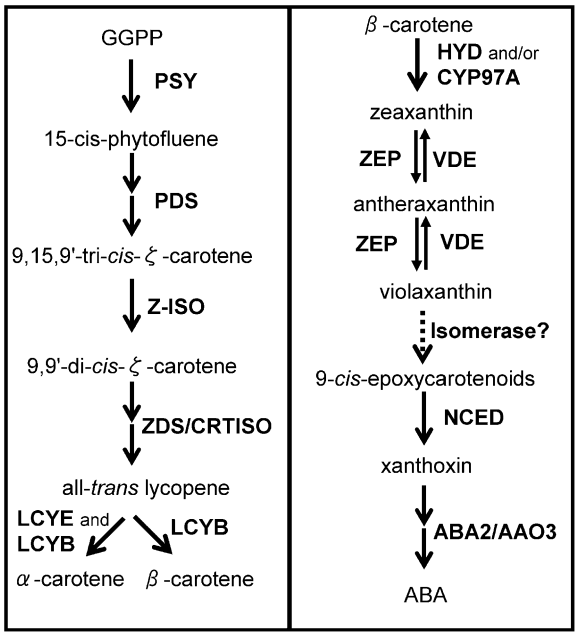

Figure 1. Carotenoid and ABA biosynthetic pathways in higher plants. Left, Carotenoid precursors of carotenes. Right, Conversion of carotenes to ABA. Enzymes are bold: LCYE, lycopene ε-cyclase; CYP97A, P450 carotene β-ring hydroxylase; VDE, violaxanthin de-epoxidase; ABA2, short-chain alcohol dehydrogenase; AAO3, ABA oxidase.

gous genes, *PvNCED1* 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.

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 *PSY1*s have a small first intron (approximately 100 bp) and a large second intron (>600 bp), but all *PSY2*s 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

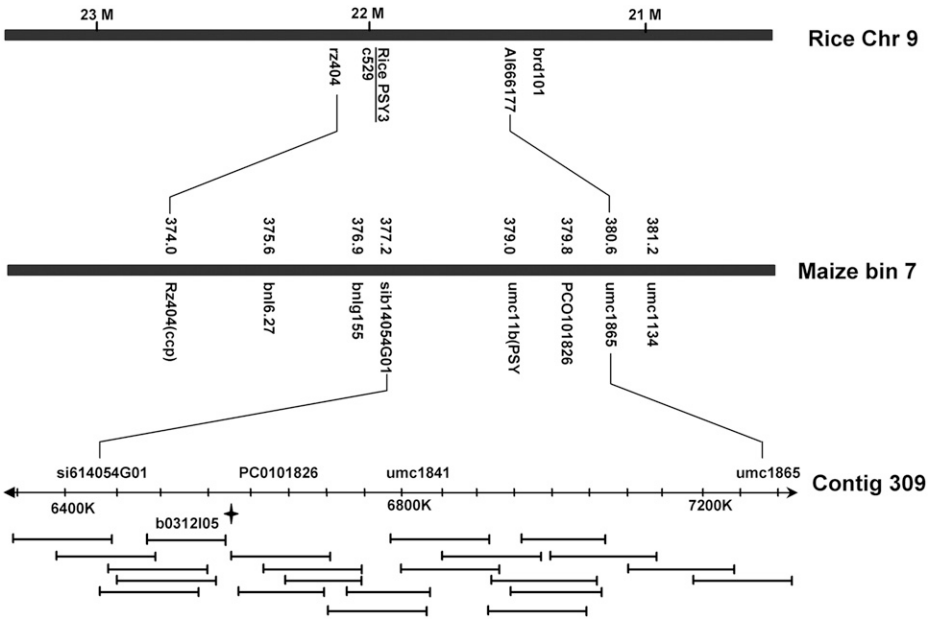

Figure 2. Cloning of maize *PSY3* using synteny between rice and maize. Rice *PSY3* (Gramene ID LOC_Os09g38320) was located on chromosome 9 between markers *AI666177* and *rz404* (top). The syntenic region on maize is bin 7 between markers *umc1865* and *Rz404* (*ccp*; middle). BAC clone b0312I05 containing maize *PSY3* was identified by PCR from contig 309 BACs that covered this region (bottom). The maize bin 7 map was adapted from IBM2 2004 neighbors 7 of MaizeGDB. Contig 309 of the Maize Agarose FPC Map was developed by the Arizona Genomic Institute.

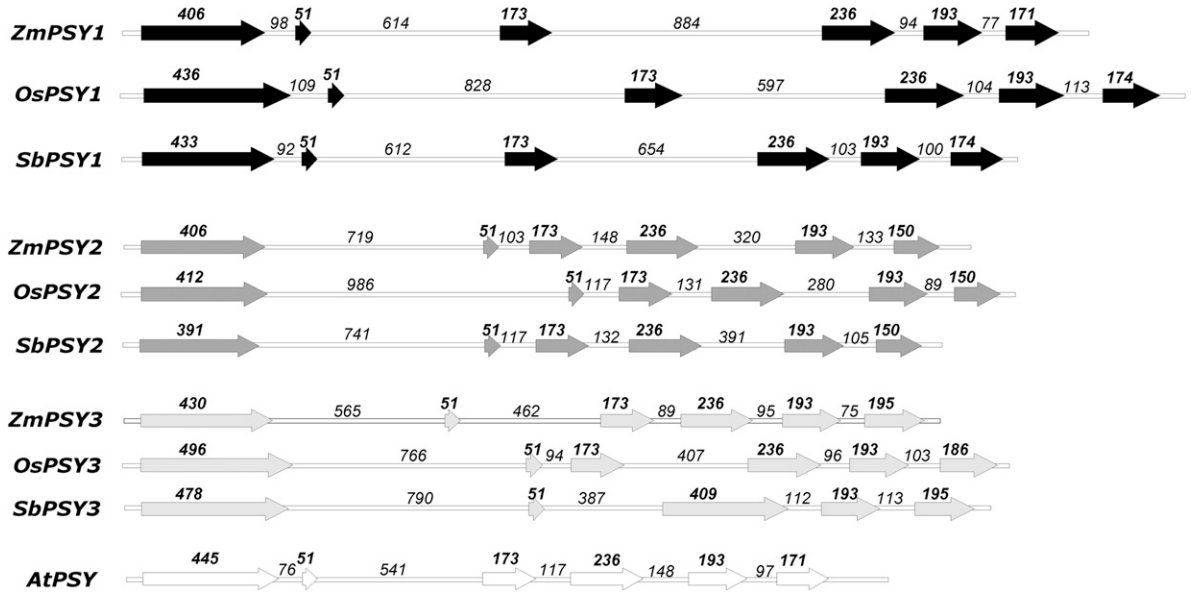

Figure 3. Gene structure of maize, rice, sorghum, and Arabidopsis *PSY* genes. Maize *PSY1* (*ZmPSY1*; GenBank AY324431); rice *PSY1* (*OsPSY1*; GenBank AP005750); sorghum *PSY1* (*SbPSY1*; PlantGDB SbGSStuc11-12-04.5154.1); maize *PSY2* (*ZmPSY2*; GenBank AY325302); rice *PSY2* (*OsPSY2*; GenBank AL831803); sorghum *PSY2* (*SbPSY2*; PlantGDB SbGSStuc11-12-04.12062.1); maize *PSY3* (*ZmPSY3*; GenBank DQ372936); rice *PSY3* (*OsPSY3*; Gramene LOC_Os09g38320); sorghum *PSY3* (*SbPSY3*; PlantGDB SbGSStuc11-12-04.766.1); Arabidopsis *PSY* (*AtPSY*; GenBank AB005238). Arrows and thin bars indicate exons (bold numbers) and introns, respectively, sizes for which are in base pairs.

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 pACCAR25Δ*crtB*, 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, pACCAR25Δ*crtB* (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

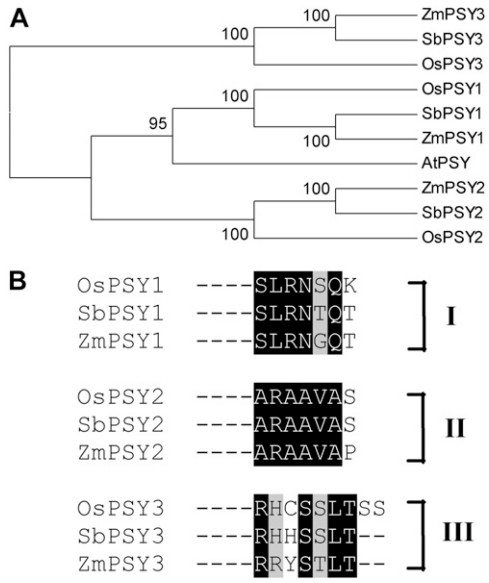

Figure 4. PSY1, PSY2, and PSY3 protein sequence comparisons. A, Phylogenetic analysis of amino acid sequences. B, Conserved carboxyl termini of PSY proteins. GenBank accessions are in parentheses. Arabidopsis (AtPSY AAA32836), rice (OsPSY1, AAS18307; OsPSY2, AAK07735; OsPSY3, DQ356431), sorghum (SbPSY1, AY705389; SbPSY2, AW679367; SbPSY3, AY705390), maize (ZmPSY1, P49085; ZmPSY2, AAQ91837; 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.

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

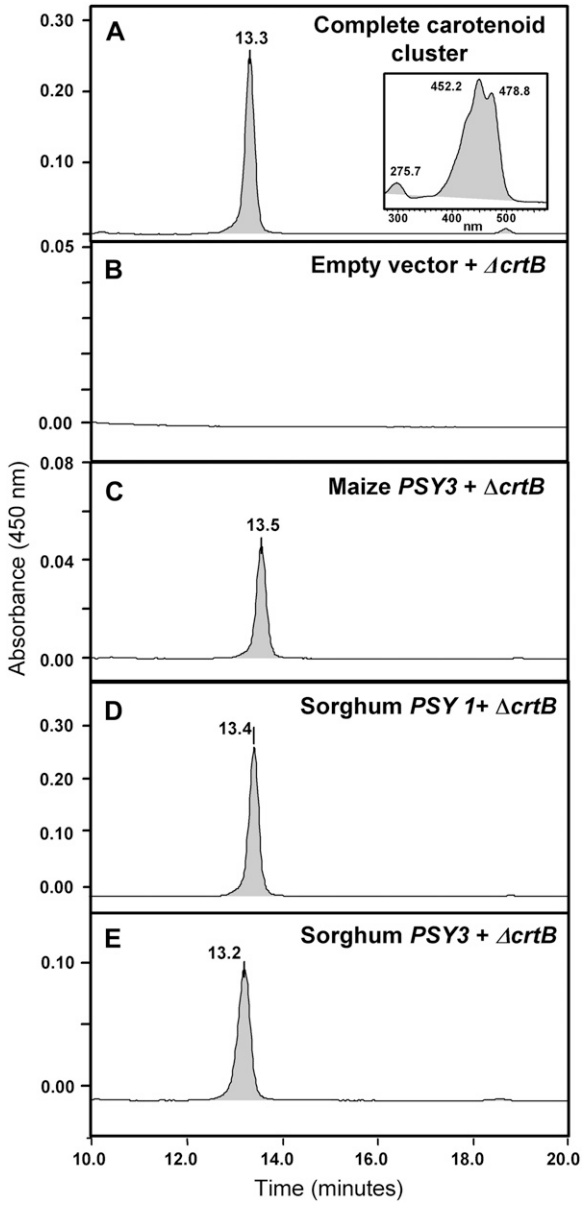

Figure 5. Functional complementation of *PSY3* from maize and sorghum. *E. coli* cells were transformed with pACCAR25 (A); pACCAR25ΔcrtB + pET23a (empty vector; B); pACCAR25ΔcrtB + maize *PSY3* (C); pACCAR25ΔcrtB + sorghum *PSY1* (D); and pACCAR25ΔcrtB + sorghum *PSY3* (E). Chromatograms show HPLC separation of extracted pigments; inset in A shows the spectral fine structure for the pathway end product, zeaxanthin diglucoside.

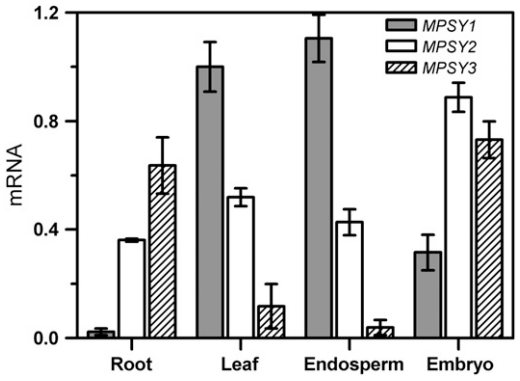

Figure 6. Transcript levels for *PSY* gene family members in different maize tissues. Endosperm and embryo were collected at 20 DAP from field-grown maize B73. Leaf and root samples were collected from B73 seedlings at the five-leaf stage. Transcript levels of maize *PSY1*, *PSY2*, and *PSY3* were normalized to levels of β-actin transcripts measured in the same samples and are shown relative to *PSY1* transcript levels in leaves (the expression level of maize *PSY1* in leaf = 1). Values represent the mean of three RT-PCR replicates ±SD from five pooled plants. *MPSY1*, maize *PSY1*; *MPSY2*, maize *PSY2*; *MPSY3*, maize *PSY3*.

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

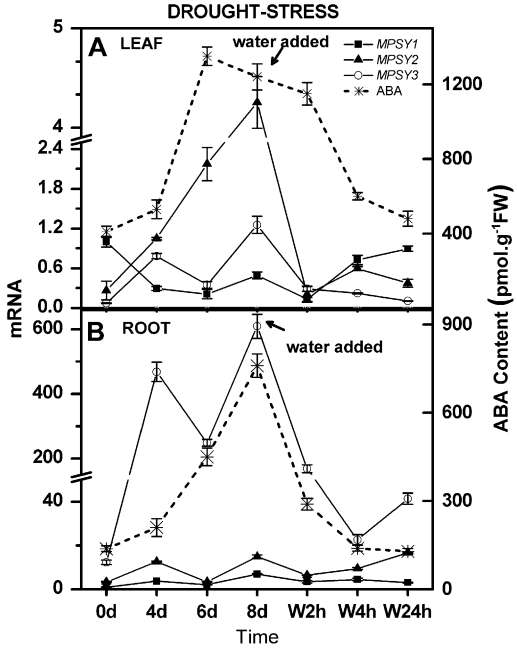

Figure 7. Effect of drought stress on maize *PSY* transcript levels. Maize B73 seedlings at the five-leaf stage were greenhouse grown, deprived of water for 8 d, followed by rewatering. Total RNA was extracted from leaves or roots at 0, 4, 6, and 8 d after water was withheld and 2, 4, and 24 h after rewatering. Transcript levels of maize *PSY1*, *PSY2*, and *PSY3* were normalized to levels of β-actin transcripts measured in the same sample, and are shown relative to *PSY1* transcript levels in leaf (A) and root (B) at 0 d. Values represent the mean of three RT-PCR replicates ±SD from five pooled plants. Genes are abbreviated as in Figure 6.

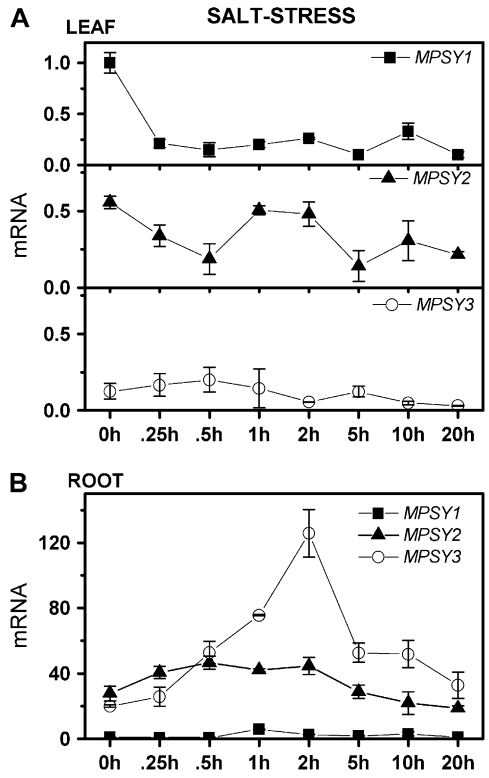

Figure 8. Effect of salt stress on maize *PSY* transcript levels. Total RNA was extracted from leaves or roots at indicated hours (h) after treatment with 250 mM NaCl. Transcript levels were normalized to levels of β-actin transcripts measured in the same sample, and are shown relative to PSY1 transcript levels in leaf (A) and root (B) at 0 h. Values represent the mean of three RT-PCR replicates ±SD from five pooled plants. Genes are abbreviated as in Figure 6.

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 ζ-carotene by ζ-carotene isomerase (Z-ISO; Li et al., 2007); homozygous mutants accumulate the Z-ISO substrate 9,15,9′ ζ-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 ζ-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.

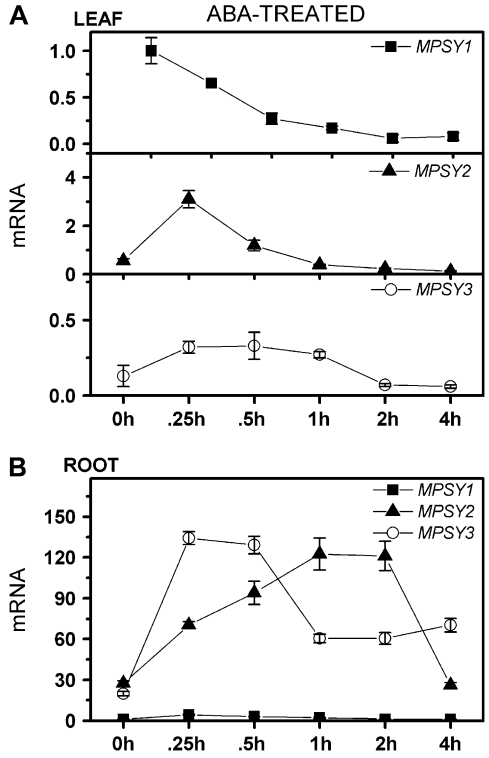

Figure 9. Effect of ABA on maize *PSY* transcript levels. Total RNA was extracted from leaves or roots at 0, 0.25, 0.5, 1, 2, and 4 h after ABA treatment. Transcript levels were normalized to levels of β-actin transcripts measured in the same sample and are shown relative to mPSY1 transcript levels in leaf (A) and root (B) at 0 h. Values represent the mean of three RT-PCR replicates ±SD from five pooled plants. Genes are abbreviated as in Figure 6.

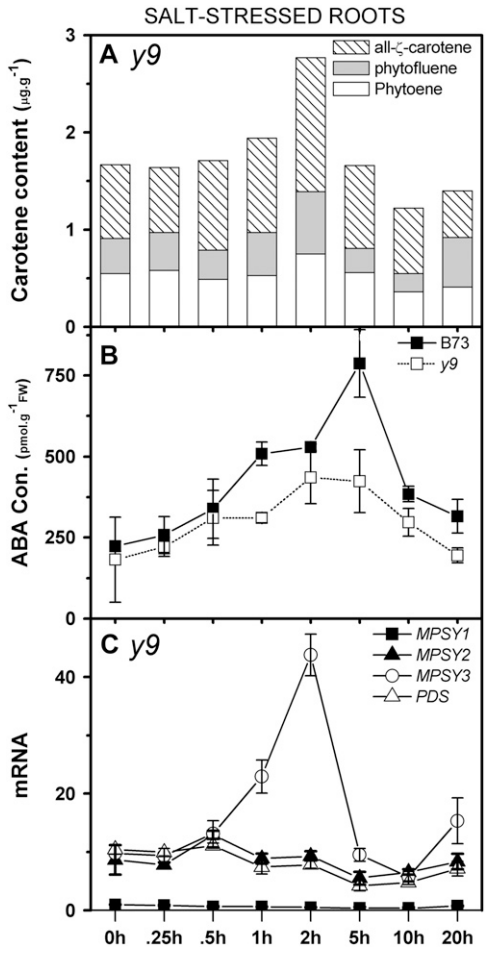

Figure 10. HPLC analysis of carotenoid content of maize *y9* root tissue after salt treatment. Maize *y9* mutant seedlings and normal B73 seedlings at the five-leaf stage were salt treated for 0, 0.25, 0.5, 1, 2, 5, 10, and 20 h. A, HPLC chromatogram (at λmax) showing carotenoid content of *y9* mutant 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 ±SD from five pooled plants. Other genes are abbreviated as in Figure 6.

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).

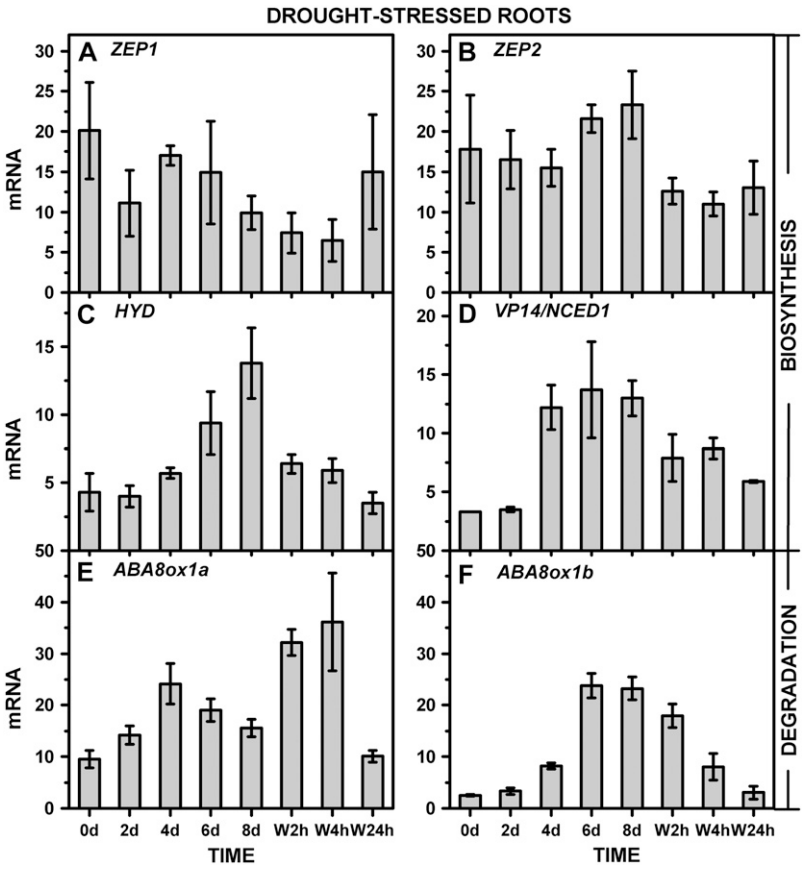

Figure 11. Drought stress-modulated levels of transcripts encoded by genes controlling biosynthesis and degradation of ABA in maize. The mRNA levels of *ZEP1* (A), GenBank DR820114, *ZEP2* (B), GenBank CO532283, *HYD* (C), GenBank BQ619575, *NCED1* (D), GenBank U95953, *ABA8ox1a* (E), GenBank DR806072, and *ABA8ox1b* (F), GenBank EE190691, were normalized to levels of β-actin transcripts measured in the same samples and are shown relative to maize *PSY1* mRNA levels in roots at 0 h. Values represent the mean of three RT-PCR replicates $\pm$SD from five pooled plants.

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 be chloroplast-localized and marked by a paralog-specific C-terminal domain, whereas PSY3 is 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). Therefore, we examined maize for the effects of salt, drought, and ABA upon transcript levels of *PSY3* and its paralogs. We discovered that drought-induced elevation of ABA in leaves and roots was accompanied by elevated leaf mRNA levels for *PSY2* and, to a lesser extent, *PSY3*, in contrast to roots where only *PSY3* transcript levels increased; salt had a similar effect on induction of *PSY3* mRNA levels in roots, but not on *PSY2* transcripts in leaves. Rehydration reversed induction of *PSY*3 transcript levels in roots, stimulated induction of transcripts for the ABA degradation enzyme, ABA8ox, and led to diminution of ABA. *PSY1* mRNA levels never showed enhancement by any of the abiotic stresses tested. Direct treatment with ABA induced elevation of both *PSY2* and *PSY3* transcripts in roots, although the *PSY3* response was earlier. The effect of abiotic stress on *PSY3* transcripts 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 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; Woitsch and Romer, 2003; Havaux et al., 2007). Nonstressed embryo, dissected 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 photosynthetic plastids, *PSY* expression could possibly relate to plant development (Sorefan et al., 2003; Booker et al., 2004) and/or apocarotenoid biosynthesis (Matusova et al., 2005).

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 overexpressed 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 similar 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. Vallabhaneni 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. ZMU32636) was used in BLAST analysis to identify a putative homolog from sorghum (*Sorghum bicolor*; GenBank accession no. BG46454; Altschul et al., 1997). Further sequence 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, CF312554, CF312553, CF307565,

AK108154, and AY078162), although none was found for maize. Among these, five ESTs (except AK108154) were drought inducible 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 AI666177 and *rz404* on chromosome 9 (Gramene ID LOC_Os09g38320). Next, we used synteny between rice and maize (http://www.tigr.org/tdb/synteny) 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 Agarose FPC Map project (http://www.genome.arizona.edu/fpc/maize). Maize B73 BAC clones within this contig were requested and screened 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 b031205 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, DQ356431, AY705389, AY705390, DQ356430): Arabidopsis (AtPSY AAA32836), rice (OsPSY1, AAS18307; OsPSY2, AAK07735; OsPSY3, DQ356431), sorghum (SbPSY1, AY705389; SbPSY2, AW679367; SbPSY3, AY705390), maize (ZmPSY1, P49085; ZmPSY2, AAQ91837; ZmPSY3, DQ356430). 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, Gramene, and PlantGDB for gene structure analysis: maize *PSY1* (*ZmPSY1*; GenBank AY324431); rice *PSY1* (*OsPSY1*; GenBank AP005750); sorghum *PSY1* (*SbPSY1*; PlantGDB SbGSStuc11-12-04.5154.1); maize *PSY2* (*ZmPSY2*; GenBank AY325302); rice *PSY2* (*OsPSY2*; GenBank AL831803); sorghum *PSY2* (*SbPSY2*; PlantGDB SbGSStuc11-12-04.12062.1); maize *PSY3* (*ZmPSY3*; GenBank DQ372936; described in this article); rice *PSY3* (*OsPSY3*; Gramene LOC_Os09g38320); sorghum *PSY3* (*SbPSY3*; PlantGDB SbGSStuc11-12-04.766.1); Arabidopsis *PSY* (*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 DQ356430) and rice *PSY3* (sequence deposited as DQ356431) 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 b031205 and rice LOC_Os09g38320, respectively. The rice *PSY3* (DQ356431) was subcloned into the pGEMT-vector (Promega) and renamed pGEMT-RPSY3 prior to sequencing of both strands and use in phylogenetic analysis. The maize *PSY3* cDNA (DQ356430) was inserted into the pET23b (+) vector between *Eco*RI and *Xho*I, named pETb-MPSY3. Sorghum *PSY1* from CD234165 and *PSY3* from BG46454 were inserted between the *Eco*RI and *Hin*dIII sites of the pET23a (+) vector, designated as pETa-SPSY1 and pETa-SPSY3, respectively.

Functional Complementation

To test the function of *PSY* gene products, a heterozygous complementation assay was carried out as previously described (Gallagher et al., 2004). Briefly, *Escherichia coli* BL21 (DE3) cells (Novagen) were transformed with combinations of pACCAR25Δ*crtB* 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 $2^{-\Delta Ct}$, 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 [(±)-ABA; catalog A1049; Sigma]. For an effective concentration of 100 mM ABA, 200 mM of the enantiomeric 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} $NaHCO_3$). The suspension was stirred 48 h at 4°C for extraction and centrifuged at 3,000*g* 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 Tris-buffered saline (50 mM Tris, 0.1 mM $MgCl_2$, and 0.15 M NaCl, pH 7.8) and ABA concentration determined using the Phytodetek ABA immunoassay kit (Idetek). Note that the monoclonal antibody is specific for ABA and does not bind to ABA-GE [2-cis-(s)-ABA-B-D-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, DQ356430, DQ356431, 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.

ACKNOWLEDGMENTS

We are grateful to Rena Quinlan for critical reading of the manuscript, Christina Murillo for technical support, and Dr. Edward Kennelly and Dr. Bei Jiang for advice on HPLC methodology.

Received October 17, 2007; accepted December 23, 2007; published December 27, 2007.

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