The Maize Phytoene Synthase Gene Family: Overlapping Roles for Carotenogenesis in Endosperm, Photomorphogenesis, and Thermal Stress Tolerance

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Carotenoids are essential for photosynthesis and photoprotection; they also serve as precursors to signaling molecules that influence plant development and biotic/abiotic stress responses. With potential to improve plant yield and nutritional quality, carotenoids are targets for metabolic breeding/engineering, particularly in the Poaceae (grass family), which includes the major food crops. Depending on genetic background, maize (Zea mays) endosperm carotenoid content varies, and therefore breeding-enhanced carotenoid levels have been of ongoing interest. The first committed step in the plastid-localized biosynthetic pathway is mediated by the nuclear-encoded phytoene synthase (PSY). The gene family in maize and other grasses contains three paralogs with specialized roles that are not well understood. Maize endosperm carotenoid accumulation requires PSY1 expression. A maize antibody was used to localize PSY1 to amyloplast envelope membranes and to determine PSY1 accumulation in relation to carotenoid accumulation in developing endosperm. To test when and if PSY transcript levels correlated with carotenoid content, advantage was taken of a maize germplasm diversity collection that exhibits genetic and chemical diversity. Total carotenoid content showed statistically significant correlation with endosperm transcript levels at 20 d after pollination for PSY1 but not PSY2 or PSY3. Timing of PSY1 transcript abundance, previously unknown, provides critical information for choosing breeding alleles or properly controlling introduced transgenes. PSY1 was unexpectedly found to have an additional role in photosynthetic tissue, where it was required for carotenogenesis in the dark and for heat stress tolerance. Leaf carotenogenesis was shown to require phytochrome-dependent and phytochrome-independent photoregulation of PSY2 plus nonphotoregulated PSY1 expression.

Carotenoids represent a diverse group of more than 750 structures found in bacteria, fungi, algae, and plants (Britton et al., 2004). In higher plants, these pigments have numerous biological roles. For example, carotenoids serve as accessory pigments in the photosynthetic apparatus where they function in light harvesting and photoprotection (Niyogi, 2000). Heat and light stress tolerance is mediated by carotenoid antioxidants that protect membranes from lipid peroxidation (Davison, 2002; Havaux et al., 2007; Johnson et al., 2007). Carotenoids are also precursors to cleavage products, such as the apocarotenoid abscisic acid (ABA), which regulates plant growth, embryo development, dormancy, and stress responses (Nambara and Marion-Poll, 2005); additional apocarotenoid products, such as strigolactone and others, have less defined roles in internal and external signaling but are essential elements that may affect plant yield (Booker et al., 2004; Akiyama and Hayashi, 2006). As plant dietary sources, carotenoids contribute to human and animal health, such as the provitamin A compound β-carotene (Fraser and Bramley, 2004). Accordingly, regulatory mechanisms for pathway induction are complex and not well understood, especially in plants of agronomic value found throughout the Poaceae (grass family).

The first committed and rate-controlling step in the plastid-localized carotenoid biosynthetic pathway (as recently revised; see Li et al., 2007) is mediated by the nuclear-encoded phytoene synthase (PSY); PSY catalyzes formation of 15-cis phytoene from two molecules of geranylgeranyl pyrophosphate (for review, see Matthews and Wurtzel, 2007). It is well known for many species that the PSY step affects flux to carot-
enoids and much, though not all of the regulation is at the transcriptional level. In the grass family, which includes maize (Zea mays), sorghum (Sorghum bicolor), and rice (Oryza sativa), there are three paralogous PSY genes (Gallagher et al., 2004; Li et al., 2008). It is unknown whether the three PSY genes in the grasses have overlapping functions in modulation of carotenogenesis in different tissues and in response to multiple developmental and/or stress signals. For efforts to metabolically engineer enhanced levels of endosperm carotenoids, it would be helpful to elucidate the timing of gene expression for pathway enzymes to determine when and if transcript levels in developing endosperm correlate with carotenoid accumulation. In the absence of such data, use of a generic endosperm-specific promoter to drive transgene expression may be less effective in providing adequate levels of enzyme at the optimal time (Ye et al., 2000). As a result, a generic endosperm promoter may be a limiting factor in metabolic engineering of enhanced carotenoid content.

PSY gene duplications in other species such as tomato (Solanum lycopersicum; Bartley and Scolnik, 1993; Giorio et al., 2008) and tobacco (Nicotiana tabacum; Busch et al., 2002) are examples of convergent evolution that have limited value in predicting roles and regulation for members of the grass PSY gene family. Pathway localization is another factor adding to complexity of carotenogenesis; pathway biogenesis may be specific to plastid types (e.g. amyloplasts, chromoplasts, chloroplasts) that differ in membrane architecture, physiological, and/or developmental state. Further examination of the grass PSY gene family will contribute data that will facilitate predictable outcomes in metabolic engineering (“predictive metabolic engineering”; Wurtzel and Grotewold, 2006) in improvement of plant yield and/or enhancement of nutritional content in maize and other related crops (Kean et al., 2007; Pozniak et al., 2007; Giuliano et al., 2008; Harjes et al., 2008).

Maize, a key food crop and target for improvement of endosperm carotenoids, is an attractive grass model having useful resources for investigating roles of the PSY gene family members in mediating carotenogenesis. Resources include pathway mutants (Wurtzel, 2004) and a genetically and chemically diverse germplasm collection (Harjes et al., 2008). The germplasm collection could be used to investigate the timing and abundance of transcripts that might correlate with carotenoid content in endosperm. For grasses lacking these tools, it may be possible to employ phylogenetic and syntenic genome analyses to identify orthologous sequences having potentially similar roles (Li et al., 2008). The three paralogs in the maize PSY gene family all encode functional enzymes. PSY3 is associated with root carotenogenesis needed for drought and salt stress-induced production of ABA (Li et al., 2008), and PSY1, encoded by the Yellow1 (Y1) locus, is required for endosperm carotenoid accumulation (Randolph and Hand, 1940; Buckner et al., 1996; Palaisa et al., 2003; Gallagher et al., 2004). The roles of the three PSY gene family members were further investigated utilizing mutant and diversity collections. Data obtained on timing of expression and correlation with carotenoid content, enzyme localization, and overlapping roles in carotenogenesis will be useful in developing strategies to provide predictable outcomes in metabolic engineering and/or breeding of enhanced carotenoid content.

RESULTS

Immunolocalization of PSY1 in Endosperm Amyloplasts

Having previously established that accumulation of PSY1 transcripts correlated with presence of endosperm carotenoids in maize and absence in rice (Buckner et al., 1996; Gallagher et al., 2004), an antiserum was developed (as described in “Materials and Methods”) to further examine PSY enzyme accumulation and localization in maize endosperm. PSY1, encoded by maize Y1, was overexpressed and purified from Escherichia coli to raise a polyclonal antiserum. To test specificity of the antiserum, protein extracted from normal Y1 and mutant y1-602C endosperm was subjected to western analysis, as shown in Figure 1. This

Figure 1. Western analysis demonstrating specificity of anti-PSY1 antiserum for PSY in maize endosperm. Proteins were extracted from yellow (Y1) and white (y1) endosperm at 20 DAP. Immunoblots were probed by anti-PSY1 antiserum (top) or anti-Shrunken1 (Sh) antiserum (bottom).
particular $y1$ allele blocks endosperm carotenogenesis but does not interfere with leaf carotenogenesis; $PSY1$ transcript accumulation is only affected in endosperm but not in leaf tissue (Gallagher et al., 2004). Sequence analysis of the $y1$-602C allele (GenBank accession no. EU306869) revealed no mutations in the open reading frame, but the 5’ regulatory region was missing an ins2 insertion, the presence of which has been strongly associated with $PSY1$ expression in endosperm (Palaisa et al., 2003). The antiserum recognized an approximately 38-kD antigen, as expected for the processed, plastid-localized protein (Gallagher et al., 2004), and detected this antigen only in protein extracted from normal $Y1$ but not from the mutant $y1$ endosperm. Antiserum specificity was tested against $PSY2$ and $PSY3$. The antiserum proved to recognize only $PSY1$, but not $PSY2$ or $PSY3$, when all three proteins were individually expressed in E. coli by introduction of the corresponding cDNAs carrying a T7 Tag at the N terminus; as a control, all E. coli-expressed proteins were detectable with the T7 Tag monoclonal antibody (see Supplemental Fig. S1). To demonstrate localization of $PSY1$ within endosperm amyloplasts, endosperm thin sections were examined using confocal microscopy for immunolocalization of $PSY1$. As shown in Figure 2A, the antiserum localizes the protein to the amyloplast envelope; no plastid signal was obtained in the absence of the primary antibody (Fig. 2B). The antiserum also detected $PSY$ antigen in isolated maize amyloplasts but not in isolated amyloplasts of rice endosperm that does not accumulate $PSY1$ transcripts (E.T. Wurtzel and J. Yu, unpublished data).

**Timing of $PSY$ Expression and Carotenoid Accumulation in Endosperm**

To better understand the relationship between temporal regulation of $PSY$ transcript accumulation as compared to carotenoid accumulation during endosperm development, quantitative reverse transcription (RT)-PCR was used to monitor transcript levels for $PSY1$, $PSY2$, and $PSY3$ in combination with spectrophotometric analysis (Kurilich and Juvik, 1999) of total carotenoid content for developing endosperms of maize inbred B73, a line that carries the dominant $Y1$ allele. As shown in Figure 3A, carotenoid content increased 26.4-fold between 10 and 28 d after pollination (DAP). At 10 DAP, the levels were not statistically different from those of the maternal ovule tissue collected from unfertilized ear. The period of 10 to 16 DAP represented the most significant increase in content, 15.6-fold, though carotenoid accumulation continued throughout development (Fig. 3A).

While $PSY2$ levels were highest in the unfertilized ovule, compared to $PSY1$ and $PSY3$, only $PSY1$ transcript levels increased significantly during endosperm carotenogenesis, starting after 12 DAP. In the B73 inbred, $PSY1$ mRNA levels declined after 22 DAP and dropped down to significantly lower levels at 28 DAP. In contrast to maize $PSY1$, $PSY2$ mRNA levels were constant during endosperm development, except between 10 and 14 DAP, when $PSY1$ transcripts increased and $PSY2$ transcripts decreased; when $PSY1$ transcript levels decreased late in endosperm development at 24 DAP, $PSY2$ transcript levels increased. Maize $PSY3$ transcripts remained at relatively low levels, which were 30-fold lower than that of maize $PSY1$ at 20 DAP (Fig. 3A); a slight reduction in $PSY3$ transcripts was also observed from 10 to 12 DAP, as seen for $PSY2$.

The $PSY1$ antiserum was used to examine temporal accumulation of $PSY1$ for comparison with transcript accumulation in developing endosperm (Fig. 3B). Early in endosperm development (10 DAP) and in unfertilized ovule tissue, transcripts were present, but no $PSY1$ antigen was detected. Even though transcript levels were about the same between 10 and 12 DAP, PSY protein was first detected at 12 DAP, suggesting that PSY accumulation might be linked to endosperm development and plastid biogenesis, as previously observed for posttranscriptional regulation of PSY destined for chloroplasts (Welsch et al., 2000). The period of 14 to 16 DAP also showed the steepest rise in accumulated carotenoids for a given time frame, following induction of PSY protein seen between 10 and 12 DAP. After 22 DAP when carotenoid accumulation was leveling off, protein levels started to be reduced, in parallel to the reduced $PSY1$ transcript levels.

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**Figure 2.** Localization of maize $PSY1$ in maize B73 endosperm. Endosperm dissected at 17 DAP was probed with: A, anti-maize $PSY1$ antiserum and anti-rabbit IgG FITC conjugate; or B, anti-rabbit IgG FITC conjugate only. Bar = 50 μm (A) and 100 μm (B). Arrow points to amyloplast within an endosperm cell.
PSY gene family transcript accumulation was also examined in maize carrying the PSY1 mutant allele, y1-602C. PSY2 transcripts increased about 3-fold between 15 and 25 DAP (Fig. 4) in comparison to more constant levels observed for PSY2 in B73, which carries the Y1 allele (Fig. 3A); PSY3 transcripts in y1-602C were at a constant and low level throughout development, as seen in B73. These results suggest that under nonmutant conditions of the Y1 allelic background, PSY1 expression (or some carotenoid intermediate/product) may have a negative feedback effect on transcript accumulation for PSY2 but not PSY3. The inverse temporal accumulation of PSY1 relative to PSY2 and PSY3 in the Y1 endosperm of B73 (Fig. 3A) is consistent with this interpretation, for which further analysis is needed to better understand the underlying mechanisms. Endosperm transcript accumulation for PSY2 in the y1 background was later (20–25 DAP) than seen for PSY1 in the Y1 background (12–18 DAP), which might suggest that endosperm carotenogenesis is linked with a specific window of gene expression that is associated with the PSY1 (Y1) gene but not with PSY2.

**Correlating Timing of PSY1 Transcript Levels with Endosperm Carotenogenesis**

Although PSY1 was recognized for its importance in endosperm carotenogenesis, it was unknown how timing of expression might influence carotenoid content. Also, maize PSY3 was a new paralog, not yet evaluated in terms of endosperm carotenogenesis (Li et al., 2008). If it were known when and if PSY transcript levels correlated with carotenoid accumulation, then timing could be applied as a factor in breeding elevated carotenoid levels; timing of expres-

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**Figure 3.** Temporal control of PSY transcripts and carotenoid accumulation in developing maize endosperm. Maize B73 inbred was pollinated and endosperms or unfertilized ovules (UN) dissected at indicated days after pollination. A, Quantitative RT-PCR amplification of transcripts for PSY gene family members compared with mature kernel carotenoid content; B, PSY1 protein levels detected with anti-PSY1 antiserum. Transcript levels of maize PSY genes were normalized to levels of β-actin transcripts measured in the same samples and made relative to the maize PSY1 transcript level at 20 DAP. Values represent the mean of three RT-PCR replicates ± SD.

**Figure 4.** PSY transcripts in developing endosperms of y1-602C plants. Endosperms were dissected at indicated days after pollination from field-grown y1-602C plants. Transcript levels were measured by quantitative RT-PCR and normalized to transcript levels of β-actin measured in the same samples and made relative to maize PSY1 transcript levels at 20 DAP in maize B73. There was no detectable signal for PSY1 transcripts in the endosperm of y1-602C allele using real-time PCR. Values represent the mean of three RT-PCR replicates ± SD.
sion could be used in screening for optimal alleles (e.g. assessing mRNA levels at specific endosperm developmental time points) or controlling temporal expression of PSY transgenes (e.g. by choice of promoter that provides transcription in a given temporal window during endosperm development). To assess whether timing of PSY transcript level correlated with carotenoid content, advantage was taken of the genetic and chemical diversity available in maize. A subset of 10 inbred lines was chosen that exhibited a range in expression in a diverse maize germplasm collection. A correlation analysis of PSY1 gene transcripts and carotenoid content quantified as described in “Materials and Methods.” Pearson correlation analysis revealed that PSY1 was the only gene whose transcripts showed significant correlation with total carotenoids (Fig. 5, A and B); this statistically significant correlation was specifically seen at 20 DAP (96% correlation [r] and P value of 0.001). Therefore, PSY1 transcript level at 20 DAP is a good predictor of carotenoid content influenced by PSY1 expression and therefore may be used in selecting optimal PSY1 alleles for breeding or designing optimal timing of transgenes.

**PSY2, the Only Paralog That Is Up-Regulated by Light in Photosynthetic Tissue**

During de- etiolation or under high-light stress, carotenoid biosynthesis is induced to assist photosynthesis and to protect against photooxidative damage. Transcript levels of several genes encoding carotenogenic enzymes, especially PSY, have been shown to be up-regulated by light, which leads to carotenoid accumulation (Bartley and Scolnik, 1993; von Lintig et al., 1997; Simkin et al., 2003). Therefore, maize PSY gene family transcripts were quantified during de-etiolation to assess paralog-specific roles in leaf carotenogenesis. Dark-grown maize B73 inbred seedlings were illuminated with white light (approximately 50 μmol m⁻² s⁻¹) for varying amounts of time (0–8 h) as described in the “Materials and Methods” section. In etiolated seedlings, the transcript level of PSY1 was about 3-fold higher than that of PSY2 (Fig. 6, 0 h). Upon illumination, it was observed that the PSY2 mRNA levels were up-regulated by light. PSY2 transcript levels started to increase after 1 h of illumination and reached the highest level at 2 h, being 6-fold higher than that of the unilluminated control. After 2 h, PSY2 transcript levels began to decline, reaching approximately the same levels observed in the unilluminated control measured after 8 h illumination. In contrast to PSY2, which exhibited light modulation of transcript levels, PSY1 transcript levels in leaves were not affected by light. Maize PSY3 transcript levels remained at very low levels during de-etiolation, suggesting that PSY3 is not involved in light-induced leaf carotenogenesis.

**Role of Phytochrome in Light-Mediated Induction of PSY2 Transcript Levels**

To determine the light quality required for photoinduction of PSY2 transcript levels, dark-grown maize B73 seedlings were exposed to 100 μmol m⁻² s⁻¹ of red, far-red, or blue light and transcripts measured by quantitative RT-PCR. All of the light regimes induced transcript elevation, although they had specific temporal signatures and effects (Fig. 7, A, C, and E). In red and far-red light (Fig. 7, A and E), PSY2 mRNA levels began increasing after 2 h and peaked at 4 h, with a 5-fold increase in PSY2 mRNA as compared to the unilluminated control; the far-red light response showed slower kinetics compared to the red-light response. In comparison, blue light (Fig. 7C) caused a more rapid and elevated accumulation of PSY2 mRNA levels; levels increased at 1 h and peaked after only 2 h in blue light, causing a 10.5-fold increase in PSY2 transcripts as compared to the unilluminated control. These light regimes were also tested with PSY1 and PSY3, and as predicted from the white light experiments (Fig. 6), photoinduction was not observed (Supplemental Figs. S2 and S3).
replicates in unilluminated seedlings. Values represent the mean of three RT-PCR same conditions and were made relative to maize PCR. All quantifications were normalized to light (50 m

Figure 6. PSY transcript profiles during white light de-etiolation of seedlings. Nine-day-old maize B73 seedlings were treated with white light (50 \mu mol m^{-2} s^{-1}) for 0 to 8 h. Total cDNAs were prepared from leaves of illuminated seedlings for use as templates for quantitative RT-PCR. All quantifications were normalized to \beta-actin amplified using the same conditions and were made relative to maize PSY1 transcript levels in unilluminated seedlings. Values represent the mean of three RT-PCR replicates \pm SD from five pooled plants. [See online article for color version of this figure.]

The observed induction by red and far-red light suggested that phytochrome was involved in mediating PSY2 photoinduction. To verify this, loss of the red and far-red light photoinduction was tested by blocking phytochrome activity. This was accomplished by using maize seedlings that were homozygous for the recessive elongated mesocotyl 1 (elm1) allele that interferes with biosynthesis of the phytochrome chromophore, thus preventing assembly of all phytochrome types (Sawers et al., 2002). Dark-grown elm1 (Y1) seedlings were subjected to the three light regimes. No increase in PSY2 transcripts was observed after illumination by red or far-red light (Fig. 7, B and F). These results were consistent with the role of phytochrome in mediating PSY2 up-regulation by red or far-red light. When elm1 plants were subjected to blue light (Fig. 7D), photoinduction was still observed and PSY2 transcript levels peaked at 2 h as seen for B73 (which carries the dominant Elm1 allele), although the down-regulation at 4 h in B73 was delayed to 6 h in elm1 plants. The blue light result in elm1 plants was also as expected, because blue light photoinduction is not phytochrome mediated but instead mediated by other photoreceptors such as phototropins or cryptochromes (Briggs and Olney, 2001; Im et al., 2006). The slight temporal change in the blue light induction may be due to some crosstalk between the red and blue light signaling. Again, PSY1 and PSY3 transcript levels were tested in the elm1 plants, and, as expected, no change was observed as compared to B73 seedlings treated with the three light regimes (Supplemental Figs. S2 and S3). In summary, carotenogenesis that accompanies photomorphogenesis involves the photoinduction of PSY2, which is mediated both by phytochrome and nonphytochrome receptors.

PSY1 Is Essential for Heat Stress-Induced Carotenogenesis in Photosynthetic Tissue

As shown above, photoinduction of PSY2 transcript levels was shown to be linked with carotenogenesis in tissue undergoing photomorphogenesis; PSY1 and PSY3 were clearly found to be unresponsive to light. Assuming that PSY1 plays no role in leaf carotenogenesis, it was puzzling to note that in etiolated seedlings that harbor proplastids and low carotenoid levels relative to light-grown seedlings (Table I), PSY1 transcripts were more abundant than those for PSY2 (Fig. 6). Moreover, in fully green, de-etiolated seedlings (Fig. 6) or in light-grown seedlings (Gallagher et al., 2004), transcript levels for PSY1 and PSY2 were equivalent; in mature leaves, the level of PSY2 transcripts actually exceeded those for PSY1 (Li et al., 2008). Although the role of PSY1 in endosperm carotenogenesis is well established, this unexpected abundance of PSY1 transcripts in green and etiolated leaves suggested that perhaps PSY1 might also have a role in green tissue carotenogenesis. To test this possibility, a PSY1 null allele of y1 was needed. Two classes of y1 alleles have been reported in maize by Robertson’s earlier studies (Robertson and Anderson, 1961; Robertson, 1987): those with white endosperm and normal green leaves, and those with white endosperm and pale-green leaves. The y1-602C allele used in earlier experiments (Fig. 1) is representative of Robertson’s class 1, which affects carotenogenesis in endosperm but not leaves, because the mutation affects only the promoter. Therefore, plants carrying y1 alleles (obtained from the Maize Genetics Stock Center, University of Illinois, Champaign-Urbana, IL) were screened to identify one carrying a null allele, which would likely fall into Robertson’s class 2 mutants. Sequence analysis revealed that in one of the PSY1 mutants, y1-8549, a G was inserted 377 bp downstream of the ATG initiator codon, which caused a frame shift and resulted in a new stop codon at 388 bp (Fig. 8A). Previous reports of this allele (Robertson and Anderson, 1961) indicated that higher temperatures exacerbated the mutant phenotype and homozygous plants were extremely weak and generally did not survive to maturity under their field conditions, although the cause was unknown at the time. Therefore, the y1-8549 null allele was used to investigate the impact of blocking PSY1 activity on carotenoid accumulation in etiolated and mature leaves.

The Y1 nonmutant and y1-8549 mutant seedlings were grown under four conditions varying for light and heat stress, where 37°C is considered heat stress and 20°C is within the optimal temperature range (Wilhelm et al., 1999). Under low temperature (20°C) in the dark, the total carotenoid levels (including both carotenes and xanthophylls) of y1-8549 seedlings were
62% of the levels found in nonmutant Y1 seedlings (Table I, values); carotenes alone were only 24% of the normal (Table I, ratios). When the temperature was increased to 37°C, Y1 seedlings grown in the dark responded with a 2.2-fold higher level of carotenoids, whereas the carotenoid level in y1-8549 seedlings was unchanged and therefore exhibited only 30% of normal levels, suggesting that the PSY1 null allele was interfering with the response to elevated temperature under dark growth conditions.

Under low temperature (20°C) in the light, y1-8549 seedlings showed only a slight difference compared to Y1 seedlings; carotenoid and chlorophyll levels in y1-8549 were 92% and 72% of the levels found in Y1 seedlings, respectively (Fig. 8, B and C; Table I). However, when grown at 37°C in the light, the PSY1 null mutant was photobleached and showed only 3% carotenones, 9% xanthophylls, and 21% chlorophyll, as compared to plants carrying the normal allele (Table I, ratios). Carotenoid levels in leaves of Y1 plants grown at 37°C in the light were approximately 6% higher than found in plants grown at 20°C in the light, as compared to a 2.2-fold elevation in carotenoids when plants were grown in the dark at 37°C. One explanation for the limited heat-stress response in the light may be due to photooxidation of pigments, which takes place at high temperatures for plants grown in the light and leads to carotenoid degradation, thus limiting carotenoid accumulation (Britton, 1995). If the role of PSY1 is to contribute to heat-stress activation of carotenoid biosynthesis, then the observed photobleaching seen in y1-8549 plants that was exacerbated by high temperatures (Robertson and Anderson, 1961) might be due to rapid degradation of carotenoids, which are insufficiently replenished due to limited biosynthetic capacity in the absence of a functional maize PSY1.

Under dark growth conditions, the mRNA levels of PSY1 in the y1-8549 mutant were 30% and 40% of the Table I.

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>Y1 Normal Values</th>
<th>y1-8549 Mutant Values</th>
<th>Mutant to Normal Ratio</th>
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<td></td>
<td>Chlorophyll</td>
<td>Carotene</td>
<td>Xanthophylls*</td>
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<td>16.30 ± 1.66</td>
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<tr>
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<tr>
<td>37°C light</td>
<td>493.24 ± 34.64</td>
<td>142.81 ± 11.53</td>
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*Xanthophylls include lutein, neoxanthin, violaxanthin, and antheraxanthin.
levels found in Y1 at 20°C and 37°C, respectively, while the mRNA levels of PSY2 were 1.5-fold greater at 20°C and 3-fold greater at 37°C in the mutant compared to Y1 (Fig. 8B). The higher accumulation of PSY2 transcripts seen in the mutant might be a result of feedback regulation of PSY1 on PSY2 expression; in the Y1 background, expression of the functional Y1 product and/or its ensuing activity somehow represses PSY2 mRNA accumulation, and in the mutant, this repression is absent such that PSY2 expression partially compensates for the PSY1 deficiency. This observation is reminiscent of the case seen in endosperm when either PSY1 transcripts were temporally reduced or absent, in the case of the y1-602C endosperm mutant (Fig. 4). When the seedling growth temperature was raised, the transcript levels of both PSY genes decreased in Y1 and y1-8549 seedlings. This decrease in PSY transcripts in etiolated Y1 plants was unexpected given the concurrent increase in carotenoid accumulation at high temperatures.

In summary, carotenoid levels doubled in etiolated Y1 seedlings subjected to heat stress as compared to normal temperature, whereas y1-8549 plants responded with only a slight increase in carotenoid accumulation; in the light, y1 mutants actually showed a severe decrease in carotenoids under heat stress conditions and appeared photobleached. The simplest explanation is that in the light, heat-stressed plants are subjected to photo-oxidative damage that can be relieved by increased carotenoid synthesis that is mediated by PSY1, a protective mechanism not available in the PSY1 null mutant seedlings. Therefore, these data support a role for PSY1 in leaf carotenogenesis, especially under heat stress conditions.

DISCUSSION

We recently established that three maize PSY paralogs arose prior to evolution of the grasses (Gallagher et al., 2004; Li et al., 2008). There remain open questions about the specific roles of these three genes that encode a key enzyme operating at the pathway entry point; the six downstream steps all appear to involve single-copy genes in maize (Li et al., 1996; Matthews et al., 2003; Li et al., 2007; Harjes et al., 2008). Whereas some species contain a single PSY gene, the PSY gene duplication in the grasses could potentially provide grasses with a fine tune control of carotenoid biosynthesis for different regulatory scenarios.

PSY1 Timing in Endosperm Carotenogenesis

From early genetic analysis to more recent association studies, the PSY1 locus has been associated with maize endosperm carotenoids (Randolph and Hand, 1940; Palaisa et al., 2003). Additionally, in maize and wheat, a major quantitative trait locus for endosperm carotenoids maps to the PSY1 locus (Wong et al., 2004; Pozniak et al., 2007). However, it was unclear what was the temporal relationship between gene expression during endosperm development and kernel carotenoid content. Efforts to develop strategies that provide pre...
dictable changes in plant chemistry, or "predictive metabolic engineering," are dependent on availability of such data. In the absence of such information, use of a tissue-specific promoter provides tissue specificity but not necessarily the optimal window of expression for maximizing pathway end-product accumulation. Therefore, endosperm carotenoid accumulation was determined for developing kernels, together with PSY transcripts and protein. Carotenoid accumulation was observed from 10 DAP to maturity. This pattern of continuous accumulation is in contrast to that seen in sorghum varieties, which exhibited a peak of accumulation mid-development followed by a steep drop (Kean et al., 2007). Next, temporal expression of PSY1 was quantified in maize B73 and nine other maize inbred lines that spanned carotenoid content and composition in the maize diversity panel (Harjes et al., 2008). By combining both transcript and carotenoid data, correlation was tested for carotenoid content and transcript levels of PSY gene family members. PSY1 transcript level at 20 DAP was found to be a good predictor of carotenoid accumulation and therefore may be useful in selecting optimal PSY1 alleles for breeding or designing optimal timing of transgenes.

The maize diversity lines are a powerful resource for gaining insight into the regulation of plant chemistry across genetically diverse germplasm. When used in association analysis, the diversity lines may provide information on polymorphisms that can be used for breeding, as in the case of improving provitamin A carotenoid content (Harjes et al., 2008). As applied here, these lines provided insight into the timing of candidate gene expression. There will likely be other steps and/or regulatory factors that may affect carotenoid accumulation and for which expression can be examined using these diversity lines. Germplasm diversity collections of other species, for which metabolic breeding holds interest, represent untapped value to examine candidate gene expression in relation to metabolite composition.

**Amyloplast Envelope Membrane Localization of PSY1**

Maize PSY1 was shown to be resident on endosperm amyloplast envelope membranes, suggesting that the envelope membrane is the site of the carotenoid biosynthetic metabolon in amyloplasts. An envelope location for the pathway may not be limited to amyloplasts. Early studies provided evidence that an envelope-localized carotenoid biosynthetic pathway might also exist in chloroplasts. Chloroplast envelope membranes were shown to contain a unique carotenoid profile compared to thylakoid membranes (Jeffrey et al., 1974; Siefermann-Harms et al., 1978); chloroplast envelope membrane extracts also exhibited enzyme activity for conversion of zeaxanthin to violaxanthin (Costes et al., 1979). More recent reports have localized PSY and other pathway enzymes to thylakoid membranes in chloroplasts (Linden et al., 1993; Bonk et al., 1997; Welsch et al., 2000). Therefore, the carotenoid pathway may actually be two pathways from a topological standpoint, potentially existing on envelope and thylakoid membranes. In amyloplasts, the pathway is likely associated with the envelope membrane while in chloroplasts, the pathway may be associated with both envelope and thylakoid membranes. In assembly of the biosynthetic metabolons, the nuclear-encoded pathway enzymes are recruited to membrane locations that may differ from one plastid type to another. Because many of the pathway enzymes are encoded by single copy genes (Li et al., 1996; Matthews et al., 2003), specificity of targeting must be controlled by factors beyond the transit sequence. Optimization of metabolic engineering/breeding of carotenoids is predicated on elucidating the mechanisms that control recruitment of pathway enzymes to metabolons on different plastid membranes in various plastid types.

**PSY2 Is Associated with Photomorphogenesis**

Given the involvement of carotenoids in photosynthetic tissue, it was expected that the PSY genes were photoregulated. For example, the single PSY gene in Arabidopsis was shown to be regulated by continuous red, far-red, and blue light regimes (von Lintig et al., 1997). However, in a Y1 genetic background, only PSY2 was photoregulated by light, mediated by phytochrome-dependent and phytochrome-independent mechanisms that involved blue light signals. Maize PSY1 and PSY3 did not respond to light of any spectral type. It was previously demonstrated that PSY3 was associated with carotenogenesis leading to drought and salt-induced ABA biosynthesis in roots (Li et al., 2008); recent work on rice PSY3 showed the rice homolog to have a similar role in rice roots (Welsch et al., 2008). Unlike maize, rice PSY1 and PSY2 are both photoregulated, although blue light was not tested (Welsch et al., 2008), and rice PSY1 is not expressed in endosperm (Gallagher et al., 2004). Therefore, the ancestral Poaceae PSY was a photoregulated gene; the duplications analyzed in Y1 maize represent losses in the photoregulatory mechanisms and evolution of other features, including the gain-of-function endosperm-specific expression seen for PSY1 in Y1 maize, but absent in its evolutionary ancestor teosinte and other subfamilies of the grasses, including species such as rice.

**A Second Role for PSY1 in Photosynthetic Tissue and Thermal Tolerance**

Examination of leaf carotenogenesis revealed involvement for both maize PSY1 and PSY2, indicating that PSY1 is not exclusively associated with endosperm carotenogenesis. In addition to endosperm function, PSY1 was shown to be required for leaf carotenogenesis, in the dark and under conditions of heat stress. When PSY1 expression was eliminated in the maize y1-8549 PSY1 null mutant, the plant had reduced carotenoids in the dark and suffered severe
photobleaching at elevated temperatures in the light. Dual roles for a supposedly “nonphotosynthetic tissue” PSY paralog are not unique to maize. The loss of the “fruit-specific” PSY1 in the tomato r,r mutant showed reduced carotenoid accumulation not only in fruit but also in leaf tissue (Fraser et al., 1999). In that case, it is not known whether the tomato PSY1 plays a role in stress resistance. While PSY1 (y1) has been labeled as the gene needed for endosperm carotenogenesis, the data presented indicate that PSY1 is essential in controlling carotenogenesis in photosynthetic tissue. Its absence has severe effects on plant vigor that are exacerbated by high temperature. It is unclear at present how PSY1-controlled carotenogenesis mediates stress resistance in leaf tissue. PSY1 is not impacting ABA biosynthesis, as carotenoids are not limiting in photosynthetic tissue as they are in roots (Li et al., 2008). Because carotenoids are known to protect against heat-stress-induced lipid peroxidation, it appears that PSY1 is the maize paralog that is essential for heat-stress-induced biosynthesis of carotenoid antioxidants that protect plastid membranes (Davison, 2002; Havaux et al., 2007). The implications of these findings are that efforts to manipulate PSY1 expression in endosperm may potentially cause changes in PSY1 expression in photosynthetic tissue if PSY1 alleles are chosen for use in breeding programs to enhance endosperm carotenoids without considering the dual role of PSY1 in endosperm and leaf stress responses. On the other hand, this aspect of PSY1 related to thermostolerance may open new avenues for breeding more stress-tolerant lines.

Posttranscriptional Regulation

Examination of gene expression in some of the mutants suggested that the various PSY genes may be subject to feedback regulation. In different tissues, absence of PSY1 transcripts was associated with elevation of PSY2 transcripts. During endosperm development, there was an inverse relationship seen between transcripts for PSY1 relative to transcripts for PSY2 or PSY3. In leaf tissue, where PSY1 was absent due to a null mutation in y1, there appeared a concomitant increase in transcripts for PSY2 under heat stress conditions. PSY1 may also be subject to posttranscriptional regulation as suggested by the absence of endosperm PSY1 protein at early developmental time points where transcripts were detected; perhaps translation is linked to amyloplast biogenesis in a similar fashion, whereby there appears to be a link between chloroplast biogenesis and translation of PSY mRNAs in Arabidopsis (von Lintig et al., 1997).

Each of the maize PSY paralogs evolved unique roles to control carotenogenesis in different tissues and in response to stress. The ongoing maize studies are greatly facilitated by the mutant and germplasm diversity collections, which generally are unavailable for research on other agronomically important grasses. However, phylogenetic identification of orthologous genes may suggest potential targets for plant improvement whether it is to enhance plant yield through stress resistance or in nutritional improvement.

MATERIALS AND METHODS

Plant Materials

Maize (Zea mays) plants used were phytochromobilin synthase mutant elm1 (Sawers et al., 2002), inbred line B73 (which carries dominant alleles for Y1 and Elm1), PSY1 (y1) recessive mutants, y1-602C and y1-8549 (602M), also known as pastel-8549 (Robertson and Anderson, 1961), and maize near-isozyme diversity lines (T. Rocheford, University of Illinois, Champaign-Urbana, IL). Plants homozygous for y1-602C have a colorless endosperm with normal leaf phenotype, and plants homozygous for y1-8549 have colorless endosperm and are temperature sensitive with pale-green leaves when grown at high temperature (Robertson and Anderson, 1961). For experiments related to endosperm, maize plants were field grown, and endosperms were dissected at various DAP and stored at -80°C until analysis.

Total Carotenoid Content Measurement

The carotenoid extraction procedure used was slightly modified from Kurilich and Juvik (1999). Tissue samples of 500 mg were ground in 6 mL of ethanol containing 0.1% butylated hydroxytoluene and were incubated in an 85°C water bath for 6 min prior to a 10-min saponification with 120 μL (1 g/μL) of KOH. All samples were vortexed once during saponification, after which they were immediately placed in ice to which 4 mL of cold deionized distilled water was added to each sample, followed by 3 mL of petroleum ether:diethyl ether (2:1, v/v), vortexed, and centrifuged for 10 min at 3,500 rpm. The upper layer was transferred to a fresh tube, and the aequous phase was twice subjected to centrifugation with 3 mL of petroleum ether:diethyl ether each time and combined the fractions. The combined fractions were topped to 10 mL, and 1 mL of aliquot was used to measure total carotenoids at optical density of 450 nm using a Lambda 25 UV/VIS spectrometer (PerkinElmer Life Sciences).

Real-Time PCR

RNA isolation and cDNA synthesis were carried out as described by Gallagher et al. (2004). Real-time PCR was performed using iQ SYBR green Supermix (Bio-Rad) with 10 ng of cDNA. Primers and PCR conditions for maize PSY1, PSY2, PSY3, and the internal control actin were described previously (Li et al., 2008). 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. The fold change of transcript abundance of target genes was first calculated as 2^{-ΔΔCt}, where ΔΔCt 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 ± s.d.

Generation of Anti-Maize PSY1 Antibodies

A partial B73 maize Y1 cDNA (clone 12A33A, GenBank no. NM_001114652), provided by Dr. Brent Buckner (Truman State University, MO), was subcloned as a BglII-XhoI fragment into the BamHI-XhoI sites of the Novagen vector pET23c (+) and renamed pY1ex1-1. After transformation of BL21(DE3) pLysS, induction with 1 mM final concentration of isopropyl-β-D-thiogalactopyranoside at early stationary phase, and lysis, approximately 800 μg of inclusion body fraction containing the 32.9-kD Y1 fusion protein (including 12 amino acids, 1.32 kD of T7 Tag encoded by the vector, and Y1 protein [amino acid nos. 124–410, 31.57 kD]) was separated by 12% SDS-PAGE and the fusion protein band (about 50% yield) was excised and immersed in 5 mL of 125 mM NaCl. Rabbits, chosen for absence of preexisting immune response to maize proteins, were injected with 100 μg of Y1 fusion protein preparation at weeks 1, 2, 3, and 5 (Lampire Biological Laboratory). For western analysis and localization experiments, blood from rabbit number 3445 (5 weeks after immunization)
Expression of Maize PSY2 and PSY3 T7 Tag Fusion Proteins

To test the specificity of anti-maize PSY1 antiserum, maize PSY2 and maize PSY3 T7 Tag fusion proteins were also expressed using the PET23 vector (Novagen). pEMP5S2-1 contained a partial B73 maize PSY2 cDNA (GenBank accession no. AY458064), encoding amino acids 109 to 462 (33.46 kD) of PSY2 (Gallagher et al., 2004) fused to 16 residues (1.67 kD) of the T7 Tag, resulting in a PSY2 fusion protein of 35.13 kD. pEMP5S3 was constructed by inserting a full-length maize PSY3 cDNA (encoding residues 1-426, 47.32 kD, GenBank DQ364330) into the PET23c(+) vector (in-frame fusion with 16 residues of a T7 Tag, encoding 1.67 kD) to produce a PSY3 fusion protein of 48.99 kD. Expression of the fusion proteins was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside for 4 h when the Escherichia coli cells reached an A600 of 0.6 at 37°C. To detect the fusion protein by immunoblot analysis, the inclusion bodies were isolated and fractionated on a 12% SDS-polyacrylamide gel. Anti-T7 Tag antibody, AP conjugated (Novagen), was used and diluted 1:10,000 in Tris-buffered saline plus Tween 20 for use in western analysis according to the manufacturer’s protocol (Novagen). Anti-maize PSY1 antibody, anti-maize PSY2 and PSY3 fusion proteins to test the antibody specificity.

Western Analysis to Detect mPSY1 Expression Level in Endosperm

Maize proteins from endosperms were extracted according to the method described by Wurtzel et al. (1987). Protein samples were separated on 12% SDS-PAGE in a Criterion Cell system (Bio-Rad) and transferred to nitrocellulose membrane for western blot in a Trans-Blot Cell (Bio-Rad). The first antibody, anti-maize PSY1, and the second antibody, goat anti-rabbit IgG, were diluted at 1:10,000 for use in western analysis according to the manufacturer’s protocol (Novagen). To control for protein loading, a duplicate western was probed with a polyclonal antiserum raised against maize Shrunken1 protein (Wurtzel et al., 1987).

Immunolocalization of PSY in Maize Endosperm

Maize B73 endosperms at 17 DAP were dissected and immersed in 50 mM sodium phosphate buffer, pH 7.2, and fixed according to Zhang and Bostock (1990) for modifications. Several slices (2 x 4 x 1 mm) for each tissue were fixed in 3% glutaraldehyde in sodium phosphate buffer either for 2 h at room temperature or overnight at 4°C. Fixed slices were washed with sodium phosphate buffer at room temperature three times for 15 min each, dehydrated at room temperature through a series of ethyl alcohol 10%, 25%, 50%, 70%, and 95% (v/v) for 10 min each, then twice in 100% ethyl alcohol for 10 min each. Slices were infiltrated in 25%, 50%, and 75% (v/v) LR white (Polysciences) in ethyl alcohol at room temperature for 2 h each and then in 100% (v/v) LR white at room temperature overnight. Slices were transferred to 100% LR white in beam capsules (Polysciences) and polymerized at 65°C for 24 h. One μm semi-thin sections were prepared using glass knives on an Ultra Microtome (Leica). Sections were collected onto precoated glass slides (Polysciences) and air dried. All steps of immunofluorescent labeling were carried out at room temperature. Slides were rehydrated in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4) for 15 min, blocked in 0.5% (w/v) bovine serum albumin (BSA) in PBS for 30 min, incubated with primary antiserum (anti-maize PSY1) diluted at 1:25 in BSA/PBS overnight in a moist chamber, washed with PBS three times (5 min each), labeled with the goat anti-rabbit IgG-FITC conjugate (Sigma Diagnostic) for 2 h, and washed with PBS three times at 5 min each. Slides were air dried and mounted with coverslips using Aqua Poly/Mount as an anti-fading agent (Polysciences). For negative controls, primary antisera were replaced with 0.5% (w/v) BSA in PBS. Slides were observed using a fluorescent microscope with a blue filter, excitable at 488 nm. Photographs were taken under a MRC-600 Laser Scanning Confocal Imaging system (Bio-Rad) at Columbia University. Images were processed using Adobe Photoshop software (Adobe Systems).

Correlation Analysis between PSY Transcript Levels and Carotenoid Content of Maize Diversity Lines

For each of 10 diverse lines, PSY transcript levels in developing endosperms at specified DAP were measured by quantitative RT-PCR as described; total kernel carotenoid content was described (Islam, 2004; Harjes et al., 2008). Correlation between transcript levels and carotenoid content was evaluated using JMP (version 5.1; SAS Institute) to perform the Pearson correlation analysis (r) and to test for statistical significance (P) of the correlation between transcript levels and carotenoid content. Statistically significant correlation was based on a P value ≤ 0.05.

De-Etiolation Experiment

Seeds of maize (B73 and elm1) were surface sterilized and grown on Murashige and Skoog agar medium (Murashige and Skoog, 1962) with 1.5% (w/v) Suc. B73 and homozygous elm1 plants were grown, until the two-leaf stage, in the dark at 28°C for 9 d and 11 d, respectively. For de-etiolation, seedlings were transferred to continuous white light (approximately 50 μmol m−2 s−1), red (100 μmol m−2 s−1), far-red (100 μmol m−2 s−1), or blue light (100 μmol m−2 s−1) and irradiated for 1, 3, 4, 6, and 8 h at 24 h and room temperature. At the end of the illumination period, leaves from five plants were randomly collected and immediately frozen for RNA extraction. Etiolated seedlings that were kept in the dark for the same time periods served as controls. Far-red light was supplied at a fluence rate of 100 μmol m−2 s−1 by Q-beam FR LED light sources (Quantum Devices) at a fluence rate of 100 μmol m−2 s−1 half bandwidth <25 nm, and blue light was supplied from 464-nm LED sources in a Percival E30-LED incubator with fluorescence at 100 μmol m−2 s−1, half bandwidth <30 nm. Light intensity and spectral output were measured with a LI-COR Li-1800 spectroradiometer.

Screening Maize PSY1 Null Mutants by Sequencing

The full-length maize PSY1 cDNA and three genomic DNA segments covering the entire open reading frame and 590 bp upstream were PCR amplified from y1 alleles requested from the Maize Genetics Cooperation Stock Center (University of Illinois, Urbana, IL). Primers were designed from the published y1 sequence (GenBank accession no. ZMU32636; Buckner et al., 1996). For primer sequences and PCR conditions, refer to Supplemental Table 1. The maize PSY1 cDNA was amplified from total cDNA synthesized from leaf genomic DNA in a Peracll E30-LED incubator with fluorescence at 100 μmol m−2 s−1 half bandwidth <35 nm. Light intensity and spectral output were measured with a LI-COR Li-1800 spectroradiometer.

Analysis of Carotenoid Composition of y1-8549 Mutant

Homozygous y1-8549 mutants were grown in the dark or in the light under either low temperature (20°C) or high temperature (37°C) conditions. The etiolated leaves (two) of dark-grown plants and the third leaves of light-grown plants were collected for chlorophyll and carotenoid analysis using HPLC. Chl a and Chl b were extracted into 80% (v/v) acetone and quantified as described (Lichtenthaler, 1987). Carotenoid extraction and HPLC analysis were carried out as described by (Quinlan et al., 2007). Lutein and β-carotene were identified by comparison with commercial standards. Neoxanthin and violaxanthin standards were isolated by thin-layer chromatography of carotenoids (Cammarata and Schmidt, 1992).

Accession Numbers

Sequence data from this article can be found in the GenBank under accession numbers EU306868 (y1-8549) and EU306889 (y1-602C).
Maize PSY Subfunctionalization


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