

The Maize Phytoene Synthase Gene Family: Overlapping Roles for Carotenogenesis in Endosperm, Photomorphogenesis, and Thermal Stress Tolerance^{1[C][W][OA]}

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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-

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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

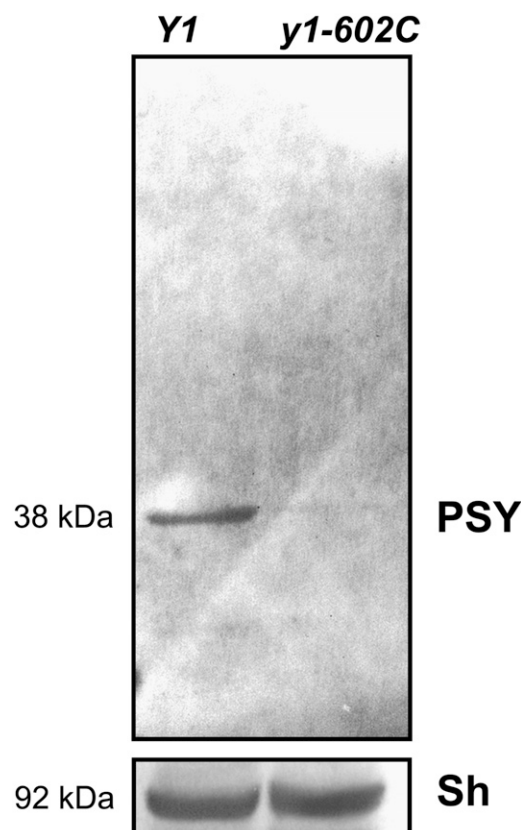


Figure 1. Western analysis demonstrating specificity of anti-*PSY1* antiserum for *PSY* in maize endosperm. Proteins were extracted from yellow (*Y1*) and white (*y1*) endosperm at 20 DAP. Immunoblots were probed by anti-*PSY1* antiserum (top) or anti-Shrunken1 (*Sh*) antiserum (bottom).

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.

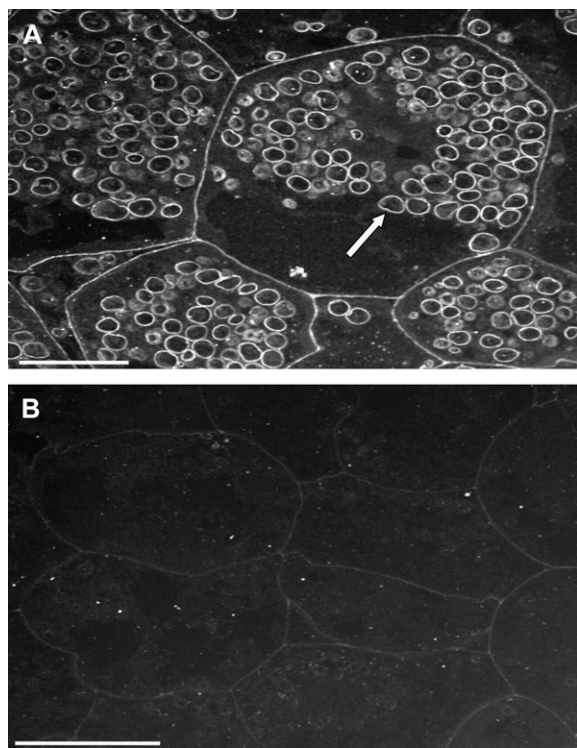


Figure 2. Localization of maize *PSY1* in maize B73 endosperm. Endosperm dissected at 17 DAP was probed with: A, anti-maize *PSY1* antiserum and anti-rabbit IgG FITC conjugate; or B, anti-rabbit IgG FITC conjugate only. Bar = 50 μ m (A) and 100 μ m (B). Arrow points to amyloplast within an endosperm cell.

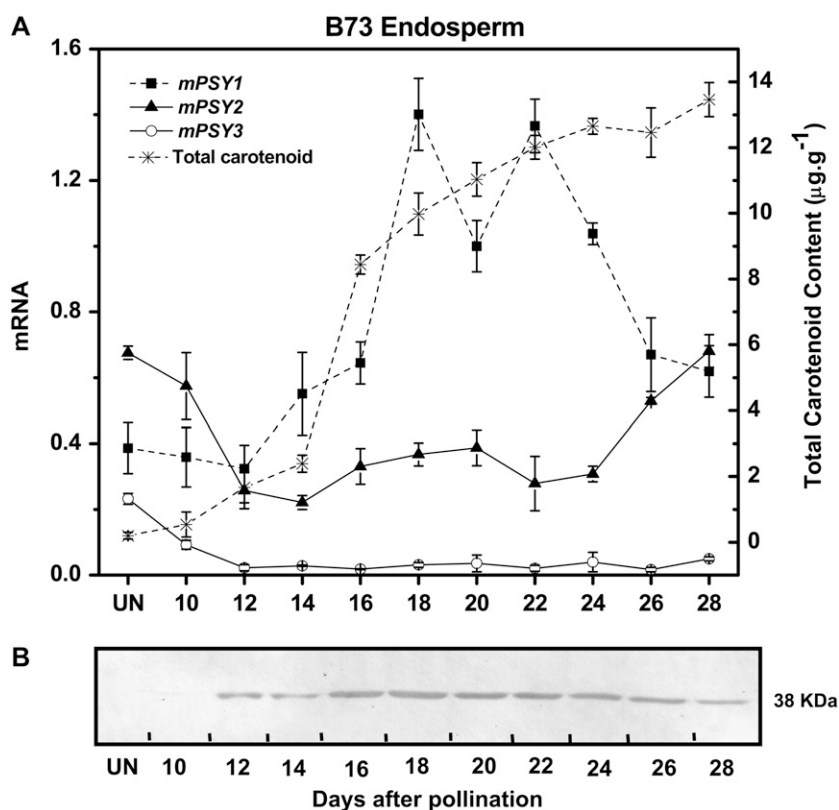


Figure 3. Temporal control of *PSY* transcripts and carotenoid accumulation in developing maize endosperm. Maize B73 inbred was pollinated and endosperms or unfertilized ovules (UN) dissected at indicated days after pollination. A, Quantitative RT-PCR amplification of transcripts for *PSY* gene family 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 $\pm$ SD.

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 con-

tent. 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-

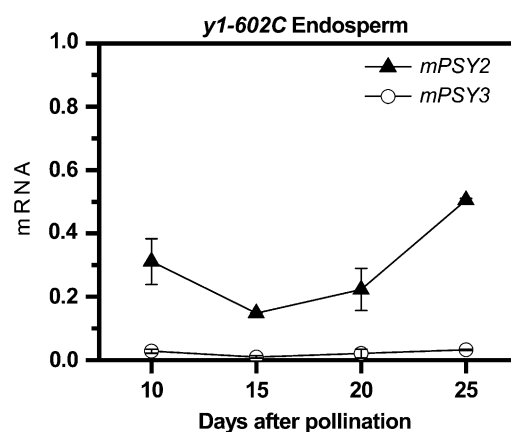


Figure 4. *PSY* transcripts in developing endosperms of *y1-602C* plants. Endosperms were dissected at indicated days after pollination from field-grown *y1-602C* plants. Transcript levels were measured by quantitative RT-PCR and normalized to transcript levels of β -actin measured in the same samples 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 $\pm$ 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 carotenoid content and composition (Harjes et al., 2008). Plants were control pollinated, endosperms dissected at 15, 20, and 25 DAP, and then *PSY* 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 (Y1) seedlings were illuminated with white light (approximately $50 \mu\text{mol m}^{-2} \text{s}^{-1}$) 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 $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ 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* mRNAs 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).

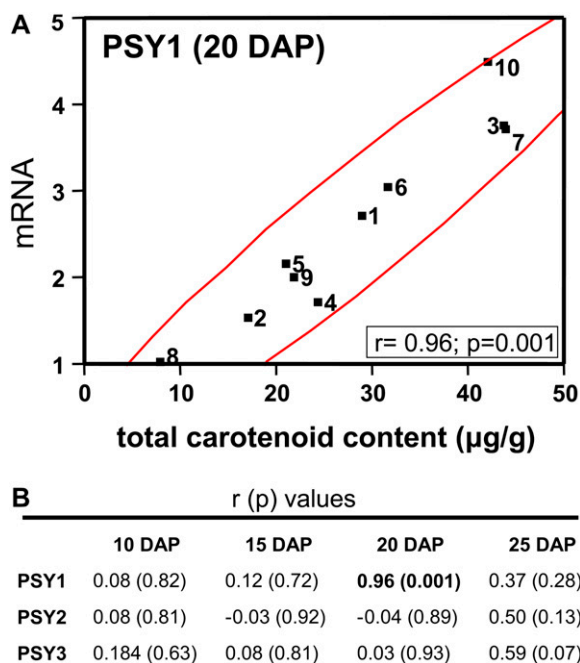


Figure 5. Timing *PSY* expression in a diverse maize germplasm collection. A, Correlation analysis of *PSY1* transcript levels at 20 DAP compared to carotenoid content of mature kernels in 10 chemically and genetically diverse lines (Harjes et al., 2008). Inbred lines are: 1, A619; 2, B73; 3, B37; 4, C17; 5, C131A; 6, DE3; 7, KUI2007; 8, NC300; 9, SD44; 10, TZ118. B, Pearson correlation (r) and statistical significance (P) for correlations made between *PSY* transcript levels at various endosperm developmental time points and carotenoid content for *PSY1*, *PSY2*, and *PSY3*. Correlation is significant for $P < 0.05$. Only *PSY1* transcript levels at 20 DAP showed significant correlation with carotenoid content. [See online article for color version of this figure.]

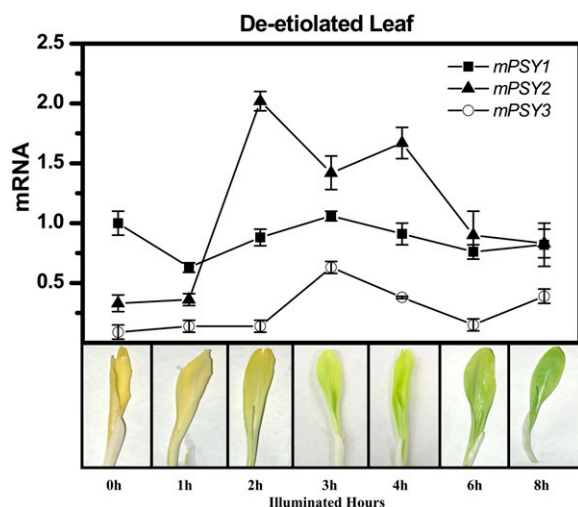


Figure 6. *PSY* transcript profiles during white light de-etiolation of seedlings. Nine-day-old maize B73 seedlings were treated with white light ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 0 to 8 h. Total cDNAs were prepared from leaves of illuminated seedlings for use as templates for quantitative RT-PCR. All quantifications were normalized to β -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 mesocotyl1* (*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 cross talk 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

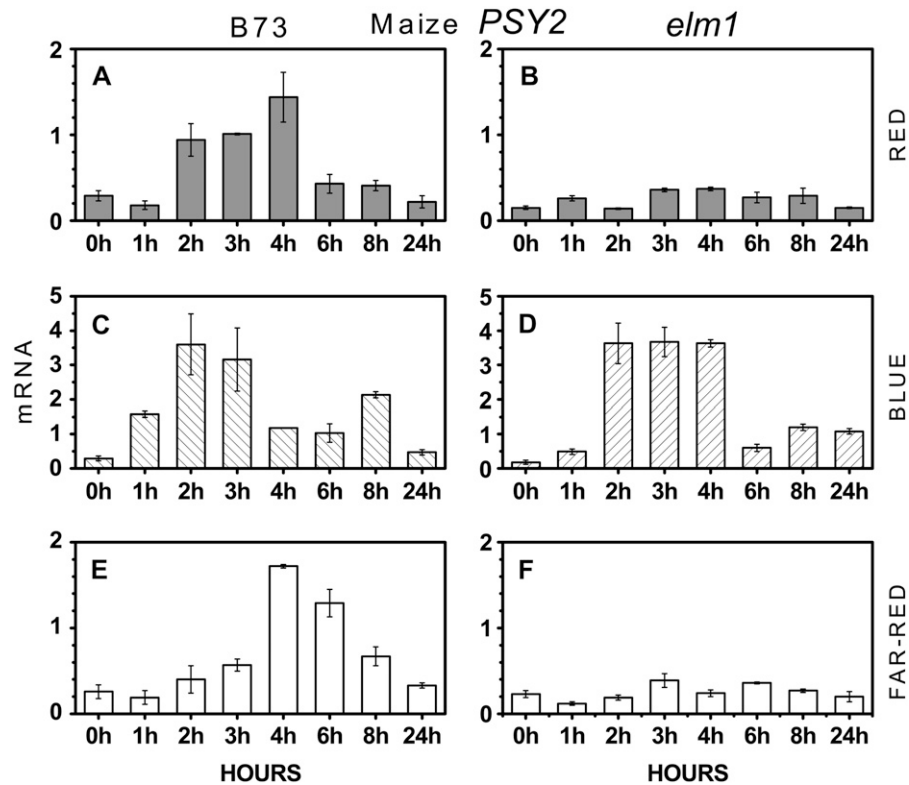
accompanies photomorphogenesis involves the photoinduction of *PSY2*, which is mediated both by phytochrome and nonphytochrome receptors.

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 1), *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

Figure 7. *PSY2* transcript levels in de-etiolating maize B73 (*Elm1*) and *elm1* mutant seedlings treated with light of various spectral qualities. Dark-grown B73 (A, C, and E, left) and *elm1* (B, D, and F, right) seedlings were illuminated with red light ($100\ \mu\text{mol m}^{-2}\text{ s}^{-1}$; A and B), blue light ($100\ \mu\text{mol m}^{-2}\text{ s}^{-1}$; C and D), or far-red light ($100\ \mu\text{mol m}^{-2}\text{ s}^{-1}$; E and F) and quantitative real-time PCR was used to determine transcript levels for maize *PSY2*. All quantifications were normalized to β -actin and shown relative to maize *PSY1* transcript levels at 0 h using the same conditions. Values represent the mean of three RT-PCR replicates $\pm$ SD from five pooled plants.



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% carotenes, 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. Chlorophyll, carotene, and xanthophyll analysis of *Y1* and *y1-8549* mutant seedlings
f.w., Fresh weight; n.a., not applicable.

Growth Condition	Y1 Normal Values			y1-8549 Mutant Values			Mutant to Normal Ratio		
	Chlorophyll	Carotene	Xanthophylls ^a	Chlorophyll	Carotene	Xanthophylls ^a	Chlorophyll	Carotene	Xanthophylls ^a
			$\mu\text{g g}^{-1}\text{ f.w.}$						
20°C dark	n.a.	2.10 $\pm$ 0.04	16.30 $\pm$ 1.66	n.a.	0.51 $\pm$ 0.05	10.89 $\pm$ 1.75	n.a.	0.24	0.67
37°C dark	n.a.	2.56 $\pm$ 0.17	37.74 $\pm$ 4.86	n.a.	0.61 $\pm$ 0.09	11.51 $\pm$ 1.19	n.a.	0.24	0.30
20°C light	535.31 $\pm$ 19.16	119.31 $\pm$ 9.49	177.40 $\pm$ 7.70	385.42 $\pm$ 26.11	119.24 $\pm$ 6.37	155.30 $\pm$ 12.31	0.72	1.00	0.88
37°C light	493.24 $\pm$ 34.64	142.81 $\pm$ 11.53	174.27 $\pm$ 10.97	105.89 $\pm$ 7.25	3.72 $\pm$ 0.86	16.40 $\pm$ 2.28	0.21	0.03	0.09

^aXanthophylls include lutein, neoxanthin, violaxanthin, and antheraxanthin.

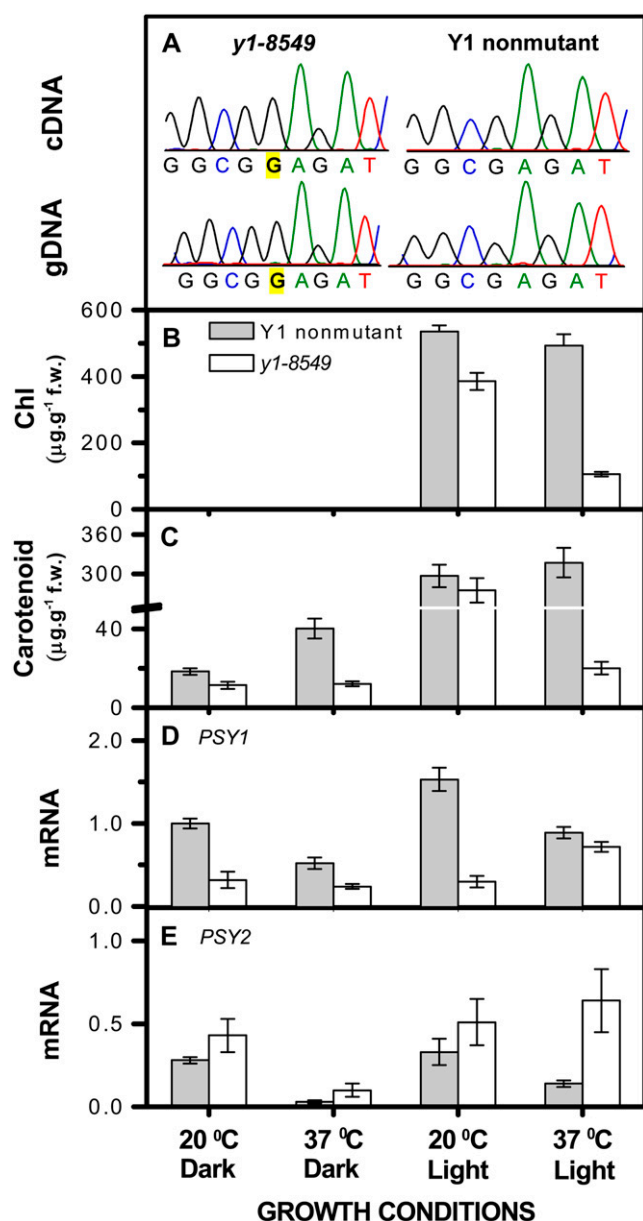


Figure 8. Effect of *PSY1* loss on maize leaf carotenogenesis. To assess the impact of functional *PSY1* loss on maize leaf carotenogenesis, the *y1-8549* mutant was grown under four conditions (dark and 20°C, dark and 37°C, light and 20°C, and light and 37°C). Chlorophyll and carotenoid content and transcript profiles of maize *PSY1* and *PSY2* of *y1-8549* mutant were analyzed. A, Sequence analysis of maize *PSY1* cDNA and genomic DNA prepared from leaf tissue of *y1-8549* mutant and B73 inbred line. The G insertion in the *PSY1* genomic DNA and cDNA prepared from *y1-8549* is bolded/highlighted. B, Chlorophyll content; C, carotenoid content. D and E, The mRNA levels of maize *PSY1* (D) and *PSY2* (E) in leaf tissue were normalized to levels of β -actin transcripts measured in the same samples and are shown relative to maize *PSY1* mRNA levels from seedlings grown in the dark at 20°C. Values represent the mean of three RT-PCR replicates $\pm$ SD from five pooled plants. [See online article for color version of this figure.]

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 thermotolerance 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 tools for research on other agronomically important grasses. However, phylogenetic identification of or-

thologous 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 germplasm 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/mL) 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 aqueous 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 $2^{-\Delta\text{Ct}}$, 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 $\pm$ SD.

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 *Bgl*III-*Xho*I fragment into the *Bam*HI-*Xho*I 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 immuniza-

tion) was used and diluted 1:10,000 in Tris-buffered saline plus Tween 20 for westerns and 1:25 for confocal experiments. Specificity of the antiserum was verified by testing for presence of the antigen in normal, but not in homozygous *y1* endosperm, using the *y1* maize COOP alleles 602C (shown in Fig. 1) and 607C (data not shown), which reduced endosperm carotenoids but did not affect leaf carotenoids.

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). pEMPSY2-1 contained a partial B73 maize PSY2 cDNA (GenBank accession no. AY450646), encoding amino acids 109 to 402 (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 product of 35.13 kD. pEMPSY3 was constructed by inserting a full-length maize PSY3 cDNA (encoding residues 1–426, 47.32 kD, GenBank DQ36430) 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 A_{600} 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 at 1:10,000 dilution to detect the induced T7 Tag fusion proteins. Then anti-maize PSY1 antiserum at 1:5,000 dilution was used to test for antigenicity with 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 Boston (1992) with modifications. Several slices ($2 \times 4 \times 1$ 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 time. 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 μ m 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 Na_2HPO_4 , 1.4 mM KH_2PO_4) 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 Diagnostics) 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 (approximately $50 \mu\text{mol m}^{-2} \text{s}^{-1}$), red ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$), far-red ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$), or blue light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) and irradiated for 1, 2, 3, 4, 6, and 8 and 24 h at 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 $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ by Q-beam FR LED light sources (Quantum Devices) with λ_{max} at 735 nm and filtered through far-red plastic resin (FRF700; Westlake Plastics) to yield a half-maximum bandwidth of <25 nm with no detectable emissions below 690 nm. Red light was obtained from 667-nm Q-beam LED sources (Quantum Devices) at a fluence rate of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ half bandwidth <25 nm, and blue light was supplied from 464-nm LED sources in a Percival E30-LED incubator with fluence rate of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, half bandwidth <30 nm. White light was obtained from cool-white fluorescent bulbs. 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 S1. The maize PSY1 cDNA was amplified from total cDNA synthesized from leaves. The PCR conditions were 94°C for 30 s, 56°C for 30 s, 72°C for 90 s, 35 cycles. The PCR conditions for isolating genomic DNA segments were 94°C for 45 s, 56°C for 45 s, 72°C for 2 min, 40 cycles with leaf genomic DNA as template. All PCR products were purified using the MinElute PCR purification kit (Qiagen) and verified by sequencing.

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. Chla and Chlb 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 EU306869 (*y1*-8549) and EU306869 (*y1*-602C).

Supplemental Data

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

Supplemental Figure S1. Western analysis to test specificity of the anti-maize PSY1 antiserum.

Supplemental Figure S2. PSY1 mRNA levels in maize treated with light of various spectral qualities.

Supplemental Figure S3. PSY3 mRNA levels in maize treated with light of various spectral qualities.

Supplemental Table S1. Primers for analyzing PSY1 mutant alleles.

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Supplementary materials

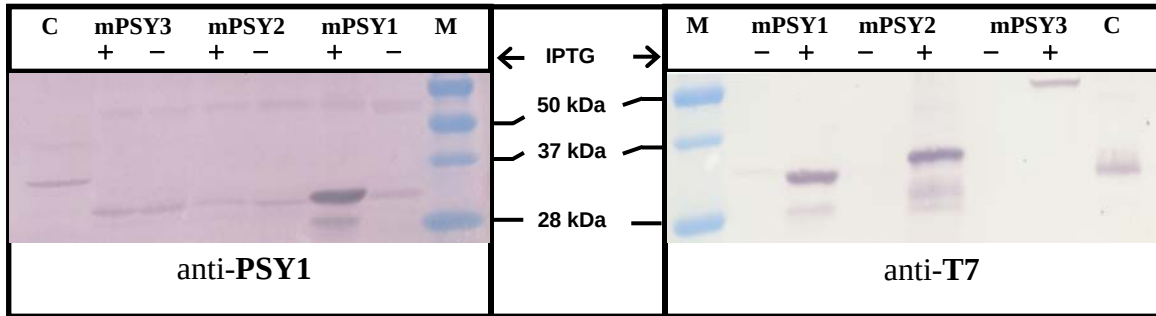


Figure 1S. Western analysis to test specificity of the anti-maize PSY1 antiserum against maize PSY2 and PSY3 fusion proteins. PSY proteins were expressed as fusion constructs with T7 tags and extracted from *E. coli* before (-) and after (+) IPTG induction. Blots were probed with anti-maize PSY1 antiserum (left) or anti-T7-Tag Antibody, AP Conjugated (right). The control “C” for anti-maize PSY1 antiserum and anti-T7 western blots were total protein extracted from 20 DAP B73 endosperm and T7.tag positive control (Novagen), respectively. M, molecular weight marker with sizes indicated in kDa.

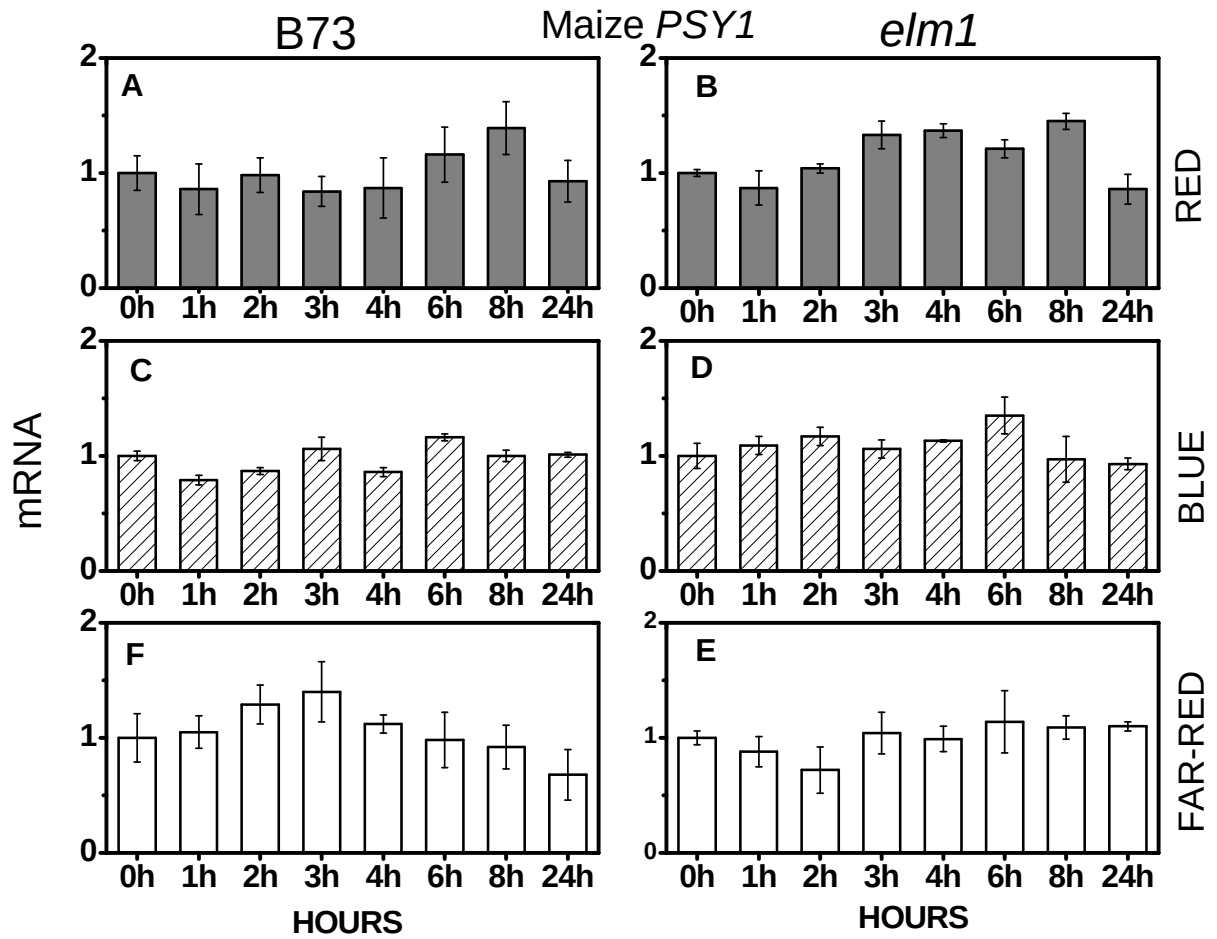


Figure 2S. *PSY1* transcript levels in de-etiolating maize B73 (*Elm1*) and *elm1* mutant seedlings treated with light of various spectral qualities. Dark-grown B73 (panels on left A,C, and E) and *elm1* (panels on right, B, D, and F) seedlings were illuminated with red light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) (panels A, B), blue light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) (panels C, D), or far-red light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) (panels E, F) and quantitative real time PCR was used to determine transcript levels for maize *PSY1*. All quantifications were normalized to β -actin and shown relative to maize *PSY1* transcript levels at 0 h using the same conditions. Values represent the mean of 3 RT-PCR replicates $\pm$ SD from five pooled plants.

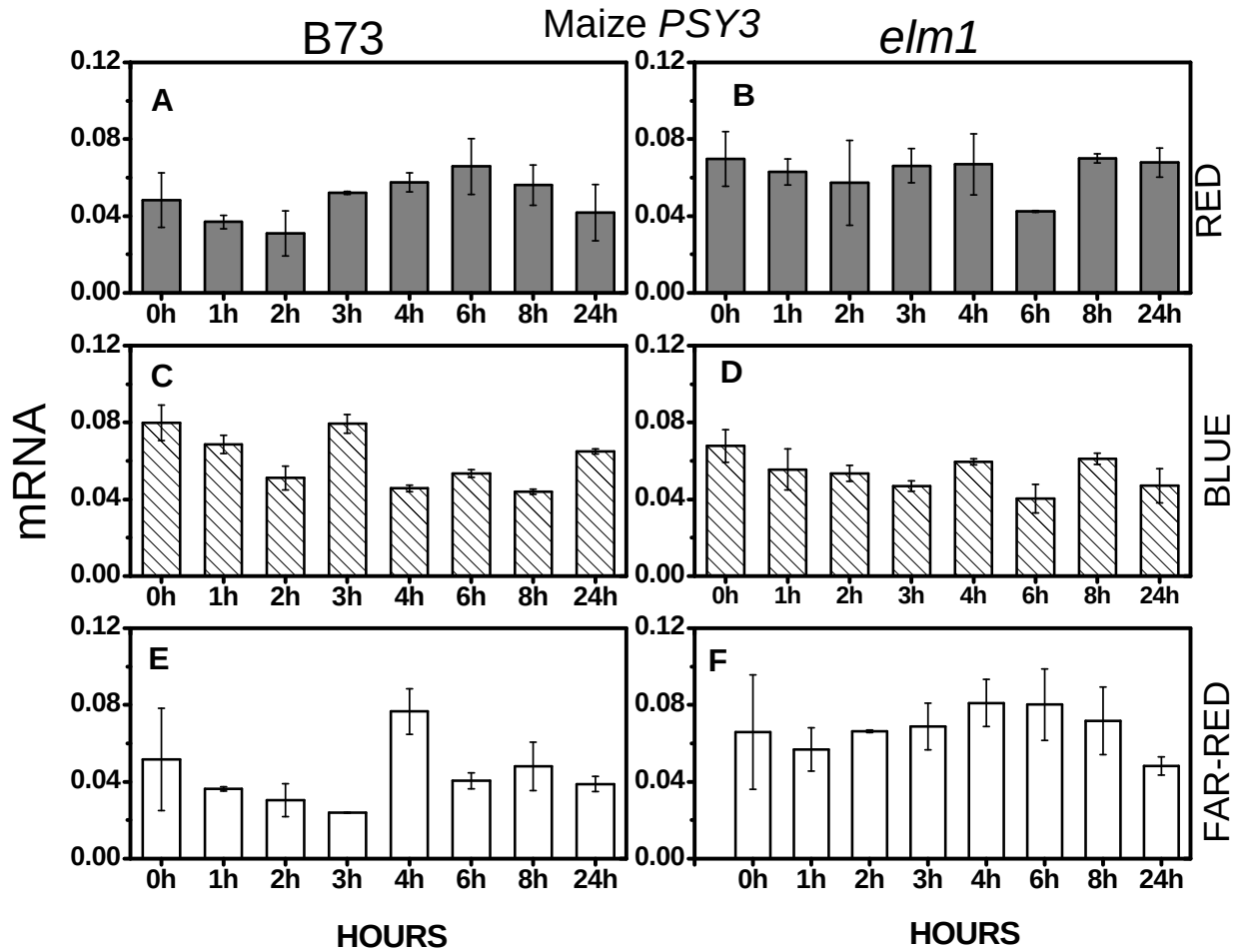


Figure 3S. *PSY3* transcript levels in de-etiolating maize B73 (*Elm1*) and *elm1* mutant seedlings treated with light of various spectral qualities. Dark-grown B73 (panels on left A,C, and E) and *elm1* (panels on right, B, D, and F) seedlings were illuminated with red light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) (panels A, B), blue light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) (panels C, D), or far-red light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) (panels E, F) and quantitative real time PCR was used to determine transcript levels for maize *PSY3*. All quantifications were normalized to β -actin and shown relative to maize *PSY1* transcript levels at 0 h using the same conditions. Values represent the mean of 3 RT-PCR replicates $\pm$ SD from five pooled plants.

Table Is. Primers for analyzing genomic DNA and cDNA of *yl-8549* and *yl-602c* maize *PSY1* mutant alleles.

Analyzed genomic DNA regions in <i>yl-8549</i> and <i>yl-602c</i> mutants		
Region	Sequence Range*	Primer sequences
1	1344-2472	# 217 GATGCCCAATGCCAAGAAATAACC # 344 GGCGCGGCGCCGCTCCTCTGTC
2	2201-3246	#509 GCATTGCTCAAACGCCAG #519 TCAGAGAGAGCGGCATCAAG
3	3229-5016	#257 TGATGCCGCTCTCTCTGATA #657 TCATCAAGGCCTCCTGATGTT
Analyzed cDNA regions in <i>yl-8549</i> and <i>yl-602c</i> mutants		
1	Whole ORF	#627 <u>AT</u> GCCATCATACTCGTACGAG #657 TCATCAAGGCCTCCTGATGTT

* Sequence ranges are based on the sequence data of U32636