

Article Addendum

The *phytoene synthase* gene family in the Grasses

Subfunctionalization provides tissue-specific control of carotenogenesis

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Abbreviations: PSY, phytoene synthase; ABA, abscisic acid

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Carotenoids are a complex class of isoprenoid pigments playing diverse roles in plants and providing nutritional value. Metabolic engineering of the biosynthetic pathway has been of interest to specifically address global vitamin A deficiency by breeding cereal crop staples in the Poaceae (Grass family) for elevated levels of provitamin A carotenoids. However, there remain open questions about the rate-controlling steps that limit predictability of metabolic engineering in plants, whether by transgenic or nontransgenic means. We decided to focus on the first committed biosynthetic step which is mediated by phytoene synthase. Our studies revealed that in the Grasses, PSY is encoded by three genes. Maize transcript profiling, together with carotenoid and ABA analysis, revealed that the three PSY copies have subfunctionalized and provide the Grasses with a fine tune control of carotenogenesis in response to various developmental and external cues. Promoter analysis supports subfunctionalization; cis-element analysis of maize *PSY1* alleles and comparison with Grass orthologs suggests that man's selection of yellow maize endosperm has occurred at the expense of a change of gene regulation in photosynthetic tissue as compared to the progenitor white endosperm *PSY1* allele.

Carotenoids represent a diverse group of naturally occurring pigments found in various taxonomies, including plants, fungi

and bacteria.¹ In higher plants, carotenoids function as accessory pigments for photosynthesis and prevent photo-oxidative damage.² Apocarotenoids, carotenoid cleavage products, include the plant hormones abscisic acid (ABA) and strigolactone.^{3,4} ABA plays an important role in regulating plant abiotic stress responses, differential growth and embryo dormancy; the recently discovered strigolactone inhibits shoot branching. For humans and animals, plant dietary carotenoids serve as precursors of vitamin A and other nutritional factors.⁵ In recent years, improvement of pro-vitamin A carotenoid content in seed endosperm of crop staples has been an important goal for alleviating global vitamin A deficiency.^{6,7} However, complexity and poorly understood pathway regulation are barriers to predictive metabolic engineering, especially in the Grass family (Poaceae) containing the major cereal crop staples (e.g., maize, sorghum, millets, rice, wheat, oats, barley, rye).

Predictable manipulation of the carotenoid biosynthetic pathway reviewed in⁸ necessitates elucidation of biosynthetic step(s) that control carotenoid accumulation in various tissues. Previous studies suggested that control of flux to carotenoids is mediated by phytoene synthase (PSY), the first committed enzyme in the plastid-localized pathway.⁹⁻¹³ PSY catalyzes the condensation of two molecules of geranylgeranyl pyrophosphate into 15-*cis* phytoene, the backbone of all C40 carotenoids and derived apocarotenoids. In Arabidopsis, tomato and other dicot plants, the nuclear-encoded *PSY* genes were shown to be upregulated during plastid development, leading to carotenoid accumulation in leaf, flower or fruit;¹⁴⁻¹⁶ transgenic over-expression of *PSY* causes elevated carotenoid content.^{6,17} However, additional studies are needed to facilitate predictable manipulation of this pathway in the many agronomically important food crops of the Poaceae. Therefore, we chose maize as a model system and the *PSY* gene as a key potential regulator of pathway flux to study carotenoid regulatory mechanisms in the Grasses. The maize B73 inbred line was the standard for study since carotenoids are found in both leaves and endosperm, and therefore comparisons of gene expression could be made among various carotenogenic tissues.

In Arabidopsis, a single *PSY* gene regulates 15-*cis* phytoene synthesis in all tissues. In contrast, most Grass species contain at least two *PSY* genes; *PSY1* expression was shown to be strongly associated with endosperm carotenoid accumulation in maize and carotenoid absence in rice.^{10,13} More recent examination of the completed rice

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and

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genome led to identification of a new *PSY* gene, *PSY3*, which was found to be present not only in rice, but also in maize and sorghum.¹⁸ All three genes were shown to encode functional enzymes in a heterologous bacterial platform.^{13,18} Quantitative transcript profiling for the three *PSY* genes revealed that the maize *Y1* locus, *PSY1*, encoded the most abundant transcript in leaf and endosperm, carotenoid-accumulating tissues. In contrast, maize *PSY3* transcripts were found predominately in root and embryo, carotenoid-limited tissues.¹⁹ The tissue-specific transcript patterns suggested that the maize *PSY* genes might be subfunctionalized and not merely redundant copies. We therefore tried to address the following questions: (1) What are internal and external cues that stimulate steady state mRNA levels of each maize *PSY* gene? (2) How do the three *PSY* genes contribute to controlling carotenogenesis for different physiological purposes? (3) Is *PSY* subfunctionalization unique to maize or is it a more general phenomenon seen in other Grasses? (4) What are the promoter *cis*-elements that may be responsible for the tissue-specific transcript patterns of the *PSY* genes?

Endosperm Carotenogenesis

In developing endosperm of the maize B73 inbred, or other genetically diverse inbreds carrying the *Y1* allele, *PSY1* was the only gene family member for which transcript levels significantly increased during endosperm development and correlated with accumulation of endosperm carotenoids;²⁰ only low levels of *PSY2* and *PSY3* transcripts were detected in endosperm. The contribution of *PSY2* and *PSY3* to endosperm carotenoid accumulation was further evaluated in a line carrying the *PSY1* allele, *y1-602C*. This allele carries a promoter mutation blocking *PSY1* expression in endosperm but not in leaves. In *y1-602C* plants, the presence of *PSY2* and *PSY3* endosperm transcripts which can potentially encode functional proteins, could not compensate for the absence of endosperm *PSY1*. These data indicated that presence of functional *PSY1* in endosperm is critical for carotenoid accumulation and the contribution of *PSY2* and *PSY3* was negligible. It could be that function of *PSY2* and *PSY3* proteins in endosperm plastids is prevented by an unknown mechanism that interferes with protein translation, protein stability or metabolon biogenesis, among other possibilities.

Leaf Carotenogenesis

The roles of maize *PSY* genes in leaf carotenogenesis were determined by monitoring transcript changes during de-etiolation. In dark-grown plants, *PSY1* represented the major transcript. *PSY2* was the only paralog for which transcript levels increased in response to illumination, suggesting that *PSY2* plays an important role in controlling leaf carotenogenesis during greening. Moreover, the photoinduction of *PSY2* by red or far-red light was repressed in the maize phytochrome deficit mutant *elongated mesocotyl1 (elm1)*,²¹ indicating that phytochromes mediate *PSY2* photoinduction by red and far-red light. In contrast, photoinduction of *PSY2* was still observed in blue light illuminated *elm1* seedlings, suggesting that another photoreceptor in addition to phytochrome, might be involved in blue light induction of *PSY2*.

Abiotic Stress: Thermotolerance

Although maize *PSY1* was unresponsive to light during greening, we could not completely exclude a role in leaf carotenogenesis

because of the high abundance of leaf *PSY1* transcripts. Thus, we used a *PSY1* frame-shift mutant, *y1-8549*, to investigate the effect on leaf carotenogenesis when *PSY1* function was eliminated. At high temperature, *y1-8549* mutant leaves were bleached due to photo-oxidative damage and chlorophyll and carotenoid levels decreased dramatically. These results suggested that functional maize *PSY1* is essential for maintaining leaf carotenoid content, particularly under heat stress growth conditions. Therefore, maize *PSY1* in a *Y1* background, has an overlapping role in controlling both endosperm and leaf carotenogenesis.

Abiotic Stress: Drought and Salt

The abundance of *PSY3* transcripts in roots suggested that *PSY3* might have a unique role in root carotenogenesis, perhaps in the role of apocarotenoid formation. Moreover, searching of GenBank revealed a link between rice *PSY3* expression and the ABA pathway or abiotic stresses, indicating that *PSY3* in the Grasses may be involved in abiotic stress-induced carotenogenesis or in regulation of ABA biosynthesis. Maize seedlings were therefore subjected to various abiotic stresses to test this hypothesis. In roots, the levels of maize *PSY3* mRNAs were induced by drought, salt and exogenous application of ABA.¹⁸ Moreover, the elevation in *PSY3* transcripts was accompanied by induced levels of carotenoid intermediates, elevation of other downstream carotenogenic genes, and followed by ABA accumulation. The levels of *PSY2* mRNAs were also affected by abiotic stress in leaves but *PSY1* mRNAs were not elevated in any tissues tested. In particular we showed that root ABA accumulation is limited by *PSY3* expression and de novo carotenogenesis in contrast to leaf ABA induction which is known to be regulated by carotenoid cleavage and not by carotenoid synthesis.²²

The prevalence of rice *PSY3* ESTs associated with abiotic stress was the rationale for us to investigate stress-induced regulation of maize *PSY3*. The upregulation of maize *PSY3* transcript levels in response to abiotic stresses suggested that *PSY3* responses were not unique to maize. Soon after we characterized the role of maize *PSY3* in root carotenogenesis, the subfunctionalization of *PSY* paralogs in rice was also described by Welsh et al.²³ Both rice *PSY2* and *PSY3* shared similar upregulation patterns with their maize orthologs; rice *PSY2* was also photoinducible and root *PSY3* mRNA levels increased during ABA formation in response to salt or drought stress. However, maize and rice *PSY1* genes were different in their tissue specificity and in light responsive pattern; transcript levels of maize *PSY1* showed endosperm developmental responses but lacked photoinduction, whereas the rice paralog showed the reverse, photoregulation without endosperm expression. We were intrigued that orthologs of only two of the three genes shared regulatory responses. We hypothesized that the progenitor allele, *y1*, found in many noncarotenogenic endosperm maize inbreds and the wild ancestor, teosinte, might actually possess photoregulation for *PSY1*; that domestication and cultivation may have led to selection of an allele exhibiting endosperm expression at the expense of light regulation.

Promoter Elements and Subfunctionalization

Examination of the *PSY1* promoters in the grasses might give some clues as to the basis for the differential gene expression among the *PSY1* alleles and across species. We hypothesized that the different expression pattern of maize *PSY1* might be due to a change

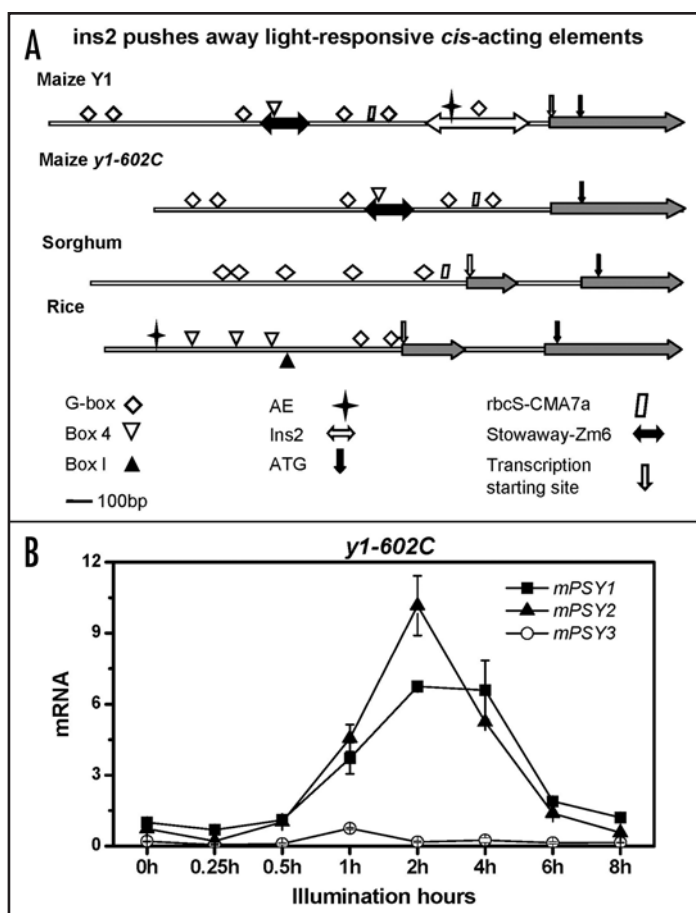


Figure 1. *ins2* transposon in maize *Y1* pushes away light-responsive cis-acting elements. (A) light-responsive cis-acting elements within maize *Y1* and *y1-602C* allele, sorghum and rice *PSY1* 5' upstream regions were predicted with PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html>). Transposon *ins2* inserts 300 bp upstream of transcript starting site in maize *Y1* but not in allele *y1-602C*. (B) light-induced *PSY1* and *PSY2* expression during de-etiolation in *y1-602C* allele. Dark-grown nine-day-old dark-grown maize *y1-602C* seedlings were illuminated with white light ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 0 to 8 h. The leaves of illuminated seedlings were harvested for cDNA preparation and used as templates for quantitative RT-PCR as carried out previously.²⁰ All quantifications were first normalized to β -actin amplified using the same conditions and were made relative to *PSY1* transcript levels in unilluminated seedlings. Values represent the mean of three RT-PCR replicates \pm SD from five pooled plants.

in regulatory components. Examination of maize and rice *PSY1* promoter regions revealed that both genes shared a similar cis-acting element arrangement except for some transposon insertions in the maize *PSY1* (*Y1*) promoter (Fig. 1A). The insertion of transposon *ins2* at 300 bp up-stream of the maize *PSY1* start codon has been shown to be statistically associated with endosperm carotenoid accumulation.¹⁰ We suspected this transposon insertion might have pushed away the cis-acting elements essential for *PSY1* photoinduction. To verify this hypothesis, we tested for light regulation of *PSY1* in the allele *y1-602C* which lacks the *ins2* element. When *y1-602C* seedlings were illuminated, it was observed that the mRNA levels of both *PSY1* and *PSY2* increased while only *PSY2* transcript levels were photoinduced in B73, which carries the *Y1* allele (Fig. 1B). These results suggested that the maize *y1-602C* allele is more similar to rice *PSY1* and the maize ancestor and that the *ins2* insertion in the

B73 *Y1* allele is responsible for the loss of light regulation of maize *PSY1* in *Y1* backgrounds. In summary, maize plants that accumulate endosperm carotenoids, do not have the capacity for *PSY1* photo-regulation in green tissue, but must rely on *PSY2* photoregulation alone.

Differential cis-acting elements in the *PSY* paralogs could also be responsible for the paralog-specific responses to various environmental cues. Besides the light responsive elements found in *PSY1* and *PSY2* promoters, an ABRE (ABA-responsive element) binding site was found in both rice and maize *PSY3* promoter regions but not in either *PSY1* or *PSY2*²³ and our unpublished data (Li F, Tsfadia O, Wurtzel ET).

In summary, the *PSY* gene triplication appears to be a general phenomenon in the Grasses. Subfunctionalization provides for fine control of carotenogenesis that serves numerous physiological purposes. Open questions remain regarding the unknown post-transcriptional mechanisms that control metabolon assembly and membrane association in various plastids found in different tissues.

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