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ORIGINAL RESEARCH

# Identification of Genomic Loci Associated with the Photochemical Reflectance Index by Genome-Wide Association Study in Soybean

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## Abstract

The photochemical reflectance index (PRI) is determined from canopy spectral reflectance measurements and can provide important information about photosynthesis. The PRI can be used to assess the epoxidation state of xanthophyll pigments, which provides information on nonphotochemical quenching (NPQ) and the amount of energy used for photosynthesis. Genome-wide association analyses were conducted to identify single-nucleotide polymorphisms (SNPs) and genomic loci associated with PRI using data from a soybean [*Glycine max* (L.) Merr.] diversity panel grown under field conditions over 2 yr. Based on a mixed linear model (MLM), 31 unique candidate SNPs that identify 15 putative loci on 11 chromosomes were identified. Several candidate genes known to be associated with NPQ, photosynthesis, and sugar transport processes were identified in the proximity of 10 putative loci. Violaxanthin de-epoxidase, one of the identified genes, is directly involved in the xanthophyll cycle, which plays a major role in NPQ. This study is the first to identify genomic loci for PRI and illustrates the potential of canopy spectral reflectance measurements for high-throughput phenotyping of a photosynthesis related trait. Significant SNPs, candidate genes, and genotypes contrasting for PRI identified in this study may prove useful for crop improvement efforts.

## Core Ideas

- Identification of the first loci for the photochemical reflectance index
- Application of high-throughput canopy spectral reflectance in identifying genetic loci for a photosynthetic trait
- Use of GWAS for the identification of canopy spectral reflectance trait loci in soybean

CONVERSION EFFICIENCIES of total solar energy to biomass observed for C3 photosynthetic crop species are commonly in the range of 1.0 to 4.6% (Beadle and Long, 1985; Monteith and Moss, 1977; Zhu et al., 2010). The low observed conversion efficiencies are in large part because more than half of the total incident solar radiation is not photosynthetically active radiation. The remaining losses are associated with reflection and transmission of incident light, energy losses at various steps associated with the light and dark reactions of photosynthesis, and losses as a result of respiration (Zhu et al., 2008). These include the losses that occur because, as illustrated by photosynthetic light response curves, leaves do not have the capacity to fully utilize the absorbed sunlight for $CO_2$ assimilation at high incident solar radiation. Thus, crops often experience saturating levels of irradiance under normal field conditions. Preventing

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**Abbreviations:** BLUP, best linear unbiased prediction; FDR, false discovery rate; GWAS, genome-wide association studies; LHC, light-harvesting complex; MLM, mixed linear model; NPQ, nonphotochemical quenching; PRI, photochemical reflectance index; SNP, single-nucleotide polymorphism; VDE, violaxanthin de-epoxidase.

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this excessive energy from damaging the photosynthetic machinery is paramount for plants. At midday, when plants normally are experiencing the highest levels of solar radiation, the efficiency of excitation capture by open photosystem II is reduced (Gamon et al., 1997). This reduction is associated with an increase in NPQ (Huang et al., 2006). Nonphotochemical quenching is the release of heat that occurs when more light is absorbed than can be used for photosynthesis (Li et al., 2009). Nonphotochemical quenching has long been associated with protecting the proteins associated with the light reactions of photosynthesis. However, this dissipation of energy is also associated with a reduction in quantum yield of photosystem II (Niyogi, 1999).

Nonphotochemical quenching is in large part a function of the xanthophyll cycle, which involves the epoxidation and de-epoxidation of the xanthophyll pigments, violaxanthin and zeaxanthin, and the intermediate antheraxanthin. The xanthophyll pigments are not only involved in photoprotection through dissipation of excess light absorption as heat (NPQ) but also through scavenging of reactive oxygen species (Bassi et al., 1993; Demmig-Adams and Adams, 1992, 1996; Thenot et al., 2002) and bind to the light-harvesting complexes (LHCs) associated with photosystems I and II (Krol et al., 1995).

Measuring NPQ traditionally involves dark adaptation of leaves and then determining the ratio of the difference between light- and dark-adapted maximum chlorophyll fluorescence measurements to the light-adapted maximum chlorophyll fluorescence (Niyogi et al., 1997). For NPQ determination, ~20 min of dark adaptation of leaf tissues are generally recommended before initiation of chlorophyll fluorescence measurements (Maxwell and Johnson, 2000). Not including dark adaptation, a single NPQ measurement can be made within ~5 min (Maxwell and Johnson, 2000). Nonetheless, when large numbers of measurements are desired, throughput can become a limiting factor for directly assessing NPQ. In addition, NPQ measurements are sensitive to time of day, thus constraining the number of measurements that can be made per day (Maxwell and Johnson, 2000).

When plants experience abiotic stress in conjunction with high light stress, NPQ becomes more advantageous than inhibitory for photosynthesis. In particular, NPQ can play an important role when plants experience abiotic stresses such as salt stress (Qiu et al., 2003), drought stress (Massacci et al., 2008), and high-temperature stress (Davison et al., 2002). Accordingly, approaches to increase photosynthetic efficiency and consequently crop photosynthesis and productivity by altering NPQ will need to account for its importance in photoprotection particularly under abiotic stress conditions. Nonetheless, fine tuning of NPQ could provide an avenue to improve crop photosynthesis and productivity. Exploration of the phenotypic diversity in NPQ in a genotypically diverse panel of accessions can be expected to provide much information on the range in NPQ that resulted from adaptation to different environmental conditions and can be leveraged to identify genotypes and genetic markers useful for breeding programs.

A high-throughput method to screen large populations of exotic genotypes and to subsequently phenotype large breeding populations in multiple locations would be beneficial for selection and breeding purposes. Measurements of canopy or leaf spectral reflectance are fast and amenable to high-throughput phenotyping. Thus, the discovery by Gamon et al. (1992) that the epoxidation state of xanthophylls influences light reflectance permits the use of canopy reflectance as a surrogate measurement of NPQ. Canopy reflectance measurements can be performed rapidly on field-grown plants and are amenable for high-throughput phenotyping under field conditions. The index developed to provide information on the relationship between xanthophyll pigments and reflectance characteristics is known as the photochemical reflectance index (PRI). The $R^2$ between the xanthophyll epoxidation state and PRI in sunflower (*Helianthus annuus* L.) was found to be 0.65 in plants grown under control conditions, 0.83 with plants grown under N deficiency, and 0.80 with plants grown under water stress (Gamon et al., 1992). An even better correlation between PRI and NPQ ($R^2 = 0.87$) was reported more recently by Rahimzadeh-Bajgiran et al. (2012) for eggplant (*Solanum melongena* L.). The photochemical reflectance index also has been used to investigate various aspects of photosynthesis including radiation use efficiency of barley (*Hordeum vulgare* L.) (Filella et al., 1996), photoprotection of water stressed durum wheat (*Triticum turgidum* L.) (Tambussi et al., 2002), and soybean grown in different water and N conditions (Inoue and Peñuelas, 2006). However, to date, despite the fact that canopy reflectance measurements can be obtained from a large number of field grown plants (Singh et al., 2013), no studies exist that have examined PRI in a large panel of soybean genotypes.

The advent of high-throughput genomic analyses and genome-wide association studies (GWAS) allows the identification of genomic regions associated with a trait of interest without being limited to the genetic differences encompassed by the parents of biparental populations. Genome-wide association studies have been employed successfully in soybean to investigate qualitative and quantitative traits including flower, hilum, and pubescence color, maturity, plant height, seed weight, seed oil, and seed protein (Hwang et al., 2014; Sonah et al., 2015; Zhang et al., 2015) and for physiological traits like C isotope ratio, N derived from atmosphere, as well as ureide and N concentrations (Dhanapal et al., 2015a,b; Ray et al., 2015).

Given the availability of an annotated genome sequence, loci identified by GWAS can be used to search for candidate genes that underlie the observed phenotypic differences. Despite the usefulness of PRI illustrated by many publications (Filella et al., 1996; Gamon et al., 1997; Guo and Trotter, 2004; Inoue and Peñuelas, 2006; Inamullah and Isoda, 2005; Porcar-Castell et al., 2012; Thenot et al., 2002), we are not aware of any studies that have used GWAS or biparental populations to identify

genomic regions contributing to PRI. However, the genetics and molecular mechanisms that contribute to NPQ have been and continue to be investigated (Greer, 1998; Li et al., 2000; Niyogi et al., 2001; Niyogi and Truong, 2013). While most of this work is conducted with model species, Kasajima et al. (2011) reported two loci that contributed to NPQ in rice (*Oryza sativa* L.).

Exotic accessions are increasingly being leveraged for genetic improvement of crops and can immediately be introduced into breeding programs to improve crop varieties. Nonetheless, to date, no studies have been conducted to examine the natural variation in a soybean diversity panel or to identify genetic markers associated with PRI or NPQ.

This study was conducted to identify genomic regions and genes associated with PRI and, by extension, NPQ in soybean. To this end, 373 diverse soybean genotypes were grown for 2 yr and canopy hyperspectral reflectance measurements were collected and used to calculate PRI. Putative loci associated with PRI were identified based on association analyses using 31,145 SNP markers. Genes potentially underlying the PRI phenotype were identified based on gene annotations in the vicinity of genomic regions surrounding the putative loci identified by GWAS.

## Material and Methods

### Field Management

Three replications of 373 maturity group IV soybean genotypes were grown under rainfed conditions in a randomized complete block design at the Bradford Research Centre near Columbia, MO, in 2009 and 2010. Before planting, tillage was conducted using one pass with a disk followed by one pass with a cultivator. Phosphorus and K were applied before field cultivation based on soil analyses and University of Missouri Extension recommendations (http://aes.missouri.edu/pfcs/soiltest.pdf). Sowing of 4.87 m long four-row plots with a row spacing of 0.76 m was conducted on 23 May 2009 and 27 May 2010 to a density of 25 seeds $m^{-2}$ and placement of seeds at 2.5 cm depth. Pre-emergence herbicide, Sethoxydim, was used for weed management using 2.6 kg a.i. $ha^{-1}$. Post emergence weed control was conducted with Lambda-cyhalothrin with 0.23 kg a.i. $ha^{-1}$.

### Soybean Genotypes

Soybean entries selected for this study were obtained from the USDA–ARS germplasm collection and originated from 11 nations. These nations and the number of genotypes include South Korea (244), China (60), Japan (41), North Korea (11), Georgia (6), Korea (4), Russia (2), Taiwan (2), India (1), Mexico (1), and Romania (1). Selection of the 373 maturity group IV genotypes was based on data from the Germplasm Resources Information Network (GRIN, www.ars-grin.gov). Genotypes were selected in two groups: Group 1 (182 genotypes) included the highest yielding (>2.5 Mg $ha^{-1}$) genotypes with good agronomic characteristics (plant height, low lodging, and shattering) while Group 2 (191 genotypes) consisted of genotypes with good agronomic characteristics but lower yields (<2.5 Mg $ha^{-1}$). To maximize the genetic diversity of Group 2, unlike with Group 1, selection of the genotypes included consideration of country and province of origin.

### Canopy Reflectance

Canopy spectral reflectance measurements were conducted at two developmental stages each season. However, for this study, only the measurements made at early to full flowering (R1–R2 stages; Fehr et al., 1971) were used. Canopy reflectance was measured as described by Singh et al. (2013). Briefly, from 54 to 57 d after planting in 2009 and from 58 to 61 d after planting in 2010, three random measurements were collected per plot with an ASD FieldSpec, FR spectroradiometer (Analytical Spectral Devices Inc.). The three measurements were obtained between 1000 and 1400 h with the optical head (25° field of view) held 0.5 m above the canopy and were averaged to represent each plot. Every 5 to 10 min, a Spectralon reference panel (white reference panel calibrated at 99% reflective) was measured and used to automatically adjust canopy reflectance to the white reference. The PRI was calculated based on these canopy reflectance measurements using the equation below (Gamon et al., 1997; Peñuelas et al., 1995):

$$\text{PRI} = (R_{531} - R_{570})/(R_{570} + R_{531})$$

where $R_{531}$ and $R_{570}$ are the reflectance at 531 and 570 nm, respectively. As described by Gamon et al. (1992), the $R_{570}$ is a reference peak and $R_{531}$ is correlated with the epoxidation state of the xanthophyll pigments.

### Statistical Analysis

All statistical analyses were conducted using SAS 9.4 (SAS Institute, 2013). Basic descriptive statistics were generated using PROC MEANS and the average of each genotype from 2009 and 2010. Analysis of variance was performed for all factors with replications nested in genotype using PROC GLM. For GWAS analysis, best linear unbiased predictions (BLUPs) were used to reduce the environmental effects. The BLUPS were created using PROC GLIMMIX with genotype as the fixed effect and all other factors as random. Broad-sense heritability ($h^2$) estimates were calculated with data using PROC GLM (Piepho and Mohring, 2007).

### Population Structure and Genome-Wide Association Study

The population structure matrix ($Q$) for 373 soybean genotypes was obtained using STRUCTURE 2.3.4 software (Hubisz et al., 2009; Pritchard et al., 2000) based on 31,145 SNPs as described by Dhanapal et al. (2015b). The kinship matrix ($K$) was generated using TASSEL 5.2.3 software based on similarity matrix as described by Endelman and Jannink, (2012). We employed MLM with $Q$ and $K$ matrix

for correction of population structure (*Q*) and genetic relatedness (*K*) (Dhanapal et al., 2015a,b). TASSEL 5.2.3 software was employed to determine associations between markers and phenotype. Association analyses of SNPs with minor allele frequency ≥5% were conducted for each of the 2 yr separately as well as for the mean across years using BLUPs derived as described above.

Markers were defined as being significantly associated with PRI when they satisfied a false discovery rate (FDR) <0.05. For multiple testing, the FDR was calculated using QVALUE R 3.1.0 employing the smoother method (Storey and Tibshirani, 2003), an extension of the FDR method (Benjamini and Hochberg, 1995). All SNPs that satisfied the multiple testing analysis had a minimum threshold of –Log10 *P*-value greater than 3.21, which was slightly greater than minimum thresholds used for soybean by others (Hao et al., 2012; Hwang et al., 2014; Zhang et al., 2015).

### Candidate Genes

To identify candidate genes, a BLAST search was conducted based on the 60-bp sequences flanking the final 31 candidate SNPs with default parameters in SoyBase (www.soybase.org) (Grant et al., 2010). Additional searches were conducted in SoyBase for PRI-related genes using keywords photochemical, chlorophyll fluorescence, monooxygenase, xanthophyll, epoxidase, and de-epoxidase. For more broadly annotated genes, the highest blast score obtained from the National Center for Biotechnology Information were reported to supplement the SoyBase annotation for the identified candidate genes.

## Results and Discussion

### Analysis of Variance, Descriptive Statistics, and Heritability

Analysis of variance using PRI BLUPs revealed strong genotype and year effects ($P < 0.0001$ for both) but no significant interaction between genotype and year ($P = 1.000$). Average PRI for the 2 yr were 0.062 (2009) and 0.053 (2010), and values for genotypes with maximum PRI were 1.95-fold and 2.66-fold those of genotypes with minimum PRI in 2009 and 2010, respectively (Fig. 1).

Only minimal information on soybean PRI has been published to date. In a greenhouse study, Inamullah and Isoda (2005) observed PRI values for soybean cultivar Tachinagaha that were between 0.02 and 0.03, which corresponds to values near or below the lower end of those determined in the present study. For one soybean cultivar grown in containers under natural light conditions, Inoue and Peñuelas (2006) observed PRI values between 0.04 to −0.04. The PRI values from both of these studies are below the mean PRI values observed for the 373 genotypes. However, although plants were exposed to natural sunlight, neither of these two studies were field experiments, as Inoue and Peñuelas (2006) grew the plants in containers and the experiments by Inamullah and Isoda (2005) were conducted in the greenhouse. The

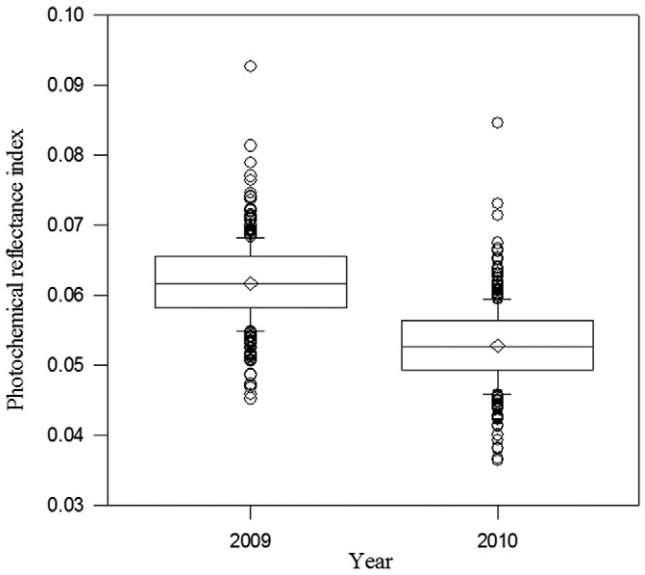


Fig. 1. Box and whisker plots of photochemical reflectance index (PRI) values for 2009 and 2010. Box edges represent the first and third quartile and the whiskers represent 1.5 times the interquartile range. Open circles represent outlier data beyond the 1.5 times the interquartile range. The mean for each year is indicated by an open diamond and the median by a solid line within the boxes.

comparatively low PRI values observed by Inamullah and Isoda (2005) were associated with low levels of NPQ, suggesting that the plants were not exposed to excess light or conditions that would require abundant energy dissipation. Cursory comparison of the PRI values with those observed for greenhouse-grown durum wheat (0.018), rice (0.06–0.12), and field-grown maize (0.075 and 0.00) suggest that the growth and light environment has a profound impact on the PRI of various crop species (Shrestha et al., 2012; Strachan et al., 2002; Tambussi et al., 2002). Nonetheless, the results from these studies indicate that the values observed in this study are within the range of previously observed PRI values.

The wide range in PRI values observed in the soybean diversity panel indicates that the genotypes included in this study required different levels of photoprotection despite having been grown under the same conditions. Based on the research by Gamon et al. (1992, 1997) and others (Gamon and Surfus, 1999; Peñuelas et al., 1994; Porcar-Castell et al., 2012), one can expect that the observed PRI values indicate different levels of epoxidation states of the xanthophyll pigments and therefore represent varying levels of NPQ for different genotypes experiencing the same growth conditions.

The reasons underlying the significant year effect on PRI are unclear. While canopy spectral reflectance was determined on clear days, and plants were not stressed, differences in prevailing environmental conditions (e.g., temperature and incident solar radiation) leading up to the measurement days may have influenced the xanthophyll pool size and NPQ requirements. However, the absence of a significant year × genotype interactions

**Table 1. Five genotypes with photochemical reflectance index (PRI) best linear unbiased prediction values that were among the lowest or highest 1.34% of all genotypes in both 2009 and 2010.**

| Genotype | Year | PRI |
|---|---|---|
| PI567767B | 2009 | 0.045 |
| | 2010 | 0.036 |
| PI574483 | 2009 | 0.047 |
| | 2010 | 0.038 |
| PI594410 | 2009 | 0.046 |
| | 2010 | 0.037 |
| PI603777 | 2009 | 0.038 |
| | 2010 | 0.047 |
| A5959 | 2009 | 0.038 |
| | 2010 | 0.047 |
| PI404199 | 2009 | 0.093 |
| | 2010 | 0.085 |
| PI417232 | 2009 | 0.076 |
| | 2010 | 0.067 |
| PI417107 | 2009 | 0.081 |
| | 2010 | 0.073 |
| PI567202 | 2009 | 0.079 |
| | 2010 | 0.071 |
| PI567532 | 2009 | 0.077 |
| | 2010 | 0.067 |

indicates that the genotypes responded consistently in both years. This is also illustrated by the fact that the five genotypes with the most consistent high or low PRI in the 2 yr (Table 1) were within the bottom or top 1.34% of all entries in both years. The genotypes with low PRI are expected to display low NPQ and high energy use for photosynthesis while those with high PRI are expected to have high NPQ and low energy use efficiency.

No estimates of heritability of PRI were found in the literature for either soybean or other plant species. Calculation using 2-yr-average BLUPs for PRI revealed a broad-sense heritability of 69% based on the soybean diversity panel used in this study. Thus, the most consistent genotypes (Table 1) may prove useful for breeding and genetic studies. However, to date, implications of low or high PRI values or capacities for NPQ for breeding have not been explored in any crop. Genotypes with low PRI may require reduced levels of photoprotection through NPQ. In contrast to genotypes with high PRI, these genotypes may be capable of using light energy more efficiently and therefore require less energy dissipation. Experiments aimed at developing a more comprehensive understanding of PRI and NPQ, and the implications and relevance of high and low PRI and NPQ with regard to crop adaptation to different environments, agronomic practices, and germplasm improvement efforts, are essential and yet to be conducted.

## Genome-Wide Association Analysis

Genome-wide association analysis of 31,145 SNP markers with PRI was conducted with a MLM–*Q*–*K* model using

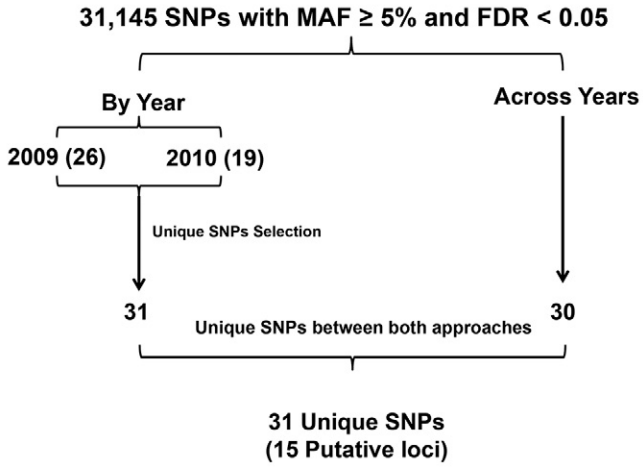


Fig. 2. Flow chart showing final single-nucleotide polymorphism (SNP) selection for photochemical reflectance index (PRI) based on analyses using a mixed linear model plus *Q* plus *K* model with 31,145 SNPs with minor allele frequency (MAF) ≥5%. For all analyses, best linear unbiased prediction means were used for association testing.

both BLUP values generated by year as well as across years (2009 and 2010). As indicated in Fig. 2, by-year and across-year analyses revealed SNPs that were unique to each analysis approach as well as SNPs that were found by both approaches. Association analysis by year resulted in the identification of 26 SNPs for 2009 and 19 SNPs for 2010. Of these 45 SNPs, 31 SNPs were unique, and 14 SNPs were common for 2009 and 2010. Association analysis based on across-year BLUPs resulted in the identification of 30 SNPs. Only one of the 31 SNPs identified based on the by-year analysis was not identified among the 30 SNPs found in the across-year analysis. The 31 unique candidate SNPs mark 14 putative loci on 11 chromosomes that were identified by both by-year and across-year approaches and one putative locus on chromosome 2 that was identified based on the by-year approach only (Table 2; Fig. 3). To our knowledge, these 15 putative loci represent the first ever reported for PRI in soybean or any other plant species.

Of the 15 loci, seven were identified by two or more closely spaced SNPs, and the remaining eight were identified by single SNPs. Two of the loci, one on chromosome 2 and one on chromosome 8, were identified by five closely spaced SNPs. Three closely spaced SNPs marked one locus each on chromosome 10 and 14; two loci on chromosome 19 and one locus each on chromosome 12 and eight were identified by two SNPs. The remaining eight loci located on chromosomes 1 (two loci), 2, 6, 7, 11, and 20 were identified by one SNP (Table 2). These SNPs will need to be verified in additional environments and by additional mapping efforts to strengthen their relevance for use in crop improvements.

## Candidate Genes Associated with Putative Loci

To identify candidate genes associated with PRI, searches based on flanking regions of the putative SNPs as well as keyword searches were conducted. The BLAST search

**Table 2. Putative unique candidate single-nucleotide polymorphisms (SNPs) identified for photochemical reflectance index (PRI) based on by-year and across-year analysis.**

| Locus | SNP ID | Chromosome† | Wm82.a1‡ | Wm82.a2§ | MAF¶ | Major Allele | Minor Allele | –Log10 *P*-value | $R^2$ | Year | Allele effect | Allele effect |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | % | | | % |
| 1 | ss715578795 | Gm01 | 2,518,132 | 2,525,149 | 0.07 | T | G | 3.72 | 3.84 | PRI_2009 | 0.00237 | 3.82 |
| | | | | | | | | 4.07 | 4.29 | PRI_2010 | 0.00236 | 4.44 |
| | | | | | | | | 3.97 | 4.16 | PRI_Across | 0.00230 | 4.11 |
| 2 | ss715579934 | Gm01 | 4,912,239 | 4,934,180 | 0.15 | G | A | 3.70 | 3.81 | PRI_2010 | –0.00157 | –2.97 |
| | | | | | | | | 3.27 | 3.29 | PRI_Across | –0.00154 | –2.69 |
| 3 | ss715582638 | Gm02 | 4,378,683 | 4,430,441 | 0.07 | C | T | 3.92 | 4.11 | PRI_2009 | –0.00443 | –7.19 |
| | | | | | | | | 3.22 | 3.26 | PRI_2010 | –0.00441 | –8.36 |
| | | | | | | | | 3.49 | 3.58 | PRI_Across | –0.00442 | –7.73 |
| | ss715582779 | Gm02 | 4,460,533 | 4,512,291 | 0.08 | T | C | 3.90 | 4.03 | PRI_2009 | –0.00393 | –6.38 |
| | | | | | | | | 3.26 | 3.27 | PRI_Across | –0.00391 | –6.83 |
| | ss715582785 | Gm02 | 4,462,589 | 4,514,347 | 0.08 | A | G | 3.89 | 4.02 | PRI_2009 | –0.00393 | –6.37 |
| | | | | | | | | 3.26 | 3.27 | PRI_Across | –0.00390 | –6.82 |
| | ss715582786 | Gm02 | 4,463,067 | 4,514,825 | 0.08 | A | G | 3.90 | 4.03 | PRI_2009 | –0.00393 | –6.38 |
| | | | | | | | | 3.26 | 3.27 | PRI_Across | –0.00391 | –6.83 |
| | ss715582789 | Gm02 | 4,465,606 | 4,517,364 | 0.08 | T | C | 3.92 | 4.06 | PRI_2009 | –0.00394 | –6.39 |
| | | | | | | | | 3.28 | 3.29 | PRI_Across | –0.00391 | –6.84 |
| 4 | ss715583720 | Gm02 | 6,250,982 | 6,327,303 | 0.30 | A | G | 3.58 | 3.65 | PRI_2009 | –0.00115 | –1.86 |
| 5 | ss715594386 | Gm06 | 40,671,903 | 41,376,571 | 0.05 | G | A | 3.76 | 3.86 | PRI_2009 | –0.00533 | –8.64 |
| | | | | | | | | 3.28 | 3.30 | PRI_2010 | –0.00529 | –9.95 |
| | | | | | | | | 3.41 | 3.45 | PRI_Across | –0.00531 | –9.28 |
| 6 | ss715598125 | Gm07 | 42,750,297 | 42,696,047 | 0.08 | A | G | 3.35 | 3.38 | PRI_2009 | –0.00322 | –5.22 |
| | | | | | | | | 3.32 | 3.17 | PRI_2010 | –0.00317 | –6.01 |
| | | | | | | | | 3.25 | 3.27 | PRI_Across | –0.00319 | –5.58 |
| 7 | ss715599924 | Gm08 | 17,386,933 | 17,321,123 | 0.05 | A | G | 4.04 | 4.21 | PRI_2009 | –0.00575 | –9.33 |
| | | | | | | | | 3.49 | 3.55 | PRI_Across | –0.00569 | –9.96 |
| | ss715599926 | Gm08 | 17,387,528 | 17,321,718 | 0.05 | A | G | 4.04 | 4.21 | PRI_2009 | –0.00575 | –9.33 |
| | | | | | | | | 3.49 | 3.55 | PRI_Across | –0.00569 | –9.96 |
| | ss715599927 | Gm08 | 17,388,190 | 17,322,380 | 0.05 | A | C | 4.04 | 4.21 | PRI_2009 | –0.00575 | –9.33 |
| | | | | | | | | 3.49 | 3.55 | PRI_Across | –0.00576 | –9.96 |
| | ss715599928 | Gm08 | 17,388,801 | 17,322,991 | 0.05 | A | G | 4.04 | 4.21 | PRI_2009 | –0.00575 | –9.33 |
| | | | | | | | | 3.49 | 3.55 | PRI_Across | –0.00569 | –9.96 |
| | ss715599931 | Gm08 | 17,399,234 | 17,333,424 | 0.05 | C | T | 4.09 | 4.30 | PRI_2009 | –0.00575 | –9.34 |
| | | | | | | | | 3.54 | 3.63 | PRI_Across | –0.00571 | –9.96 |
| 8 | ss715601929 | Gm08 | 41,503,963 | 42,145,795 | 0.20 | T | C | 3.24 | 3.33 | PRI_2009 | –0.00216 | –3.51 |
| | | | | | | | | 3.48 | 3.61 | PRI_2010 | –0.00214 | –4.07 |
| | | | | | | | | 3.44 | 3.57 | PRI_Across | –0.00215 | –3.77 |
| | ss715601931 | Gm08 | 41,504,420 | 42,146,252 | 0.22 | T | C | 3.37 | 3.00 | PRI_2009 | –0.00203 | –3.31 |
| | | | | | | | | 3.23 | 3.25 | PRI_2010 | –0.00202 | –3.84 |
| | | | | | | | | 3.28 | 3.29 | PRI_Across | –0.00202 | –3.55 |
| 9 | ss715606303 | Gm10 | 34,620,283 | 35,024,662 | 0.15 | G | A | 3.33 | 3.27 | PRI_2009 | –0.00352 | –5.73 |
| | | | | | | | | 3.25 | 3.37 | PRI_2010 | –0.00348 | –6.62 |
| | | | | | | | | 3.34 | 3.48 | PRI_Across | –0.00350 | –6.14 |
| | ss715606310 | Gm10 | 34,805,035 | 35,325,979 | 0.15 | A | G | 3.50 | 3.62 | PRI_2009 | –0.00351 | –5.72 |
| | | | | | | | | 3.59 | 3.74 | PRI_2010 | –0.00352 | –6.66 |
| | | | | | | | | 3.67 | 3.84 | PRI_Across | –0.00351 | –6.15 |
| | ss715606317 | Gm10 | 34,990,419 | 35,521,790 | 0.15 | T | C | 3.82 | 3.97 | PRI_2009 | –0.00368 | –5.99 |
| | | | | | | | | 3.84 | 4.00 | PRI_2010 | –0.00364 | –6.93 |
| | | | | | | | | 4.00 | 4.19 | PRI_Across | –0.00366 | –6.43 |
| 10 | ss715611302 | Gm11 | 9,393,129 | 9,421,766 | 0.10 | G | A | 3.67 | 3.88 | PRI_2010 | –0.00397 | –7.58 |
| | | | | | | | | 3.39 | 3.55 | PRI_Across | –0.00399 | –6.97 |

*(cont'd.)*

**Table 2. Continued.**

| Locus | SNP ID | Chromosome† | Wm82.a1‡ | Wm82.a2§ | MAF¶ | Major Allele | Minor Allele | –Log10 *P*-value | $R^2$ | Year | Allele effect | Allele effect |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | % | | | % |
| 11 | ss715612967 | Gm12 | 39,447,867 | 39,423,587 | 0.09 | T | C | 3.54 | 3.61 | PRI_2009 | –0.00359 | –5.83 |
| | | | | | | | | 3.39 | 3.55 | PRI_Across | –0.00354 | –6.19 |
| | ss715612968 | Gm12 | 39,451,471 | 39,427,191 | 0.09 | A | G | 3.51 | 3.58 | PRI_2009 | –0.00366 | –5.94 |
| | | | | | | | | 3.27 | 3.29 | PRI_Across | –0.00359 | –6.28 |
| 12 | ss715617369 | Gm14 | 10,298,490 | 10,091,235 | 0.17 | C | T | 3.23 | 3.24 | PRI_2010 | –0.00325 | –5.31 |
| | | | | | | | | 3.32 | 3.35 | PRI_Across | –0.00327 | –5.75 |
| | ss715617372 | Gm14 | 10,301,411 | 10,094,156 | 0.17 | T | C | 3.24 | 3.26 | PRI_2010 | –0.00324 | –5.28 |
| | | | | | | | | 3.34 | 3.48 | PRI_Across | –0.00326 | –5.74 |
| | ss715617376 | Gm14 | 10,304,752 | 10,097,497 | 0.17 | T | C | 3.45 | 3.51 | PRI_2010 | –0.00341 | –6.51 |
| | | | | | | | | 3.32 | 3.35 | PRI_Across | –0.00338 | –5.95 |
| 13 | ss715635548 | Gm19 | 45,882,216 | 45,999,915 | 0.09 | A | G | 3.55 | 3.61 | PRI_2009 | 0.00386 | 6.21 |
| | | | | | | | | 3.45 | 3.51 | PRI_2010 | 0.00393 | 7.38 |
| | | | | | | | | 3.58 | 3.66 | PRI_Across | 0.00389 | 6.74 |
| | ss715635553 | Gm19 | 45,999,263 | 46,116,996 | 0.10 | C | T | 3.72 | 3.84 | PRI_2009 | 0.00391 | 6.29 |
| | | | | | | | | 3.51 | 3.60 | PRI_2010 | 0.00401 | 7.51 |
| | | | | | | | | 3.70 | 3.82 | PRI_Across | 0.00396 | 6.85 |
| 14 | ss715635555 | Gm19 | 46,017,148 | 46,134,881 | 0.10 | A | G | 3.62 | 3.70 | PRI_2009 | 0.00371 | 5.97 |
| | | | | | | | | 3.39 | 3.43 | PRI_2010 | 0.00381 | 7.13 |
| | | | | | | | | 3.55 | 3.62 | PRI_Across | 0.00376 | 6.51 |
| | ss715635838 | Gm19 | 48,328,052 | 48,451,378 | 0.13 | T | C | 3.63 | 3.74 | PRI_2009 | 0.00289 | 4.64 |
| | | | | | | | | 3.45 | 3.54 | PRI_2010 | 0.00293 | 5.49 |
| | | | | | | | | 3.64 | 3.76 | PRI_Across | 0.00291 | 5.03 |
| 15 | ss715637535 | Gm20 | 34,077,356 | 35,216,761 | 0.23 | T | C | 3.33 | 2.98 | PRI_2009 | 0.00243 | 3.89 |
| | | | | | | | | 3.43 | 3.16 | PRI_2010 | 0.00251 | 4.69 |
| | | | | | | | | 3.26 | 3.28 | PRI_Across | 0.00247 | 4.26 |

† Gm, *Glycine max* chromosome.

‡ Wm82.a1, Williams 82 assembly 1 position

§ Wm82.a2, Williams 82 assembly 2 position

¶ MAF, minor allele frequency.

based on the flanking regions of the final 31 candidate SNPs revealed that 13 SNPs were present in introns or coding regions of a gene. In addition, for all SNPs not located in a gene, the gene closest to the SNP was identified in SoyBase; however, none of these genes have any obvious functional relationship with PRI (data not shown). Therefore, to investigate whether any annotated genes of interest may be located near the 31 putative candidate SNPs, the terms photochemical, chlorophyll fluorescence, monooxygenase, xanthophyll, epoxidase and de-epoxidase were used to search SoyBase for PRI related genes located within ±2 Mb of the candidate SNPs.

Based on these search terms, nine of all the annotated genes located near the loci were genes that are known to be directly involved with NPQ, and six of the genes located near the loci were genes more broadly related to photosynthetic processes that contribute to NPQ (Table 3). These genes were found in the vicinity of 10 of the 15 putative loci (Fig. 3). Five PRI-related genes were found near one of the two loci on chromosome 2, four near one of the two loci on chromosome 8 and near the locus on chromosome 12, and three PRI-related genes each near the loci on chromosome 14 and 20. Two PRI-related genes were discovered in the vicinity of one of the two loci on chromosome 2 as well as near loci on chromosomes 7, 11, and 19. A single PRI-related gene was found near the locus on chromosome 10 (Table 3; Fig. 3).

Among all the genes identified, the one most obviously related to energy dissipation is a gene near (~1.2 Mb) locus 14 on chromosome 19 that was annotated as violaxanthin de-epoxidase (VDE). Violaxanthin de-epoxidase is directly involved in the xanthophyll cycle, which contributes to energy dissipation associated with NPQ (Havaux et al., 2000; Niyogi et al., 1998). It modulates the levels of the epoxidated xanthophyll pigments and thus is expected to directly contribute to the PRI signature. In addition to VDE, two additional genes involved in the biosynthetic pathway of carotenoids were identified near locus 15 on chromosome 20. These genes, Glyma20g23930 and Glyma20g24140, were annotated by SoyBase to have monooxygenase activity. The last step in the biosynthesis of zeaxanthin requires the hydroxylation of the β rings of β-carotene. This step has been shown to be catalyzed by both a β-hydroxylase and a monooxygenase (da Silva Messias et al., 2014).

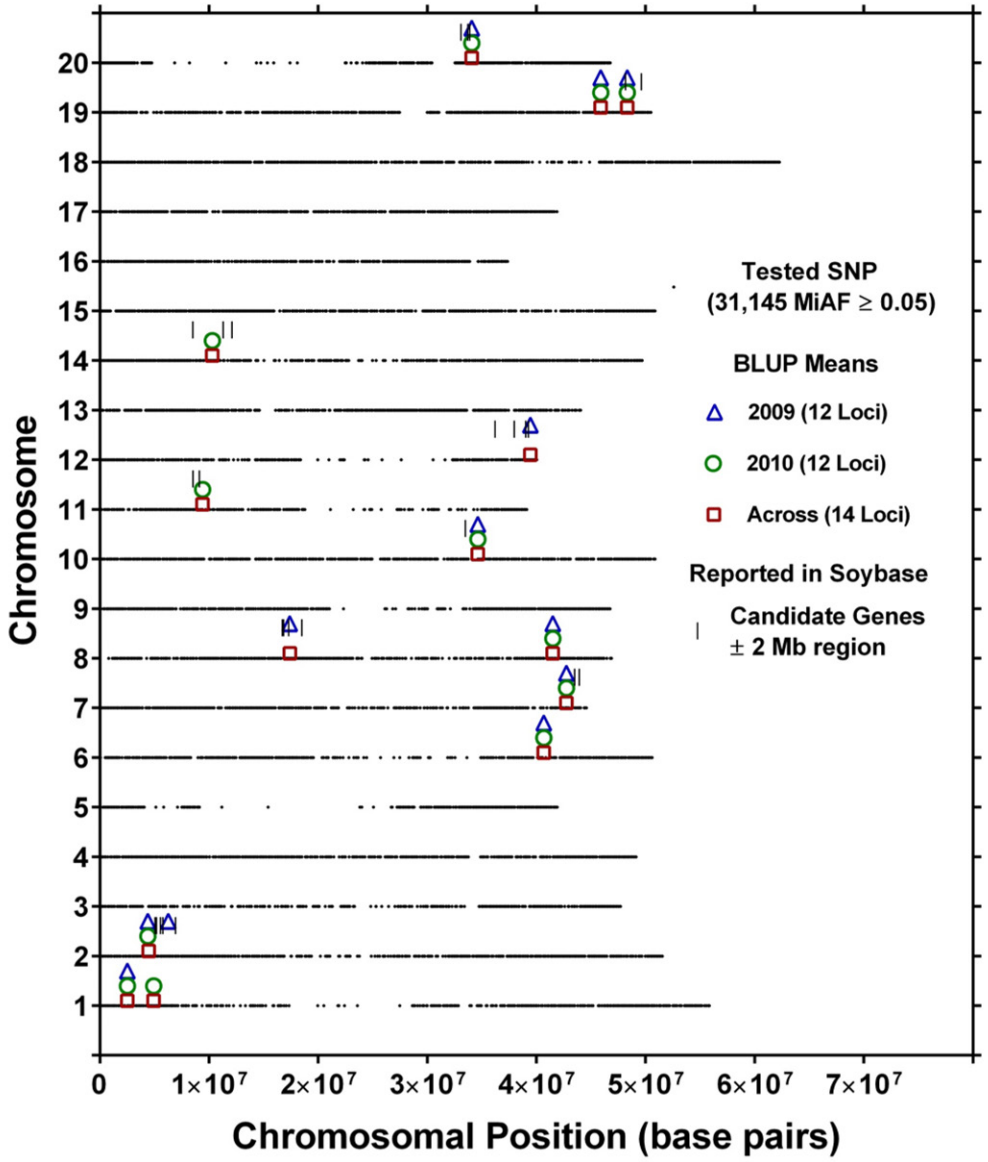


Fig. 3. Location of putative loci significantly associated with photochemical reflectance index (PRI) identified based on an mixed linear model plus *Q* plus *K* model and by-year and across-year analysis. Loci are indicated by squares, circles, or triangles positioned above the respective chromosome. Putative PRI-related genes located in the vicinity (±2 Mb) of the loci are marked by black lines. For each chromosome, the black dots represent the location of a single-nucleotide polymorphism evaluated for association with PRI.

Most other candidate genes are sugar transporters or structural proteins of photosystem II and light harvesting complexes for both photosystems (Table 3). Two of the genes (Glyma20g23100 and Glyma02g06830) are important for the function of the oxygen-evolving complex of PSII. One of them (Glyma20g23100), found near locus 15, encodes a PsbP-like protein. The PsbP protein is critical for the regulation of the oxygen-evolving complex of PSII through modulation of the $Ca^{+2}$ and $Cl^{-}$ ions (Bricker et al., 2012). Using transgenic tobacco (*Nicotiana tabacum* L.), Ifuku et al. (2005) showed that reducing the expression of PsbP leads to reduced efficiency of the light reactions. The other gene (Glyma02g06830) was found in the vicinity of locus three on chromosome 2 and encodes a protein with homology to the PsbO of PSII (Table 3), which influences the activity of the Mn cluster (Ghanotakis and Yocum, 1985; Miyao and Murata, 1984). In *Arabidopsis thaliana* (L.) Heynh., two PsbO genes encode two proteins (PsbO1 and PsbO2) and mutant plants that lacked PsbO1 exhibit more severely reduced growth than wild-type (Murakami et al., 2002). Characterization of this mutant revealed reduced photochemical efficiency compared with the wild-type. However, NPQ or carotenoid pigments were not investigated in these mutants to determine if the reduced photochemical efficiency leads to increased NPQ and consorted effects on the epoxidation state of the xanthophyll pigments.

Four genes each were annotated to encode LHC proteins and sugar transporters (Table 3). Two of the LHC genes were associated with photosystem II LHC (locus 4), one was associated with photosystem I LHC (locus 3), and one as light-harvesting chlorophyll *b* binding protein

**Table 3. Candidate genes putatively associated with photochemical reflectance index (PRI) identified in Soybase (www.soybase.org) based on the 31 unique putative single-nucleotide polymorphisms.**

| Locus† | Name of Gene | Chromosome | Search region | Start | End | Soybase | Functional Annotation |
|---|---|---|---|---|---|---|---|
| 3 | Glyma02g06280 | Gm02 | 1Mb | 4,997,892 | 5,003,300 | Glyma 1.1 | Sugar (and other) transporter |
| | Glyma02g06291 | Gm02 | 1Mb | 5,005,340 | 5,011,814 | Glyma 1.1 | Sugar (and other) transporter |
| | Glyma02g06460 | Gm02 | 1Mb | 5,138,734 | 5,141,672 | Glyma 1.1 | Sugar (and other) transporter |
| | Glyma02g07180 | Gm02 | 1Mb | 5,722,611 | 5,725,271 | Glyma 1.1 | Photosystem I light harvesting complex gene 1‡ |
| | Glyma02g06830 | Gm02 | 2Mb | 5,492,393 | 5,495,034 | Glyma 1.1 | Oxygen evolving complex subunit 33 Kda |
| 4 | Glyma02g08890 | Gm02 | 2Mb | 6,881,087 | 6,881,308 | Glyma 1.1 | Photosystem II light harvesting complex gene 1.5‡ |
| | Glyma02g08910 | Gm02 | 2Mb | 6,889,026 | 6,889,815 | Glyma 1.1 | Photosystem II light harvesting complex gene 1.5‡ |
| 6 | Glyma07g38750 | Gm07 | 1Mb | 43,444,133 | 43,449,352 | Glyma 1.1 | Carbohydrate kinase |
| | Glyma07g39380 | Gm07 | 2Mb | 43,872,963 | 43,879,402 | Glyma 1.1 | Pyrophosphate-dependent phosphofructo-1-kinase |
| 7 | Glyma08g21900 | Gm08 | 1Mb | 16,666,495 | 16,668,102 | Glyma 1.1 | Photosystem I reaction center subunit H-2‡ |
| | Glyma08g22120 | Gm08 | 1Mb | 16,790,216 | 16,794,551 | Glyma 1.1 | STAS domain\|sulfate transporter family |
| | Glyma08g22740 | Gm08 | 1Mb | 17,249,861 | 17,253,087 | Glyma 1.1 | Post-illumination chlorophyll fluorescence increase |
| | Glyma08g24280 | Gm08 | 2Mb | 18,456,056 | 18,468,337 | Glyma 1.1 | High chlorophyll fluorescence phenotype 173 protein |
| 9 | Glyma10g25531 | Gm10 | 1Mb | 33,429,232 | 33,436,184 | Glyma 1.1 | Starch Synthase |
| 10 | Glyma11g11900 | Gm11 | 1Mb | 8,486,399 | 8,489,322 | Glyma 1.1 | Fructose-bisphosphate aldolase activity |
| | Glyma11g12720 | Gm11 | 1Mb | 9,062,945 | 9,066,126 | Glyma 1.1 | Sugar (and other) transporter |
| 11 | Glyma12g35840 | Gm12 | 1Mb | 38,963,191 | 38,969,318 | Glyma 1.1 | Phosphoenolpyruvate carboxylase activity |
| | Glyma12g36132 | Gm12 | 1Mb | 39,216,769 | 39,217,288 | Glyma 1.1 | Photosystem II reaction center protein E |
| | Glyma12g32680 | Gm12 | 2Mb | 36,163,876 | 36,167,610 | Glyma 1.1 | Thylakoid formation protein |
| | Glyma12g34770 | Gm12 | 2Mb | 37,914,750 | 37,916,901 | Glyma 1.1 | Light-harvesting chlorophyll B-binding protein 3‡ |
| 12 | Glyma14g12470 | Gm14 | 1Mb | 11,248,308 | 11,251,712 | Glyma 1.1 | Phosphoglucan, water dikinase (PWD) |
| | Glyma14g10290 | Gm14 | 2Mb | 8,457,372 | 8,461,959 | Glyma 1.1 | Squalene monooxygenase activity |
| | Glyma14g13000 | Gm14 | 2Mb | 12,046,724 | 12,048,795 | Glyma 1.1 | Sucrose synthase |
| 14 | Glyma19g42010 | Gm19 | 1Mb | 48,106,697 | 48,108,489 | Glyma 1.1 | Alpha/beta hydrolase superfamily protein |
| | Glyma19g44010 | Gm19 | 2Mb | 49,571,464 | 49,574,835 | Glyma 1.1 | Violaxanthin de-epoxidase activity |
| 15 | Glyma20g23100 | Gm20 | 1Mb | 33,041,663 | 33,044,038 | Glyma 1.1 | PsbP-like protein 2 |
| | Glyma20g23930 | Gm20 | 1Mb | 33,674,172 | 33,676,446 | Glyma 1.1 | Carotenoid biosynthetic process (Monoxygenases) |
| | Glyma20g24140 | Gm20 | 1Mb | 33,845,918 | 33,848,845 | Glyma 1.1 | Carotenoid biosynthetic process (Monoxygenases) |

† Locus number based on Table 2.

‡ Soybase annotation refined with highest scoring blast results from the National Center for Biotechnology Information.

(locus 11). Because NPQ occurs within the LHCs, several studies have targeted the mechanisms that control and influence NPQ within the LHC to develop molecular models of how the xanthophylls take part in heat dissipation of excited chlorophylls (Bassi et al., 1993; Connelly et al., 1997; Verhoeven et al., 1999). These studies have revealed that each LHC binds different species and numbers of carotenoid pigments. For instance, in vitro experiments revealed that LHCB1 retained the lowest levels of violaxanthin of five LHCs tested (Bassi and Caffarri, 2000). Of the two candidates annotated as photosystem II LHC Gene 1.5, Glyma02g08910 has high homology with the *Arabidopsis* LHCB1 subunit (AT2G34430), and the other, Glyma02g08890, with LHCB2 (AT2G05070). Antisense knock down of both LHCB1 and LHCB2 expression in *Arabidopsis* resulted in reduced ability for NPQ (Andersson et al., 2003).

The influence that sugar transporters have on NPQ is less obvious than for VDE or photosystem and LHC associated proteins. Nonetheless, the keyword search revealed four sugar transporters located near two putative loci for PRI. Three of the sugar transporter genes were identified near locus three on chromosome 2 and the remaining sugar transporter gene was located near locus 10 on chromosome 11. Three of the genes (Glyma02g06280, Glyma02g06291 and Glyma02g06460) are homologous with the putative sugar transporter ERD6. The last identified candidate gene annotated as sugar transporter, Glyma11g12720, was most highly matched by SoyBase to a polyol/monosaccharide transporter. Efficient transport of photoassimilates can drastically impact photosynthesis as a result of feedback inhibition (Van Oosten et al., 1997). Source–sink manipulations have narrowed down some of the effects that lead to reduced photosynthesis to the repression of photosynthetic genes by glucose (Sheen, 1990). Increased feedback inhibition would lead to over excitation of chlorophyll and would require increased NPQ for protection from photoinhibition (Greer, 1998; Pammenter et al., 1993).

The functional annotation of several of the other candidate genes also reveals carbohydrate metabolism related roles (Glyma07g38750, Glyma14g13000). Similar to the effect sugar transporters may have on NPQ, it is plausible that carbohydrate metabolism genes may influence NPQ through feedback inhibition of photosynthesis. In addition to these carbohydrate metabolism

related genes, a gene annotated as high chlorophyll fluorescence 173 (HCF173) was identified near locus seven on chromosome 8. In *Arabidopsis*, HCF173 is involved with the translation of PsbA messenger RNA. Mutant plants that displayed the HCF173 phenotype were found to be severely deficient in PSII accumulation (Schult et al., 2007). While NPQ of these mutants was not characterized, it is likely that extensive NPQ would be required for successful growth of these plants. Therefore, differences in HCF173 may exist among soybean genotypes, which, in turn, could lead to differences in PSII abundance.

The proximity to the putative loci and their functional annotations indicate that the genes listed in Table 3 may contribute to the observed PRI phenotypes. Allelic variation for these genes, such as that observed for PsbO in *Arabidopsis*, may be present in soybean and may alter energy dissipation and conversion efficiency of absorbed light. Follow-up genetic and detailed physiological studies are needed to confirm the linkage between the loci and genes identified in this study and PRI and to investigate their relevance for NPQ and energy conversion efficiency.

### Allele Effects of Putative Loci with Photochemical Reflectance Index

Allele effects for PRI ranged from −0.00596 (−9.96%) to 0.00401 (7.51%) (Table 2). For four of the 15 identified loci, the major allele was associated with a higher PRI (loci 1, 13, 14, and 15; Table 2), and for the remaining 11 loci, the major allele was associated with a lower PRI (loci 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12; Table 2). Among the loci for which SNPs were significant in 2009, 2010, and across years, the four with the largest absolute allele effects were located on chromosomes 2, 6, and 19 (Loci 3, 5, 13, and 14; Table 2; Fig. 2). Five putative PRI-related genes were found near locus 3: three encode sugar transporters (Glyma02g06280, Glyma02g06291, and Glyma02g06460), one is a photosystem I light harvesting complex gene (Glyma02g07180), and one a photosystem II oxygen evolving complex gene (Glyma02g06830). Two genes of interest were found near locus 14: one encodes a protein with violaxanthin de-epoxidase activity (Glyma19g44010) and the other one with $\alpha/\beta$ hydrolase activity (Glyma19g42010). The overall greatest absolute allele effect was observed for locus 7 on chromosome 8, although significant SNPs were not identified in 2010. Four putatively PRI-related genes were found in the vicinity of locus 7 including two genes related with chlorophyll fluorescence (Glyma08g22740, Glyma08g24280), one gene encoding a photosystem I reaction center subunit (Glyma08g21900), and one gene in the sulfate transporter family (Glyma08g22120).

## Conclusions

Canopy reflectance measurement-based assessment of PRI revealed significant genotypic variation among 373 diverse maturity group IV soybean genotypes. Genome-wide association analysis resulted in the identification of 15 putative loci associated with the PRI. The PRI is impacted by the epoxidation state of the xanthophyll cycle pigments, which are critical for NPQ. Examination of the genomic regions in the vicinity of the putative loci revealed several genes whose annotation indicates a function in NPQ. Physiological and genetic studies are necessary to elucidate the relevance of low or high canopy-based PRI for crop improvement and adaptation to different environmental conditions.

This study illustrates that canopy spectral reflectance, which is amenable for high-throughput phenotyping under field conditions, can be used successfully to identify genomic regions associated with photosynthetic traits in field-grown crops. The genotypic variation, putative loci, and candidate genes identified in this study may be useful for genetic improvement targeting photosynthetic characteristics in soybean.


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### Competing Interests

The authors of the manuscript declare that they have no competing interests.

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