Reflective Films and Expression of Light-regulated Genes in Field-grown Apple

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ADDITIONAL INDEX WORDS. light perception, orchard management, photosynthesis, phytochrome, reverse transcription–polymerase chain reaction

ABSTRACT. Reflective films are used in orchard management to improve fruit coloration. Numerous physiological studies on the effects of application of these films have been conducted, including variation of angles of light incidence and reflection, spectral determination of reflected light, and effects on photosynthesis and pigment development. At present there have been no studies on the effects of these treatments on gene expression, particularly with regard to genetic factors controlling light perception and genes targeted by specific regulators. We sampled a fully developed leaf from apple (Malus ×domestica) branches of mature trees in 2 consecutive years. The grass between the tree rows was left untreated, sprayed with a formulation of a reflective particle film (RPF), or covered with a single layer of an aluminized plastic film (APF). Leaves at the same branch position were sampled between 1000 and 1200 HR on sunny days. Nine different gene transcripts associated with light perception, sorbitol transport, and actin (control) were measured by semiquantitative reverse transcription–polymerase chain reaction and five by quantitative reverse transcription–polymerase chain reaction. Transcript abundance of most genes was unaffected by treatment or slightly enhanced by RPF. These results are consistent with earlier observations indicating that application of this film stimulated carbon partitioning to the fruit likely through the reduced red/far-red ratio of reflected light. In contrast, the level of several gene transcripts, including MdPif3, MdPhyB, and MdSot3, was reduced by application of the APF. Only one gene, MdElip1A/B, was significantly and dramatically elevated by APF.

Received for publication 3 Mar. 2014. Accepted for publication 5 May 2014.

We thank Sharon Jones and Jing Ma for their expert assistance in sampling, preparing RNA, and conducting the gene expression analyses.

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responsible for the shade avoidance syndrome. Phytochrome interacting factors 3 through 5 (PIFs3–5) mediate this response (Supplemental Fig. 1). In addition, the phytochrome interacting factor, PIL1, is rapidly activated by exposure to low R/FR light (Salter et al., 2003), and this response can be maintained under long periods of FR exposure (Roig-Villanueva et al., 2006). Blue light and ultraviolet-A responses are perceived by a variety of receptors. Cryptochromes are flavin-containing proteins similar in sequence to the DNA repair photolyases (Chaves et al., 2011); they perceive both blue light and ultraviolet-A wavelengths. Phototropins are serine/threonine receptor kinases that phosphorylate a variety of proteins (Tokutomi et al., 2008). All of the light receptors are modulated or influenced by the Zeitlupe family of receptors controlling circadian rhythms.

One of the central components of light signaling is the leucine zipper transcription factor, HY5 [elongated hypocotyl 5 (Supplemental Fig. 1)]. Transcription of Hy5 is influenced by all of the different light receptor types, including the UVR8 protein (Brown et al., 2005), CRY1 (Vandenbussche et al., 2007), and phytochromes/PIFs. HY5 has over 3000 downstream targets, including genes associated with photosynthesis, photomorphogenesis, or, in some cases, stress response (Lee et al., 2007). Among them are genes encoding the small subunit of Rubisco (RbcS), which represents the key step in carbon fixation and various chlorophyll a/b binding proteins, which are essential for the light reactions of photosynthesis. In addition, early light-induced protein (Elip) genes encoding proteins similar to chlorophyll a/b binding proteins are also targets of HY5 and are believed to protect developing seedlings from environmental stresses such as photo-oxidation (Hutin et al., 2003) and heat shock (Harari-Steinberg et al., 2001).

Most studies of light perception and transduction at the molecular level have been conducted on etiolated seedlings of herbaceous plants, and little is known about light signaling in mature, field-grown woody plants. Furthermore, many orchard management protocols directly affect tree light perception. For example, opening up the canopy of fruit trees to increase light exposure to the internal portion of the canopy is a common management practice among many types of fruit trees (Marini and Barden, 1987). Ground covers, row and tree spacing as well as materials sprayed on or around the trees can also have an effect on light reaching the canopy. The ultimate goal of horticultural molecular biology is to transfer information derived from studies of genes and gene products to useful field applications for the maintenance/improvement of productivity. The objective of the current study was to measure the effects on expression of genes associated with different aspects of light perception and transduction in apple leaves exposed to two groundcover treatments that increase canopy light levels but with different light quality. Based on light wavelengths reflected by the treatments, genes representing different aspects of the phytochrome light induction pathway were chosen to represent events ranging from perception to transduction to activation of light-responsive targets. In addition, the sorbitol transporter was included as a representative gene involved in carbon allocation, and actin served as a light-insensitive control.

Materials and Methods

Collection of Plant Material. Trees of Malus × domestica ‘Empire’/‘M.7A’ (M.7A) were used in this study. The apple orchard was planted at Kearneysville, WV (lat. 39.3° N, long. 77.9° W) in 1992 in a north–south orientation in a moderate density configuration (500 trees/ha at 6.0 × 3.3-m spacing). The trees were not irrigated and were sprayed with conventional pesticides. A separate group of trees was used in each year (2005 and 2006). The experimental design and treatments were established in 2002 as described previously (Glenn and Puterka, 2007). The focus of the current study was on reflective materials [i.e., aluminized film strips (Clarke Ag Plastics, Greenwood, VA)] and a reflective particle formulation [95% rain-resistant calcined kaolin (Engelhard Corp., Iselin, NJ)], that were designated as APF and RPF treatments in Glenn and Puterka (2007), respectively.

Leaves were collected from branches at the outer portion of the canopy on the east side of the tree at working height (∼2 m). The approximate 12th leaf from the shoot apex was sampled from four branches that were 18 to 22 cm long. Four leaves from each of six replicated trees were collected, pooled, rinsed with deionized water, and immediately placed in liquid N2. Leaves were stored at −80 °C until used for RNA extraction.

Leaves at the 12th position from the shoot apex wereused in this study. The apple expressed sequence tags (ESTs) for select genes when available were downloaded from GenBank [National Center for Biotechnology Information (NCBI)] translated and aligned with the contig assembly program [CAP3 (Huang and Madan, 1999)] to potential A. thaliana homologs. Where no EST was available, the A. thaliana protein sequence was used to search the apple genome (Velasco et al., 2010) for the closest sequence match. The cognate nucleic acid sequence was in turn used in a BLASTn (basic local alignment search tool, NCBI) search against the apple genome, and the derived polypeptides from the resulting sequence(s) were aligned with A. thaliana sequences using COBALT (NCBI). The alignment results are shown in Supplemental Table 1. MdSot3 [apple sorbitol transporter (GenBank accession no. AB125646)] and genes obtained from the alignments were used to design primers for the semiquantitative reverse transcription–polymerase chain reaction and quantitative reverse transcription–polymerase chain reactions using Primer 3 (Rozen and Skaltsky, 1998) and Primer 3Plus software (Untergasser et al., 2007).

Expression analysis by semiquantitative RT-PCR. Leaves from individual trees were ground in liquid N2, and total RNA was extracted according to the manufacturer’s protocol using a Concert Plant RNA Reagent kit (Invitrogen, Carlsbad, CA). Total RNA was treated with DNase (RNA Clean and Concentrator-25; Zymo Research, Irvine, CA). The treated RNA was assayed for chromosomal DNA contamination using primers for the subunit7 gene of the peach (Prunus persica) 26S proteosome (GenBank accession no. AF041258), a highly conserved component of the common protein-degradation pathway. Equal amounts of DNA-free total RNA from the pooled leaf samples were converted to cDNA using SuperScript III according to the manufacturer’s directions (Invitrogen). One microlitr of each cDNA was separated
on 1.8% agarose gels, stained with SYBR®Gold (Life Technologies, Applied Biosystems, Grand Island, NY), and the image scanned on a STORM or TYPHOON Image Analyzer (GE Healthcare, Piscataway, NJ). Scanned images were analyzed with ImageQuant TL Version 7 software (GE Healthcare) to determine the relative quantity of each cDNA (Madlung et al., 2002). These were subsequently adjusted by dilution so that equal amounts of the cDNAs served as templates in the polymerase chain reaction (PCR) reactions.

The annealing temperatures of the PCR reactions were optimized for each primer set against ‘Empire’ apple genomic DNA. Primers used in this study are listed in Supplemental Table 2 and were verified by sequencing amplicons generated from genomic DNA templates. PCR reactions used HotStarTaq plus Master Mix (Qiagen, Valencia, CA) and an equal volume of water + 0.2 μL each primer (100 μM). Cycling conditions were 95 °C for 5 min followed by 35 cycles of 1 min at 95 °C, 1 min at the primer optimal annealing temperature (55 to 60 °C), and 1.5 min at 72 °C. At the end of the 35 cycles, the reaction was kept at 72 °C for 10 min to complete product extension. cDNA templates that appeared saturated under these cycling conditions were diluted to a concentration designed to yield approximately equal product intensities similar to the less abundant genes and rerun. MdAct, encoding an apple actin gene, served as a control based on a previous study indicating that it does not respond to light (Lu et al., 2002) and to our results indicating that it did not differ by treatment.

Products of each reaction were separated on 1% (w/v) agarose gels in TAE (40 mM Tris acetate, pH 8, 1 mM ethylenediamine tetraacetic acid) buffer and stained with ethidium bromide for 20 min with SYBR®Gold (10,000×) (Life Technologies) diluted 1:30000 with deionized water. Images were captured and analyzed as before. Analysis of bands included total area and intensity. The data were subjected to statistical analysis using the Ryan-Einot-Gabriel-Welsch multiple range test. In addition, simple linear correlations were calculated across treatments and years for all genes (r > 0.273 for P = 0.05) measured by semiquantitative RT-PCR and qRT-PCR methodology.

**Results**

To follow the potential effects of reflective film treatment on expression of light-responsive genes, we selected genes representative of different light spectral responses, as well as those associated with different steps in the light signaling pathways (Supplemental Fig. 1). These genes encoded apple genes related to *A. thaliana* phytochromes B and E (MdPhyB, MdPhyE), long hypocotyl 5 (MdHyc5), phytochrome interacting factor 3 (MdPif3), MdRbcS (small subunit of ribulose-bis-phosphate carboxylase), MdLhcb2-2 (light harvesting complex 2, type 2 chlorophyll a/b binding protein gene), MdSot3 (sorbit transport; Watari et al., 2004), and MdElip1A/B and MdElip1D.

Identification of apple genes equivalent to *A. thaliana* genes used apple ESTs ['Royal Gala', (Wisniewski et al., 2008) and ‘Goldrush’ (S. Korban, NCBI EST database)] where available. If no ESTs were present in the gene bank databases, we used *A. thaliana* sequences to search the apple genome (Sanzol, 2010; Velasco et al., 2010). Apple sequences with significant E values (< 1 × 10⁻⁸) were used to search the genome a second time. All candidate genes were translated and aligned to the *A. thaliana* sequences. The apple sequence with the best alignment score using COBALT (NCBI) and BLASTp was chosen as the putative apple equivalent (Supplemental Table 1). Primers for semiquantitative and qRT-PCR were designed from these templates (Supplemental Table 2).

All cDNAs from leaves of mature trees were first assessed by semiquantitative RT-PCR using primers for genes with known responses to light. MdAct was tested as a "constitutive" control to monitor variation in gel loading, dilution series, and expression under field conditions. In 2005 only transcript accumulation of *MdElip1D* and *MdSot3* was significantly different from controls by semiquantitative RT-PCR analysis, being highest in leaves sampled from RPF-treated trees (Table 1). Levels of *MdElip1D* were not significantly different in 2006 (Fig. 1A).

| Gene         | 2005       | 2006       | 2005       | 2006       | 2005       | 2006       |
|--------------|------------|------------|------------|------------|------------|------------|
|              | RPF*       | APF*       | RPF         | APF         | RPF         | APF         |
| MdAct        | 0*         | 0          | 0          | 0          | 0          | 0          |
| MdPhyB       | 0          | +          | 0          | 0          | 0          | 0          |
| MdPhyE       | 0          | 0          | 0          | 0          | 0          | 0          |
| MdHyc5       | 0          | 0          | 0          | 0          | 0          | 0          |
| MdPif3       | 0          | +          | 0          | 0          | 0          | 0          |
| MdLhcb2-2    | 0          | 0          | 0          | 0          | 0          | 0          |
| MdRbcS       | 0          | 0          | 0          | 0          | 0          | 0          |
| MdElip1A/B   | 0          | +          | 0          | 0          | 0          | 0++        |
| MdElip1D     | +          | 0          | 0          | 0          | +          | +          |
| MdSot3       | +          | +          | -          | -          | +          | -          |

RPF = reverse transcription–polymerase chain reaction; RPF = reflective particle film (Engelhard Corp., Iselin, NJ); APF = aluminaized plastic film (Clarke Ag Plastics, Greenwood, VA).

| Gene         | 2005       | 2006       | 2005       | 2006       | 2005       | 2006       |
|--------------|------------|------------|------------|------------|------------|------------|
|              | RPF*       | APF*       | RPF         | APF         | RPF         | APF         |
| MdAct        | 0*         | 0          | 0          | 0          | 0          | 0          |
| MdPhyB       | 0          | +          | 0          | 0          | 0          | 0          |
| MdPhyE       | 0          | 0          | 0          | 0          | 0          | 0          |
| MdHyc5       | 0          | 0          | 0          | 0          | 0          | 0          |
| MdPif3       | 0          | +          | 0          | 0          | 0          | 0          |
| MdLhcb2-2    | 0          | 0          | 0          | 0          | 0          | 0          |
| MdRbcS       | 0          | 0          | 0          | 0          | 0          | 0          |
| MdElip1A/B   | 0          | +          | 0          | 0          | 0          | 0++        |
| MdElip1D     | +          | 0          | 0          | 0          | +          | +          |
| MdSot3       | +          | +          | -          | -          | +          | -          |

Significant differences are indicated by symbols + and –: + = significantly increased over control; ++ = greater than 10 times increased over control; – = significantly decreased relative to control; 0 no significant difference compared with control; blank cell = not done.

Table 1. Summary of light-regulated gene responses in ‘Empire’ apple leaves to different groundcover treatments in 2005 and 2006 using two assay methods.
however, *MdSot3* transcript abundance was significantly different from the control, being highest under RPF treatment and lowest under APF (Fig. 1B). In 2006, like in 2005, *MdAct*, *MdLhcb2-2*, and *MdHy5* continued to show no difference in expression by treatment. In contrast to the 2005 results, expression of *MdPif3*, *MdPhyB*, and *MdElip1A/B* was highest in the 2006 RPF treatment; in fact, the APF treatment clearly suppressed *MdRbcS* (Fig. 1C) and *MdPif3* (Fig. 1B) transcript abundance as it did in both years for *MdSot3* transcripts.

Semiquantitative RT-PCR was sufficiently reproducible to evaluate transcript accumulation of *MdAct*, *MdHy5*, *MdLhcb2-2*, and *MdSot3* in 2005–06 (compare Table 1 and Fig. 1A–B). However, the variability of results using this technique with respect to the abundance of *MdPif3*, *MdElip1A/B*, and *MdElip1D* mRNAs led us to analyze their expression using the more accurate qRT-PCR methodology with actin serving as an internal normalization control and *MdHy5* expression as a light-responsive control, because their abundance was unaffected by treatment [Table 1; Fig. 1A (semiquantitative RT-PCR)]. Quantitative RT-PCR analysis of *MdPif3* and *MdHy5* for years 2005–06 (Table 1; Fig. 2) was similar, revealing no differences between treatments and controls. Expression of *MdElip1A/B* (in 2005) and *MdElip1D* (in 2006) was also not significantly different by treatment. On the other hand, expression of *MdElip1D* was elevated by APF in 2005 but not in 2006; in contrast, *Elip1A/B* showed a dramatic increase in transcript abundance in response to the APF treatment in 2006, but not in 2005.

Correlation of expression by semiquantitative RT-PCR of the apple genes used in this study provided a dimension of relatedness among genes. Expression of *MdLhcb2-2* and *MdSot3* was significantly correlated with expression of the apple *PhyB*, *Hy5* and the *Elip* genes (Tables 2 and 3). Interestingly, *MdRbcS* expression only correlated with *MdAct* and *MdPif3* expression. Analysis of expression of the genes determined by qRT-PCR indicated that the *MdElip* genes were correlated with each other and were the only correlation detected.

**Discussion**

Most studies of light perception and the regulation of light-activated genes have focused on young, herbaceous plants, and very little information is available regarding studies in woody species. Even less is known about how these genes respond to...
July (long days) where, like grape, both apple phytochromes
2009). Although our experiments were conducted in June and
MdPhyB
PhyA
2003). In contrast, in field-grown grape (Vitis vinifera
A.
herbaceous plants, yet it is likely that these genes would behave
and on key transcription regulatory proteins have centered on
studies conducted on the phytochromes and interacting factors
not respond appreciably to light (Lu et al., 2002). Most of the
MdRbcS
MdAct

| Variablea | MdAct | MdRbcS | MdPif3 | MdLhcb2–2 | MdPhyB | MdElip1A/B | MdElip1D | MdHy5 | MdSot |
|-----------|-------|--------|--------|-----------|--------|------------|----------|--------|-------|
| MdAct     |       | 1      |        |           |        |            |          |        |       |
| MdRbcS    | 0.4329|        | 1      |           |        |            |          |        |       |
| MdPif3    | 0.2411| 0.4303 |        | 1         |        |            |          |        |       |
| MdLhcb2–2 | −0.0777| −0.3395| 0.2952 |          | 0.8393 |            |          |        |       |
| MdPhyB    | 0.1046| −0.1572| 0.4424 | 1         |        |            |          |        |       |
| MdElip1A/B| −0.0152| −0.4149| 0.258  | 0.8744    | 0.8416 |            |          |        |       |
| MdElip1D  | 0.172 | −0.1441| 0.3495 | 0.6682    | 0.7383 | 0.7535     |          |        |       |
| MdHy5     | 0.1828| −0.2251| 0.2396 | 0.7485    | 0.8763 | 0.7979     | 0.7106   | 1      | 0.7683|
| MdSot     | −0.1237| −0.4321| 0.244  | 0.8333    | 0.8133 | 0.9683     | 0.7133   | 0.7683| 1      |

*Values greater than 0.273 indicate significant correlation of gene expression at the P = 0.05 level (highlighted in gray).

light regulation in the field where many different environmental factors can modulate their expression. Previous studies of apple trees treated with particle films as groundcovers or groundcover supplements have shown beneficial effects on fruit color and fruit size (Glenn and Puterka, 2007), but little or no information is available as to how these treatments affect light transduction in leaves at the molecular level. Because leaves are the primary "source" organs that ultimately affect fruit size, it is important to understand how the expression of light-regulated genes in leaves is affected by different groundcover treatments used under field conditions. Such studies will be useful in the future for designing strategies for improvement of agricultural production systems, particularly with regard to maintenance of orchard productivity.

The following genes whether analyzed by semiquantitative RT-PCR or qRT-PCR showed few, if any, differences by treatment or year compared with untreated controls: MdAct, MdPhy B (and MdPhyE), MdHy5, MdPif3, MdLhcb2-2, and MdRbcS. The lack of response regarding MdAct expression is consistent with previous studies indicating that this gene does not respond appreciably to light (Lu et al., 2002). Most of the studies conducted on the phytochromes and interacting factors and on key transcription regulatory proteins have centered on herbaceous plants, yet it is likely that these genes would behave somewhat differently in woody species. For example, in A. thaliana, PhyA transcripts are regulated diurnally under short days, but not in plants grown under long days (Mockler et al., 2003). In contrast, in field-grown grape (Vitis vinifera), both PhyA and B were controlled by a diurnal rhythm under long days (December, long days = 14 h 40 min light in the southern hemisphere) but were uniformly at maximum abundance under short days (April, short days = 11 h 12 min light) (Kühn et al., 2009). Although our experiments were conducted in June and July (long days) where, like grape, both apple phytochromes could have been oscillating, this is probably not a contributing factor to the field variation we observed, because collection times were always in full sun at the same time of day.

It is well known that phytochromes are predominantly regulated at the post-translational level, existing in two forms and intracellular locations, depending on the ratio between R and FR light (Kircher et al., 1999; Sakamoto and Nagatani, 1996). Therefore, the lack of effect on transcript accumulation resulting from reflective particle treatment in our study may be more pronounced at the protein level or in the receptor's ability to translocate between the cytoplasm and nucleus given that the R/FR of RPF reflected light (0.53) is considerably different from that of the APF (1.0) groundcover.

Both HY5 and PIF3 are transcription factors that are part of a light-regulated cascade. The expression of Hy5 in A. thaliana is regulated directly or indirectly by PhyA and B (Koornneef et al., 1980; Oyama et al., 1997) by UVR8, the photoreceptor of ultraviolet-B light (Brown et al., 2005; Ulm et al., 2004) and by CRY1 (Liu et al., 2011). The fact that MdHy5 expression was not significantly different by treatment in either sampling year is somewhat surprising because Hy5 is induced by narrow band ultraviolet-B, which is almost completely reflected by APF [81% of PAR (D.M. Glenn, unpublished data)]. Ultraviolet-B exposure is divided into two stages: a narrowband, very rapid (minutes) photomorphogenic effect and a broadband slower stress response (Favory et al., 2009; Ulm et al., 2004). In A. thaliana, Athy5 transcript levels rise quickly (within 2 h of dawn) in natural light (Sellaro et al., 2011). However, because our sampling times were well after dawn, MdHy5 levels were likely at maximum and would probably have shown little difference in response to the two treatments.

PIF3 is a transcription factor involved in both photomorphogenesis and shade avoidance (Supplemental Fig. 2). MdPif3 levels were not different by treatment using qRT-PCR analysis in either 2005 or 2006. The lack of treatment differences in MdPif3 transcripts is consistent with the expression of MdHy5 and may reflect the functional relationship between the two transcription factors.

Lhcb2-2 and RbcS are downstream targets of light regulation associated with the light reaction of photosynthesis and carbon fixation, respectively. The expression of both genes in apple leaves in 2005 was unaffected by treatment. In 2006, both genes were elevated by APF treatment; however, only RbcS expression differences were significant, although small.

Reflection from the mylar film increases the R/FR ratio impinging on much of the leaf canopy undersurface to a ratio
close to that of sunlight (1.1 vs. 1.35) compared with leaf undersurfaces above the untreated grass control (0.33). Although weather patterns for the sampling times in 2005 and 2006 were similar, as was the PAR measured at the time of sampling, it is possible that cloudy days before sampling in 2005 vs. 2006 lowered the APF R/FR ratio and suppressed its cumulative effect.

At least four different Elip genes (designated MdElip1A–1D) reside on the apple genome. Semiquantitative primer pairs, MdELIPF2 and R2-3, and qPCR primers, qMdELIPF2 and R2, would not amplify MdElip1C or MdElip1D under the conditions used but would amplify both MdElip1A and 1B (Supplemental Table 2). On the other hand, primer pairs MdELIPF1 and R1, as well as qMdELIPF1 and R2, would selectively amplify MdElip1D.

MdElip1D expression was most consistent in 2006 where no difference by treatment was observed. In 2005 significant differences were determined but they were not consistent by treatment or by method. MdElip1A/B expression was not significantly different by treatment in 2005 but was increased by APF in 2006 nearly 14 times over the control by qRT-PCR analysis. Interestingly, expression of both MdElip1A/B and D correlated with each other in both semiquantitative and quantitative analyses. In contrast, although Elip genes are targets of Hy5, neither correlated with this transcription factor. This suggests that APF reflective film (higher ultraviolet reflection) may have uncoupled the normal interaction between HY5 and the Elip1 promoters, although this seems unlikely because both Hy5 and Elip1 are increased in A. thaliana shortly after ultraviolet treatment (Ulm et al., 2004). Another possibility is that APF triggers Elip1 induction through a HY5-independent pathway. This seems more likely because, as noted previously, Elip1A/B may be subject to heat shock induction, and films like APF can add as much as 2 °C to the air temperature in their immediate environment (Layne et al., 2001).

The promoters of both MdElip1A and B genes are very similar, having several elements related to light regulation (Supplemental Fig. 3). Both genes also have a long repeat element upstream of the consensus TATA box. MdElip1A has two Pf3 response elements, whereas Elip1B has none. MdElip1B has an upstream consensus TATA element close to an osmoregulatory element, both of which are absent from the MdElip1A promoter. Heat shock elements in both promoters are located much further upstream than the sequence shown in Supplemental Figure 3. Along these same lines, the promoter of MdElip1D (not shown), although lacking in heat shock response elements within the first 1500 bases upstream of the ATG translation start codon, possesses 10 ACGT cores, elements related to both light and hormone responses that could indicate potential regulation by other types of abiotic stress.

ELIPs are associated with a number of abiotic stress-responsive pathways. In A. thaliana, R, FR, blue, and ultraviolet-A light positively regulate AtElip1 and 2 expression (Adamska et al., 1992; Harari-Steinberg et al., 2001), and this regulation seems to be independent of light intensity with respect to R and blue light (Adamska, 1995). Although only AtElip1 induction is promoted by HY5 in light, heat shock induction of both AtElip1 and 2 is independent of both light and HY5 activity (Harari-Steinberg et al., 2001).

Another interesting observation was reported by Mishra et al. (2012) in A. thaliana. AtELIP proteins 1 and 2 were only expressed in field-grown plants, not in plants grown in controlled chambers under different light regimes. The highest light intensity used on growth chamber-grown plants was 600 μmol·m⁻²·s⁻¹ quanta, whereas the leaves of field-grown plants were harvested at noon in full sunlight (≈1900 μmol·m⁻²·s⁻¹ quanta at the latitude indicated). This result indicates that the high light intensity and ultraviolet content of full sunlight can induce these photoprotective proteins.

Sorbitol is the primary transport sugar in apple. It is synthesized in leaves (source) and transported to other tissues (sink) as required. Multiple sequences corresponding to the apple sorbitol transporter were found in the apple genome. However, the primer pair used to assay expression would have only recognized MdSot3 (Watari et al., 2004). This gene was selected on the assumption that it would show little or no light regulation. Surprisingly, MdSot3 abundance was increased by RPF treatment but decreased by APF. Both changes were statistically significant in both years and may reflect differences in ultraviolet-B wavelengths reflected by the two materials or R/FR effects on sorbitol translocation. Expression of MdSot3 by semiquantitative RT-PCR correlated with Hy5 transcript abundance but not with Pf3; this is consistent with the role of HY5 in activation of downstream genes. Glenn and Puterka (2007) found that RPF increased fruit size in all years in contrast with APF and hypothesized that the reduced R/FR reflection increased carbon transport to fruit as was demonstrated by others (Fortnum and Kasperbauer, 1992; Kasperbauer, 1987, 2000; Kasperbauer and Hunt, 1998; Kasperbauer and Karlen, 1986; Matheny et al., 1992). Their interpretation is supported by the consistent upregulation of the sucrose transporter gene MdSot3 (Table 1).

Despite some year-to-year differences in response to treatment, several general conclusions can be drawn. Most of the genes analyzed in this study were either unchanged by the groundcover treatments or were elevated (e.g., MdSot3, MdPif3, and MdPhyB) by a reflective particle film applied to the groundcover. The aluminized plastic film suppressed expression of MdPif3, MdRbcS, and MdSot3. However, APF treatment evoked the strongest positive response on MdElip1A/B expression. We conclude that treatment with RPF was either stimulatory or not different from untreated controls regarding the expression of genes associated with light regulation. In contrast, the expression of several light-responsive genes was decreased by APF treatment with the exception of the early light-induced protein, MdElip1A/B, which was significantly stimulated by APF treatment. These results parallel the field study of Glenn and Puterka (2007) regarding the separation of gene responses; i.e., RPF significantly increased fruit size and also increased MdSot3 expression. Increased sorbitol transport is likely related to increased carbon partitioning to the fruit. The increase in MdPif3 and MdPhyB in leaves may be a consequence of the altered R/FR ratio of the RPF light quality, which is associated with modest improvement in fruit color. Conversely, APF significantly increased fruit color (Glenn and Puterka, 2007) and MdElip1A/B in our study, both observations of which are primarily ultraviolet-B light-dependent responses. Ultraviolet-B improvement in fruit color is likely the result of enhanced transcription of pigment synthesis genes for protection, similar to the MdElip1A/B response, which is also associated with protection against ultraviolet and high light-intensity damage. This study illustrates that gene expression can be tied to field responses and used to identify critical pathways influenced by cultural practices. It is speculated that further experimentation to refine the results presented here
could lead to the development of strategies to exploit light stress (e.g., ultraviolet, high light intensity, and modifying R/FR) to improve the quality or effectiveness of tree fruit production systems (e.g., see Wargent and Jordan, 2013).

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Supplemental Table 1. Comparison of apple genes to *Arabidopsis thaliana*, barrel medic, *Medicago truncatula* Gaertn., and grape, *Vitis vinifera* L., genes.

| Locus tag          | Gene  | Source                   | E value | Total score |
|--------------------|-------|--------------------------|---------|-------------|
| MDP0000290263      |       | BLAST match apple, chr12 |         |             |
| At2g46970          | Pil1  | *Arabidopsis thaliana*   | 7e-42   | 187         |
| At3g62090          | Pil2  | *Arabidopsis thaliana*   | 7e-28   | 182         |
| At1g09530          | Pil3  | *Arabidopsis thaliana*   | 3e-78   | 251         |
| MTR1g084980        | Pil3  | *Medicago truncatula*    | <1e-179 | 532         |
| MDP0000205358      |       | BLAST match apple, chr4  |         |             |
| At2g46970          | Pil1  | *Arabidopsis thaliana*   | 1e-39   | 197         |
| At3g62090          | Pil2  | *Arabidopsis thaliana*   | 7e-25   | 170         |
| At1g09530          | Pil3  | *Arabidopsis thaliana*   | 5e-76   | 245         |
| MTR1g084980        | Pil3  | *Medicago truncatula*    | <1e-179 | 540         |
| MDP0000896307      |       | BLAST match apple, chr12 |         |             |
| At2g16120          | Pmt1  | *Arabidopsis thaliana*   | <1e-179 | 676         |
| MDP0000264514      |       | BLAST match apple genome, chr12 | |             |
| AT5g11260          | Hy5   | *Arabidopsis thaliana*   | 5e-77   | 221         |
| AGN75071           | Hy5   | *Vitis vinifera*         |         |             |
| MDP0000586302      |       | BLAST match apple genome, chr12 | |             |
| At5g11260          | Hy5   | *Arabidopsis thaliana*   | 2e-71   | 203         |
| AGN75071           | Hy5   | *Vitis vinifera*         | 2e-78   | 221         |
| MDP0000840536      |       | BLAST match apple genome, chr9 | |             |
| MD0000542944       | MdElip1A | BLAST match apple genome, chr16 | |             |
| At3g22840          | AtLil1| *Arabidopsis thaliana*   | 9e-70   | 201         |
| At4g14690          | AtLil2| *Arabidopsis thaliana*   | 5e-65   | 189         |
| MDP0000542944      |       | BLAST match apple genome, chr16 | |             |
| At3g22840          | AtLil1| *Arabidopsis thaliana*   | 2e-70   | 203         |
| At4g14690          | AtLil2| *Arabidopsis thaliana*   | 4e-64   | 187         |
| MDP0000872569      |       | BLAST match apple genome, chr14 | |             |
| MDP0000840536      |       | BLAST match apple genome, chr13 | |             |
| At2g37620          | AtAct1| *Arabidopsis thaliana*   | <e-179  | 759         |
| At3g18789          | AtAct2| *Arabidopsis thaliana*   | <e-179  | 706         |
| At3g35750          | AtAct3| *Arabidopsis thaliana*   | <e-179  | 759         |
| At5g59370          | AtAct4| *Arabidopsis thaliana*   | <e-179  | 749         |
| At5g09810          | AtAct7| *Arabidopsis thaliana*   | <e-179  | 774         |
| At1g49240          | AtAct8| *Arabidopsis thaliana*   | <e-179  | 747         |
| At2g42090          | AtAct9| *Arabidopsis thaliana*   | <e-179  | 566         |
| At3g46520          | AtAct12| *Arabidopsis thaliana*  | <e-179  | 751         |
| MDP0000572047      |       | BLAST match apple genome, chr1 | |             |
| MDP0000774288      |       | BLAST match apple genome, chr13 | |             |
| At2g37620          | AtAct1| *Arabidopsis thaliana*   | <e-179  | 759         |
| At3g18789          | AtAct2| *Arabidopsis thaliana*   | <e-179  | 701         |
| At3g35750          | AtAct3| *Arabidopsis thaliana*   | <e-179  | 759         |
| At5g59370          | AtAct4| *Arabidopsis thaliana*   | <e-179  | 748         |
| At5g09810          | AtAct7| *Arabidopsis thaliana*   | <e-179  | 772         |
| At1g49240          | AtAct8| *Arabidopsis thaliana*   | <e-179  | 741         |

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Supplemental Table 1. Continued.

| Locus tag    | Gene          | Source                          | E value | Total score |
|--------------|---------------|---------------------------------|---------|-------------|
| At2g42090    | AtAct9        | Arabidopsis thaliana           | <e-179  | 563         |
| At3g46520    | AtAct12       | Arabidopsis thaliana           | <e-179  | 749         |
| MDP0000316929| MdRbcS        | BLAST match apple genome, chr13 | —       | —           |
| AT5g38430    | AtRbcS1B      | Arabidopsis thaliana           | 2e-44   | 174         |
| AT5g38420    | AtRbcS2B      | Arabidopsis thaliana           | 6e-44   | 190         |
| AT5g38410    | AtRbcS3B      | Arabidopsis thaliana           | 2e-43   | 172         |
| AT1g67090    | AtRbc1A       | Arabidopsis thaliana           | 7e-08   | 70          |
| MDP0000181339| MdRbcS        | BLAST match apple genome, chr13 | —       | —           |
| MDP0000316929| MdRbcS        | —                               | <e-179  | 1157        |
| AT5g38430    | AtRbcS1B      | Arabidopsis thaliana           | 1e-61   | 223         |
| AT5g38420    | AtRbcS2B      | Arabidopsis thaliana           | 2e-61   | 239         |
| AT5g38410    | AtRbcS3B      | Arabidopsis thaliana           | 4e-61   | 223         |
| AT1g67090    | AtRbc1A       | Arabidopsis thaliana           | 1e-23   | 99          |
| MDP0000221485| MdLhcb2       | Perfect match to MaldoLhcb2.2   | —       | —           |
| MDP0000285110| MdPhyB        | BLAST match apple genome, chr16 | —       | —           |
| At2g18790    | AtPhyB        | Arabidopsis thaliana           | <e-179  | 1859        |
| At4g16250    | AtPhyD        | Arabidopsis thaliana           | <e-179  | 1752        |

<e— = E value and Total score are perfect match to self.

<sup>1</sup>MDP0000304059 on chr7 (not shown) was a match to the other two apple actins, but there were seven mismatched nucleotides (seven of 24 = 30%) in the MdActF1 primer, making it unlikely that RNA from this locus would be detected with reverse transcription–polymerase chain reaction.

<sup>2</sup>Sanzol (2010).
Supplemental Table 2. Primers used in RT-PCR reactions.

| Gene ID | Primer ID | Primer sequence | Primer Tm | Apple genome seq ID |
|---------|-----------|-----------------|-----------|---------------------|
| **Quantitative RT-PCR primers** | | | | |
| **MdPif3** | MdPil1chr12 5F | 5'-ACT GAG GCC AGA TTC AGT AA-3' | 55.3 °C | MDP0000290263 and MDP0000205358 |
| | MdPil1chr12 5R | 5'-CAC ACC GTT GTG CTG AAC AT-3' | 55.4 °C | MDP0000586302 and MDP0000264514 |
| **MdHy5** | qMdHy5F2 | 5'-ACT GAG GCC AGA TTC AGT AA-3' | 55.3 °C | MDP0000739699 and MDP0000872569 |
| | qMdHy5R2 | 5'-CAC ACC GTT GTG CTG AAC AT-3' | 55.4 °C | MDP0000586302 and MDP0000264514 |
| **MdElip1D** | qMdELIP1F2 | 5'-ACT GAG GCC AGA TTC AGT AA-3' | 55.3 °C | MDP0000739699 and MDP0000872569 |
| | qMdELIP1R2 | 5'-CAC ACC GTT GTG CTG AAC AT-3' | 55.4 °C | MDP0000586302 and MDP0000264514 |
| **MdAct** | qMdActpF | 5'-ACT GAG GCC AGA TTC AGT AA-3' | 55.3 °C | MDP0000739699 and MDP0000872569 |
| | qMdActpR | 5'-CAC ACC GTT GTG CTG AAC AT-3' | 55.4 °C | MDP0000586302 and MDP0000264514 |
| **Semiquantitative RT-PCR primers** | | | | |
| **MdElip1A and B** | MdELIPF2 | 5'-CTACTCTTCTCTCTCGTCCC-3' | 67.3 °C | MDP0000840536 and MDP0000542944 |
| | MdELIPR2-3 | 5'-GCAAACCTTCCATTCCAGAGC-3' | 70.8 °C | MDP0000840536 and MDP0000542944 |
| **MdRbcS** | RBSS(apple)F1 | 5'-AACTCTCTCGTCAAGATGGA-3' | 53.8 °C | MDP0000181339 and MDP0000221483 |
| | RBSS(apple)R1 | 5'-AACTCTCTCGTCAAGATGGA-3' | 53.8 °C | MDP0000181339 and MDP0000221483 |
| **MdLhcb2** | PCH108KO | 5'-GCTGGACTATCCGCAGACCCTGAGAC-3' | 55.0 °C | MDP0000221483 |
| | Pch108SA | 5'-GCTGGACTATCCGCAGACCCTGAGAC-3' | 55.0 °C | MDP0000221483 |
| **MdSot3** | MdSOT3F | 5'-GCAAATCGGCGGAGAATCG-3' | 55.0 °C | MDP0000221483 |
| | MdSOT3R | 5'-GCAAATCGGCGGAGAATCG-3' | 55.0 °C | MDP0000221483 |
| **MdAct** | MdActF1 | 5'-CTACTCTTCTCTCGTCAAGATGGA-3' | 55.0 °C | MDP0000221483 |
| | MdActR1 | 5'-CTACTCTTCTCTCGTCAAGATGGA-3' | 55.0 °C | MDP0000221483 |
| **MdPhyB** | MdPhyBF1 | 5'-CTACTCTTCTCTCGTCAAGATGGA-3' | 55.0 °C | MDP0000221483 |
| | MdPhyBR1 | 5'-CTACTCTTCTCTCGTCAAGATGGA-3' | 55.0 °C | MDP0000221483 |
| **MdHy5** | MdHy5F1 | 5'-CTACTCTTCTCTCGTCAAGATGGA-3' | 55.0 °C | MDP0000221483 |
| | MdHy5R1 | 5'-CTACTCTTCTCTCGTCAAGATGGA-3' | 55.0 °C | MDP0000221483 |
| **MdPif3** | MdPil1chr12 5F | 5'-ACT GAG GCC AGA TTC AGT AA-3' | 55.3 °C | MDP0000290263 and MDP0000205358 |
| | MdPil1chr12 5R | 5'-CAC ACC GTT GTG CTG AAC AT-3' | 55.4 °C | MDP0000586302 and MDP0000264514 |

*There are two nearly identical Pif3 genes in the apple genome: one on chr12 and one on chr4. Primer pair is specific for Pif3 on chr12 but may also hybridize to a lesser degree with transcripts from Pif3 on chr4.

*yTwo nearly identical genes match the forward and reverse primers. MDP0000840536 is a perfect match but MDP0000542944 is mismatched by two nucleotides in both primer pairs.

*zBecause the melting temperatures of the primer pair for this gene differ significantly, initial RT-PCR reactions were run in a gradient thermocycler to optimize the annealing temperature (59 °C) for subsequent semiquantitative RT-PCR reactions.

RT-PCR = reverse transcription–polymerase chain reaction.
Supplemental Fig. 1. Light regulation through HY5. HY5 acts as a central regulator in light signaling pathways driven by red, blue/UV-A, and UV-B. COP1, an E3 ligase, targets HY5 for degradation in the dark. Under red or blue light, complexes formed between PHYA–E and PIFs (red) or CRY1 and SPA1 (blue/UV-A) prevent the degradation of HY5 and allow the activation of its target genes. UV-B light is perceived by the UVR8 receptor, which can bind directly to HY5 to promote its transcription. Although UVR8 also binds COP1, it is not clear whether this activity contributes to HY5 activation by preventing its degradation. UV = ultraviolet.
Supplemental Fig. 2. Shade avoidance mechanisms. Under the far-red light, PHYb is in its inactive form and the PIF transcription factors are free to transcribe \textit{PIL1} and other genes to activate the shade avoidance response. Under red (PIF4/5) and prolonged red light (PIF3/7), the PIF transcription factors bind to the active (Pfr) form of PHYB (Leivar et al., 2008). The PHYB:PIF complex facilitates degradation of the PIFs by the 26S proteosome, thus preventing activation of \textit{PIL1} by the PIF transcription factors and causing the shade avoidance response to be blocked. An alternative pathway involves the binding of HFR1 (long hypocotyl in far-red light) to the PIF factors (Hornitschek et al., 2009; Lorrain et al., 2008). This complex also favors degradation of the PIFs and blocks the shade avoidance response. No direct interaction with PHYB is proposed.
Supplemental Fig. 3. Promoter comparison of MdElip1A and MdElip1B. Promoters (approximately 1000 bp upstream of the translation start site (bold) were analyzed by PLACE and Plant CARE. Bases identical to both promoters are indicated by asterisks. Consensus TATA boxes are bold-underlined; an upstream consensus TATA box is bold-dotted underlined. Relevant cis-acting elements on the plus strand are indicated as follows: green highlight = light regulation, different binding sites [GCCAC/G, ACGT, GATA] collectively boxed = PIF3 element; yellow highlight = putative hormone (e.g., ABA) and light response element core (note that two of the Pif3 elements have an ACGT core); gray highlight = Myb/Myc element (CANNTG consensus) (note that these elements overlap two of the Pif3 elements); dark blue highlight = tandem repeat of unknown function; light blue highlight = low temperature/CBF element (note that in MdElip1A, it has been altered); magenta highlight = osmoregulatory element. R = A or G; Y = C or T. The TATCCAC box (double underlined in MdElip1B but altered in MdElip1A) is associated with GA response and requires the GA response element (upstream of illustration) and a pyrimidine box (CCTTTT and TTTTTTCC) also upstream of illustration. MdElip1A has two heat shock elements much further upstream of the consensus TATA box; MdElip1B has a single HSE in a similar position in the promoter (not shown).