

A Constitutive Shade-Avoidance Mutant Implicates TIR-NBS-LRR Proteins in *Arabidopsis* Photomorphogenic Development ^W

Ana Faigón-Soverna,^a Franklin G. Harmon,^{b,1} Leonardo Storani,^{a,c} Elizabeth Karayekov,^a Roberto J. Staneloni,^c Walter Gassmann,^d Paloma Más,^e Jorge J. Casal,^a Steve A. Kay,^b and Marcelo J. Yanovsky^{a,2}

^aIFEVA-Facultad de Agronomía, Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas, 1417 Buenos Aires, Argentina

^bDepartment of Cell Biology and Institute for Childhood and Neglected Diseases, Scripps Research Institute, La Jolla, California 92037

^cInstituto de Investigaciones Bioquímicas, Fundación Instituto Leloir, 1405 Buenos Aires, Argentina

^dDivision of Plant Sciences, University of Missouri, Columbia, Missouri 65211-7310

^eConsorci Consejo Superior de Investigaciones Científicas, Institut de Recerca i Teconologia Agroalimentaries, Laboratori de Genètica Molecular Vegetal, Institut de Biologia Molecular de Barcelona/Jordi Girona, Barcelona 08034, Spain

In plants, light signals caused by the presence of neighbors accelerate stem growth and flowering and induce a more erect position of the leaves, a developmental strategy known as shade-avoidance syndrome. In addition, mutations in the photoreceptors that mediate shade-avoidance responses enhance disease susceptibility in *Arabidopsis thaliana*. Here, we describe the *Arabidopsis constitutive shade-avoidance1 (csa1)* mutant, which shows a shade-avoidance phenotype in the absence of shade and enhanced growth of a bacterial pathogen. The *csa1* mutant has a T-DNA inserted within the second exon of a *Toll/Interleukin1 receptor–nucleotide binding site–leucine-rich repeat (TIR-NBS-LRR)* gene, which leads to the production of a truncated mRNA. *Arabidopsis* plants transformed with the truncated *TIR-NBS-LRR* gene recapitulate the mutant phenotype, indicating that *csa1* is a dominant-negative mutation that interferes with phytochrome signaling. TIR-NBS-LRR proteins have been implicated in defense responses in plants. *RPS4*, the closest homolog of *CSA1*, confers resistance to *Pseudomonas syringae* and complements the *csa1* mutant phenotype, indicating that responses to pathogens and neighbors share core-signaling components in *Arabidopsis*. In *Drosophila melanogaster* and *Caenorhabditis elegans*, TIR domain proteins are implicated in both development and immunity. Thus, the dual role of the TIR domain is conserved across kingdoms.

INTRODUCTION

Plants respond to the presence of potential competitors thanks to the perception of the low proportions of red compared with far red of the light transmitted or reflected by green tissues of neighbor plants (Ballaré, 1999; Smith, 2000; Franklin and Whitelam, 2005). Low red:far-red ratios are perceived by phytochromes, which exist in two photointerconvertible forms, an inactive red-absorbing form (Pr) and an active far-red-absorbing form (Pfr) (Quail, 2002). The high red:far-red ratio characteristic of environments with low plant density moves the phytochrome system toward the Pfr form, which inhibits stem growth, hyponasty (upward movement of leaves), and flowering (Ballaré, 1999; Smith, 2000). Conversely, in dense canopies, the red:far-red ratio is reduced, and the photoequilibrium is dominated by inactive

phytochrome. This releases stem growth, hyponasty, and flowering, causing tall plants with erect leaves to less likely become shaded by neighbors (Ballaré, 1999; Smith, 2000; Franklin and Whitelam, 2005).

Phytochrome B (phyB) is the member of the phytochrome family that contributes most strongly to shade-avoidance responses (Yanovsky et al., 1995; Franklin et al., 2003). The inactive form of phyB is localized in the cytosol, whereas the active form accumulates in the nucleus (Nagatani, 2004) and affects the expression of many genes (Devlin et al., 2003; Tepperman et al., 2004).

A number of components mediating phyB signaling have been identified through forward or reverse genetic screens, including transcription factors, kinases, phosphatases, and proteins with unknown biochemical functions (Chen et al., 2004). However, the molecular mechanism through which phyB exerts its activity is still unclear.

Here, we describe the *Arabidopsis thaliana constitutive shade-avoidance1 (csa1)* mutant, which displays shade-avoidance responses in the absence of shade. The *csa1* mutant phenotype is caused by a mutation in a *Toll/Interleukin1 receptor–nucleotide binding site–leucine-rich repeat (TIR-NBS-LRR)*-type gene. *TIR-NBS-LRRs* are plant disease resistance genes of particular interest due to their homology to Toll-like receptors that trigger

¹Current address: Plant Gene Expression Center, Albany, CA 94710.

²To whom correspondence should be addressed. E-mail yanovsky@ifeva.edu.ar; fax 54-11-4514-8134.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Marcelo J. Yanovsky (yanovsky@ifeva.edu.ar).

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innate immune responses in animals (Holt et al., 2003). In plants, TIR-NBS-LRRs confer resistance against specific pathogen strains in accordance to the gene-for-gene model (Flor, 1956). The closest homolog of *CSA1*, *RPS4*, confers resistance to *Pseudomonas syringae* pv *tomato* strain DC3000 expressing *avrRps4* (Gassmann et al., 1999). Interestingly, *RPS4* complements the *csa1-1* mutant phenotype, indicating that the ability to respond to pathogens and neighbors is mediated, at least in part, through common signaling components in *Arabidopsis*.

RESULTS AND DISCUSSION

Isolation and Physiological Characterization of *csa1*

The *csa1* mutant was isolated in a screening of activation-tagged lines (Weigel et al., 2000) for seedlings displaying a shade-avoidance phenotype (long stem) under high red:far-red ratios (i.e., in the absence of a shade signal). *csa1* also showed long petioles, erect and pale (low chlorophyll content) leaves, and early flowering under short (noninductive) photoperiods (Figure 1), resembling the *phyB* mutant.

To test whether shade-avoidance responses were impaired in the *csa1* mutant, we compared the leaf angle of wild-type and *csa1* plants placed for 5 h either under sunlight (red:far-red ratio = 1.1) or under a dense canopy (red:far-red ratio = 0.2) of the grass *Cynodon dactylon*. While leaf angle dramatically decreased in wild-type plants placed under the grass canopy compared with plants left under sunlight, no significant response was observed for *csa1* (Figure 2A).

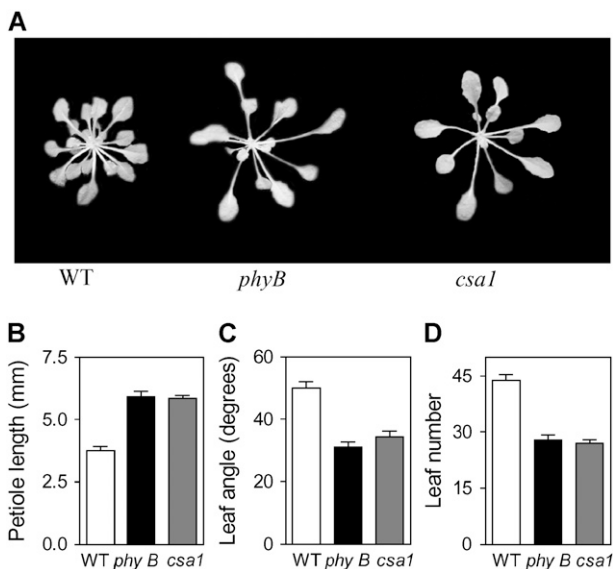


Figure 1. *csa1* Shows the Shade-Avoidance Developmental Pattern under High Red:Far-Red Ratios and Resembles the *phyB* Mutant.

- (A) Representative 30-d-old plants.
 (B) Petiole length.
 (C) Leaf angle.
 (D) Rosette leaf number (reduced leaf number indicates early flowering).

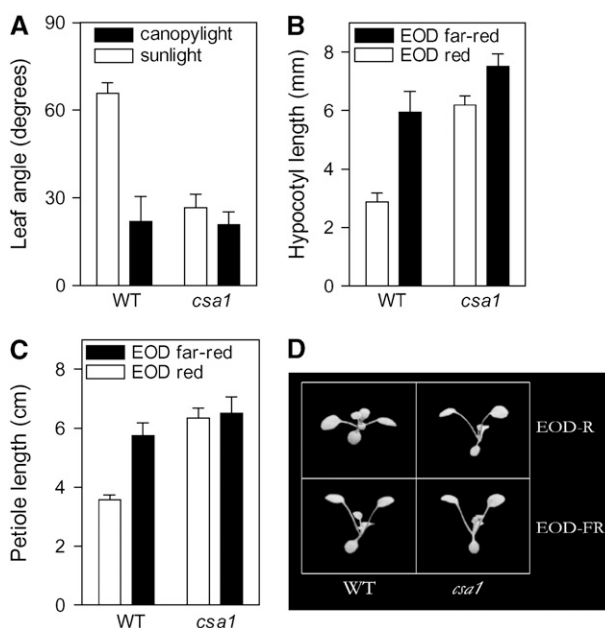


Figure 2. *csa1* Growth and Development under Natural and Simulated Shade.

(A) The response to natural shade. Leaf angle of seedlings grown for 14 d under fluorescent white light and placed on the morning of the 15th day in the field under either sunlight or canopy shade light for 5 h.

(B) Response to simulated shade. Hypocotyl length of seedlings grown for 3 d under short days (8 h white light/16 h darkness) ended with a 5-min red or far-red light pulse.

(C) and (D) Response to simulated shade. Wild-type and *csa1* seedlings grown for 2 weeks under short days (8 h white light/16 h darkness) ended with a 5-min pulse of either red or far-red light.

The shade imposed by the grass canopy simultaneously reduces the red:far-red ratio and total fluence rate of light (Franklin and Whitelam, 2005). To analyze the specific response to changes in light quality, wild-type and *csa1* seedlings were grown under white-light photoperiods (8 h) ended with a pulse (5 min) of either red or far red, which establish high or low Pfr levels during the subsequent dark period, respectively (Fankhauser and Casal, 2004). End-of-day (EOD) far red significantly promoted hypocotyl (stem) and petiole growth in wild-type plants, while responses to EOD far red were significantly reduced (Figure 2B) or absent (Figure 2C) in the *csa1* mutant.

In seedlings that grow on seed reserves in complete darkness, exposure to light signals a major shift in developmental pattern, including the arrest of hypocotyl growth (see wild type in Figure 3A). *csa1* showed poor inhibition of hypocotyl growth under red light, the spectral region that most efficiently activates phyB, but it retained normal responses to far-red or blue light compared with darkness (Figures 3A and 3B), which are mediated mainly by another member of the phytochrome family (phyA; Quail, 2002) and cryptochromes (Cashmore et al., 1999). This indicates that *csa1* is not generally defective in light regulation of cell growth, but it is specifically impaired in red light signaling.

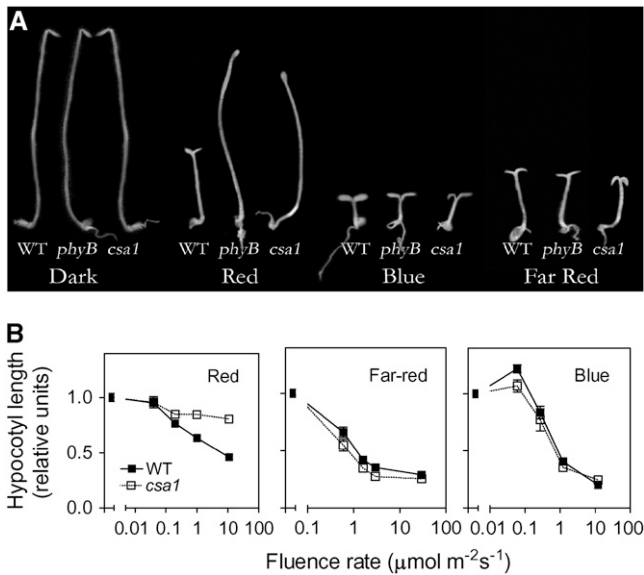


Figure 3. The Long-Hypocotyl Phenotype of *csa1* Seedlings Is Red Light Specific.

(A) Wild-type, *phyB*, and *csa1* mutant seedlings grown under continuous red, far-red, blue light, or darkness for 3 d.

(B) Hypocotyl-length fluence-rate response curves of wild-type and *csa1* mutant seedlings.

csa1 Specifically Impairs *phyB* Signaling

The *csa1* mutant strongly resembles the *phyB* mutant under white light (Figure 1) and also showed reduced responses to changes in the red:far-red ratio (Figure 2) and to red light signals (Figure 3), typically perceived by *phyB*. To address the relationship between *PHYB* and *CSA1*, we compared the behavior of the *phyB* single mutant with that of the *phyB csa1* double mutant. We found that while the *csa1* mutation alone significantly promoted hypocotyl elongation and a more erect position of the leaves under short photoperiods of white light with high red:far-red ratios, the *csa1* mutation had no additional effect on these traits when present in a *phyB* mutant background (Figure 4). These observations indicate that the *csa1* mutation alters *Arabidopsis* development by specifically impairing the *phyB* signaling pathway.

The *csa1* Phenotype Is Caused by a T-DNA Insertion in a TIR-NBS-LRR-Type Gene

The T-DNA insertion, which includes a multimerized 35S enhancer sequence from the *Cauliflower mosaic virus* (CaMV), cosegregated with the mutant phenotype and with resistance to the selection marker Basta (data not shown). Cloning of flanking DNA revealed that the T-DNA was inserted within the second exon of *At5g17880*, a gene encoding a member of the TIR-NBS-LRR gene family (Figure 5A).

Since the T-DNA insert in *csa1* contains a strong enhancer, the mutant phenotype could be the result of (1) overexpression of a gene flanking *At5g17880*, (2) lack of function of the protein

encoded by the *At5g17880* gene, or (3) a dominant-negative effect on TIR-NBS-LRR proteins caused by the expression of a truncated *At5g17880* protein.

The expression of neighbor genes *At5g17870* and *At5g17890* was not significantly different between the wild type and *csa1* (Figure 5B). Using primers that amplify either the first or the fourth exon of *At5g17880*, we could detect mRNA expression of this gene upstream but not downstream of the T-DNA insertion in the mutant, while expression of both exons was detected in the wild type (Figure 5B). This clearly indicates that an mRNA with a truncated *At5g17880* open reading frame, and most likely a truncated *At5g17880* protein, is being expressed in *csa1*.

A T-DNA mutant (SALK_023219) carrying an insertion (without enhancer sequences) within the fourth exon of the *At5g17880* gene enhanced hypocotyl growth under red light (Figure 6A), providing independent genetic evidence for the role of the *CSA1* gene in light signaling. However, this allele that we named *csa1-2* did not have the strong *csa1* phenotype when grown in white light with a high red:far-red ratio (data not shown) and showed a weaker phenotype than *csa1-1* under red light (Figure 6A) in spite of the fact that the *CSA1* mRNA was almost undetectable (Figure 6B). Thus, only a small part of the *csa1-1* phenotype can be accounted for by lack of *At5g17880* function.

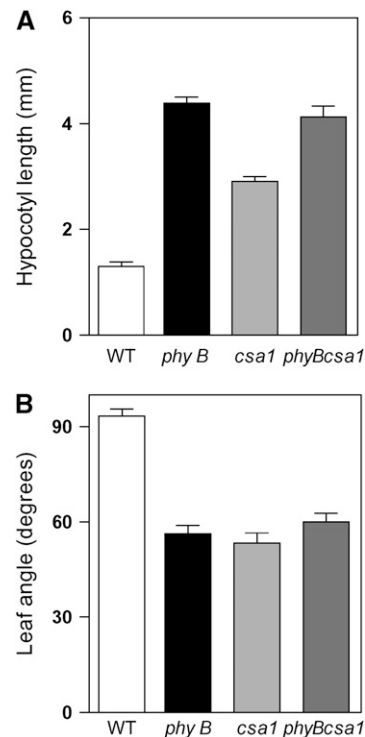


Figure 4. *csa1* Specifically Impairs *phyB* Signaling.

(A) Hypocotyl length of wild-type, *csa1*, *phyB*, and *phyB csa1* seedlings grown for 4 d under short days (8 h white light/16 h darkness).

(B) Leaf angle of wild-type, *csa1*, *phyB*, and *phyB csa1* seedlings grown for 14 d under short days (8 h white light/16 h darkness).

T-DNA Insertions in Other TIR-NBS-LRR Proteins Impair Deetiolation in Red Light

There are ~90 *TIR-NBS-LRR* genes in *Arabidopsis*, and many of them act as early components of the core-sensing mechanism that mediates pathogen perception in plants (Holt et al., 2003). *TIR-NBS-LRR* genes that are highly related to *At5g17880* (Meyers et al., 2003) include *RPS4*, a gene that has been implicated in defense responses against a bacterial pathogen (Gassmann et al., 1999), and *At5g44870*, a gene whose expression has been shown to be regulated by phyA and phyB (Tepperman et al., 2001, 2004). Thus, the lack of a strong phenotype in the *csa1-2* mutant could result from redundancy with other family members. In support of this idea, small but significant reductions in the inhibition of hypocotyl elongation by red light were also observed in null mutants carrying T-DNA insertions within the *At5g17880* homologs *RPS4* and *At5g44870* (Figure 6), which share 64% and 63%, respectively, of sequence similarity with *CSA1* across the entire protein.

Expression of a Truncated *CSA1* Gene Phenocopies the *csa1* Mutant

The observation that the *csa1-1* phenotype is semidominant (see Supplemental Figure 1 online), together with the lack of a strong phenotype in the *csa1-2* allele, support the idea that part of the light signaling defects of *csa1-1* results from expression of the truncated *TIR-NBS-LRR* gene (Figure 5B). To test this idea, we transformed *Arabidopsis* wild-type plants with a construct containing the truncated *At5g17880* gene under the control of the strong and constitutive CaMV 35S promoter. Seedlings from independent transgenic lines expressing the truncated *TIR-NBS-LRR* gene showed significantly reduced red, but not blue light,

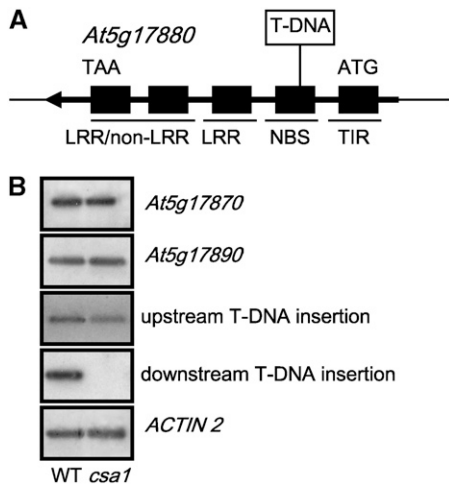


Figure 5. *csa1* Results from a T-DNA Insertion in a *TIR-NBS-LRR*-Type Gene.

(A) Schematic representation of the *TIR-NBS-LRR* gene *At5g17880* and the insertion of the T-DNA within its second exon. (B) Expression analysis of genes flanking *At5g17880* and of the first and fourth exons of *At5g17880* by RT-PCR in wild-type and *csa1* seedlings.

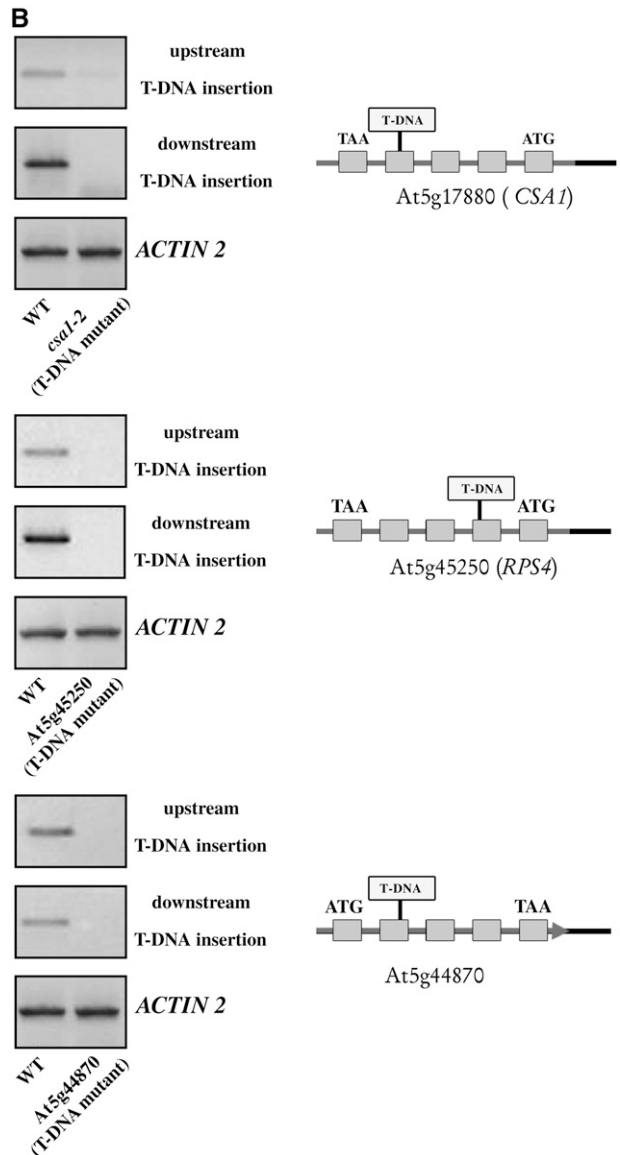
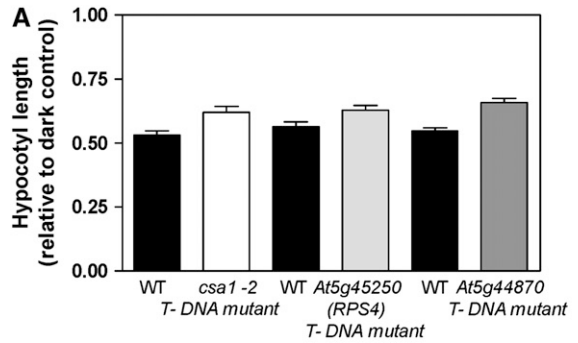


Figure 6. Reduced Sensitivity to Red Light in *TIR-NBS-LRR* Mutants.

(A) T-DNA insertion alleles in *CSA1* (*csa1-2*) and other *TIR-NBS-LRR* loci show reduced inhibition of hypocotyl growth under red light. (B) Expression levels of *CSA1*, *RPS4*, and *At5g44870* determined by RT-PCR in wild-type and T-DNA insertion mutants. T-DNA insertion sites for each mutant are shown.

inhibition of hypocotyl elongation (Figure 7A). Another phyB-mediated response, the red-light inhibition of hypocotyl negative gravitropism, was also impaired in these plants (see Supplemental Figure 5 online). Finally, these transgenic plants also showed long hypocotyls under white light with a high red:far-red ratio (i.e., in the absence of shade signals) (Figure 7B). Taken together, the above data indicate that a significant part of the *csa1* mutant phenotype is the result of a dominant-negative effect caused by the expression of a truncated TIR-NBS-LRR protein of ~212 amino acids, completely lacking the LRR and part of the NBS domains.

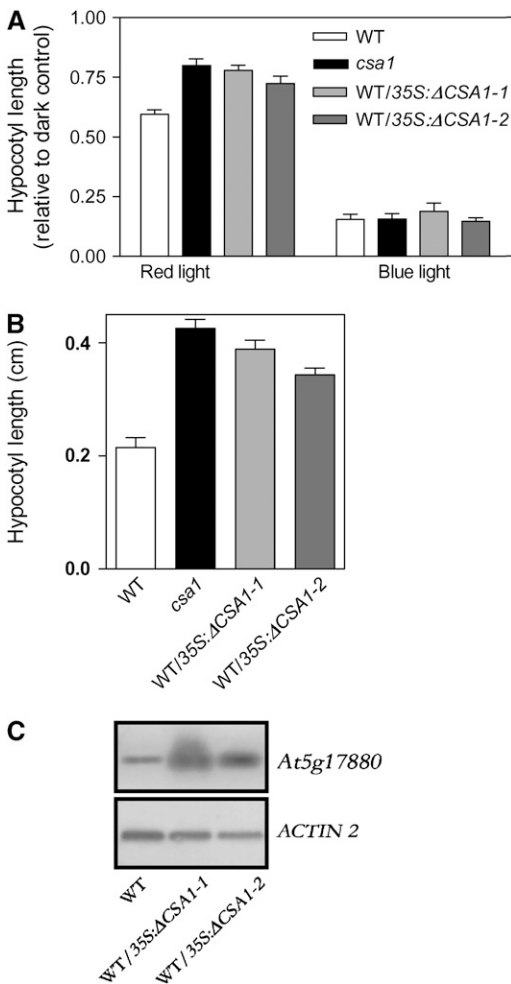


Figure 7. Expression of 35S:ΔCSA1 in the Wild-Type Background Phenocopies the *csa1* Plant.

(A) Hypocotyl length of wild-type and two independent transgenic plants expressing the 35S:Δ*csa1* construct, grown for 4 d under red or blue light.

(B) Hypocotyl length of plants grown for 4 d under white light with a high red:far-red ratio (8 h light/16 h darkness).

(C) Expression levels of CSA1 in wild-type plants and two independent transgenic plants expressing the 35S:Δ*csa1* construct determined by RT-PCR using primers that amplify the first exon.

The molecular analysis of transcripts encoding TIR-NBS-LRR proteins indicates the existence of alternative spliced forms, some of which partially resemble the truncated CSA1 form (Jordan et al., 2002). However, we were not able to detect alternative spliced forms of CSA1 (E. Petrillo, M.J. Yanovsky, and A. Kornblihtt, unpublished data). Whether the alternative spliced forms of other TIR-NBS-LRRs, which resemble the truncated CSA1 gene, modulate light signaling remains to be determined.

The TIR-NBS-LRR Gene *RPS4* Rescues the *csa1* Phenotype

In animals, Toll-like receptors signal through homodimerization of TIR domains as well as heterodimerization with the TIR domain of adapter proteins (Yamamoto et al., 2004). Interestingly, truncated proteins containing only the TIR domain function as dominant-negative forms that block signaling downstream of TIR domain receptors (Dupraz et al., 2000). By analogy with the animal model, the truncated form of CSA1 that retains intact only the TIR domain could form nonproductive heterodimers with other proteins bearing TIR domains (Meyers et al., 2002). RPS4 is the TIR-NBS-LRR protein with the highest degree of sequence similarity to CSA1. If the truncated form of CSA1 interferes with RPS4 function, increasing RPS4 levels should attenuate the mutant phenotype. Indeed, transgenic lines overexpressing RPS4 in a *csa1-1* background showed a wild-type phenotype when grown under white light with a high red:far-red ratio (e.g., short petioles, nonerect dark green leaves, and normal flowering time) (Figure 8A; see Supplemental Figure 4 online). Overexpression of RPS4 also restored the ability of *csa1-1* plants to show differential responses to EOD red compared with far-red light pulses, similar to those present in wild-type plants (Figure 8B). Furthermore, hypocotyl length of *csa1* plants transformed with the RPS4 gene was not different from that of wild-type seedlings under red light (Figure 8C) or darkness (wild type, 1.14 ± 0.06 cm; *csa1-1/35S:RPS4-1*, 1.22 ± 0.09 cm; and *csa1-1/35S:RPS4-2*, 1.14 ± 0.03 cm), indicating that overexpression of RPS4 in a *csa1* background did not produce constitutively dwarf plants, but plants with normal responses to red light. Indeed, overexpression of RPS4 also rescued another response to red light, the phyB-mediated inhibition of negative gravitropism (see Supplemental Figure 5 online). Finally, RPS4 had no effect on red light inhibition of hypocotyl elongation when overexpressed in a wild-type background (Figure 8C) at levels comparable with those obtained in a *csa1-1* background (Figure 8D), suggesting that RPS4 overexpression enhanced red light sensitivity in the *csa1-1* mutant attenuating the dominant-negative effect caused by the truncated CSA1, rather than through a nonspecific effect of RPS4 on cell growth. Taken together, our results strongly suggest that *csa1* impairs phytochrome signaling by interfering with the action of RPS4 and possibly other related TIR-NBS-LRR proteins.

TIR-NBS-LRRs and Phytochrome Signaling

The negative effect of *csa1* on phyB signaling could be due to a reduction in phyB levels, to an effect on phyB subcellular localization, to a reduction in the stability of the Pfr form (i.e., enhanced reversion to the Pr form), or to an effect on a component

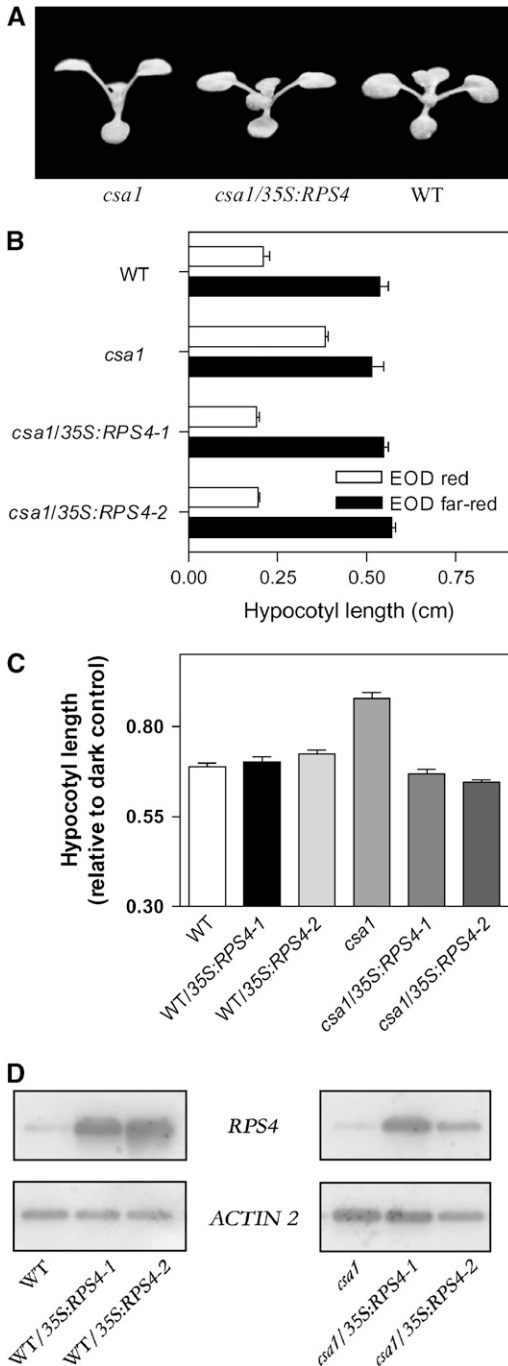


Figure 8. Overexpression of *RPS4* in *csa1* Rescues the *csa1-1* Mutant Phenotype.

(A) *csa1*, *csa1/35S:RPS4*, and wild-type plants grown under white light. (B) *csa1/35S:RPS4* seedlings behave as wild-type plants in response to EOD red and far-red treatments. (C) Hypocotyl length of wild-type, two independent wild-type/*35S:RPS4* transgenic lines, *csa1*, and two independent *csa1/35S:RPS4* transgenic lines grown for 4 d under red light. (D) Expression levels of *RPS4* determined by RT-PCR.

of the *phyB* signaling pathway that acts downstream from the photoreceptor. We could not detect effects of *csa1* on *PHYB* levels (Figure 9A) or nuclear localization (see Supplemental Figure 2 online). In addition, if the negative effect of *csa1* on *phyB* signaling were due to an increased dark reversion of the active Pfr form to the inactive Pr form, the difference in hypocotyl length between the *csa1* mutant and the wild type should be reduced under very high fluences of red light because the Pfr form will be regenerated at a higher rate. In contrast with the above prediction, the hypocotyl length difference between the *csa1* mutant and wild-type plants remained the same even at extremely high fluences of red light (see Supplemental Figure 3 online), suggesting that the defect of the *csa1* mutant is not associated with an enhanced Pfr-to-Pr dark reversion.

A dramatic increase in the levels of expression of the homeodomain transcription factor *HAT4* gene (also known as *ATHB-2*) is one of the earliest responses to a reduction in the red:far-red ratio (Carabelli et al., 1996; Devlin et al., 2003). Indeed, *HAT4/ATHB-2* is a direct primary target of phytochrome action in the

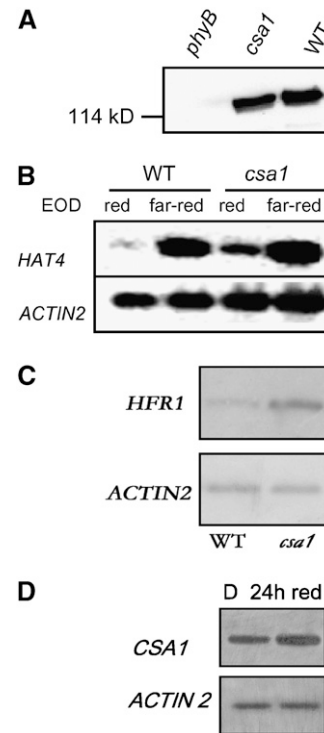


Figure 9. Molecular Analysis of *csa1* and *CSA1* Expression.

(A) *PHYB* levels detected by monoclonal antibodies are normal in *csa1* seedlings grown under white light. (B) *HAT4* expression detected by RT-PCR is enhanced in *csa1* compared with wild-type seedlings grown under white light and exposed to EOD red light. (C) *HFR1* expression determined by RT-PCR is enhanced in *csa1* compared with wild-type seedlings grown under white light. (D) *CSA1* expression detected by RT-PCR is enhanced in etiolated wild-type seedlings exposed for 24 h to continuous red light compared with plants kept in darkness (D).

shade-avoidance syndrome because its expression increases rapidly under low red:far-red ratios even in the absence of protein synthesis (Roig-Villanova et al., 2006). In the *csa1* mutant, *HAT4* expression is already elevated under high red:far-red ratios (Figure 9B). A substantial response to the EOD far-red treatment remains in *csa1*, but this also occurs in *phyB* mutants (Carabelli et al., 1996).

Low red:far-red ratios also increase the expression of the basic helix-loop-helix gene *HFR1* that antagonizes shade-avoidance responses (Sessa et al., 2005). *HFR1* mRNA levels were already increased in the *csa1* mutant compared with wild-type plants under high red:far-red ratios, indicating that both positive (*HAT4/ATHB2*) and negative (*HFR1*) arms of the shade-avoidance signaling cascade are constitutively elevated in *csa1* mutant plants (Figure 9C).

The levels of expression of *CSA1* are somewhat elevated in seedlings exposed to 24 h of red light compared with the dark controls (Figure 9D). Red and far-red light have also been shown to promote the expression of the *CSA1* homolog *At5g44870* after exposing etiolated seedlings to those light signals for several hours (Tepperman et al., 2001, 2004). However, this is not necessarily the main mechanism by which light controls TIR-NBS-LRR activity. In particular, 1 h of irradiation with red light in etiolated seedlings causes a rapid reduction in *HAT4* expression without detectable reductions in the expression of *At5g17880* or its homologs *RPS4* and *At5g44870* (Monte et al., 2004; data not shown).

TIR-NBS-LRR proteins like *RPS4* are generally predicted to be cytosolic (Gassmann et al., 1999). *PhyB* is mainly localized in the cytosol in darkness and is translocated into the nucleus under red light. *PhyB* activity occurs mainly in the nucleus, but the possibility that some *phyB* activity occurs in the cytoplasm cannot be excluded (Nagatani, 2004). We have not been able to detect physical interactions between *CSA1* and *phyB* in the yeast two-hybrid system (data not shown), strengthening the idea that TIR-NBS-LRR proteins affect *phyB* signaling downstream from the photoreceptor. An α importin homolog, which mediates the import of specific proteins to the nucleus, has recently been shown to be required for the function of a TIR-NBS-LRR protein in *Arabidopsis* (Palma et al., 2005). Thus, TIR-NBS-LRR could affect *phyB* signaling through their effect on another protein that is translocated to the nucleus and interacts with *phyB* or *phyB* signaling components.

Plant hormones like ethylene and auxin have been implicated in shade-avoidance responses. However, ethylene appears to mediate shade-avoidance responses to reductions in incident blue light rather than to reductions in the red:far-red ratio (Franklin and Whitelam, 2005). Since responses to blue light are not affected in *csa1-1*, it is unlikely that the constitutive shade-avoidance phenotype of *csa1* is due to alterations in ethylene levels or signaling. In addition, the *red1* mutant is involved in the control of auxin homeostasis (Hoecker et al., 2004) and shows elongated hypocotyls and leaf petioles, but it does not flower early as the *phyB* and *csa1* mutants do (Wagner et al., 1997), suggesting that *csa1* affects a signaling step upstream of auxin.

csa1-1 is the mutant that most closely resembles the *phyB* mutant phenotype. The *gigantea* mutant has elongated hypocotyls under red light but flowers late rather than early (Huq et al., 2000). The *elf3* (Hicks et al., 1996), *elf4* (Khanna et al., 2003), and

srr1 (Staiger et al., 2003) mutants show morphological and flowering phenotypes that resemble that of *phyB* but they also show either complete disruption of circadian rhythms or a severe alteration in period length, phenotypes that are not obviously observed in *phyB* (Salomé et al., 2002) or in *csa1-1* mutants (data not shown). Although *ELF3* interacts physically with *phyB*, genetic experiments suggest that they do not act on the same pathway in the control of hypocotyl growth and could be part of a complex interplay between light and clock signaling components (Yanovsky and Kay, 2001).

Thus, taken together, the above results strongly suggest that TIR-NBS-LRR proteins like *CSA1* and *RPS4* mediate or modulate *phyB* signaling by either acting as early components of the pathway or by modulating the levels or activity of an early step of this signaling cascade.

Light Signaling and Defense Responses Are Linked in *Arabidopsis*

Recent microarray studies indicate that the expression of many plant disease resistance genes is modulated by the activity of phytochromes (Tepperman et al., 2001; Devlin et al., 2003). Interestingly, the growth of an incompatible strain of *P. syringae* is actually enhanced in the *phyA phyB* double mutant (Genoud et al., 2002). Furthermore, the *psi2* mutant of *Arabidopsis*, isolated for its exaggerated responses to light, develops light-dependent necrotic lesions that mimic those induced during the hypersensitive response triggered by plant pathogens during incompatible interactions (Genoud et al., 1998).

We found that the growth of an incompatible strain of *P. syringae* was also enhanced in *csa1* mutant plants grown under high red:far-red ratios (Figure 10). This observation, together with the fact that the *csa1* mutant phenotype can be reduced through expression of the disease resistance gene *RPS4*, strongly suggests that photomorphogenic and defense responses interact very early in their signaling cascades by sharing core-signaling components.

Thus, our finding constitutes a first step toward understanding the molecular mechanisms underlying the crosstalk observed

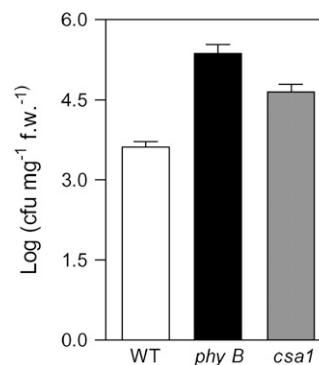


Figure 10. Growth of an Avirulent Strain of *P. syringae* 3 d after Plant Inoculation.

Data are expressed as colony forming units (cfu) per milligram of fresh weight (f.w.).

between photomorphogenic and defense responses. In the future, the identification and characterization of proteins that interact with CSA1, as well as transcriptomic analysis of *csa1* mutants, will help us understand more precisely how TIR-NBS-LRRs participate in light signaling. It is worth mentioning, however, that the initial step through which TIR-NBS-LRR proteins mediate defense responses in plants is still poorly understood.

TIR Domain Proteins Play Roles in Immunity and Development in Plants and Animals

A dual role for TIR domain proteins in development and immunity has been reported in the animal kingdom. Although mammalian Toll-like receptors are known exclusively for their roles as mediators of innate immunity (Iwasaki and Medzhitov, 2004), the prototypic receptor Toll was originally identified in *Drosophila melanogaster* for its role in dorsoventral patterning and only later shown to be involved in the *D. melanogaster* innate immune system (Imler and Hoffmann, 2002). In *Caenorhabditis elegans*, TIR domain proteins also play roles in immunity and development (Pujol et al., 2001; Couillault et al., 2004; Liberati et al., 2004). How this dual role arose in evolution is a matter of extensive debate (Johnson et al., 2003). The presence of related proteins with similar functions in plants and animals suggests an ancient evolutionary origin for these receptors. It has been postulated that protists, the common ancestors of plants and animals, may have used similar receptors to sense the presence of competitors (Magor and Magor, 2001). Thus, our observation that TIR domain proteins play a role in developmental responses to neighbor plants may reflect the conservation of an ancient role for this protein family.

METHODS

Genetic Screening

Activation-tagged lines generated in the Columbia (Col) accession of *Arabidopsis thaliana* (Weigel et al., 2000) were obtained from the ABRC (stock no. CS21995). Seeds were surface sterilized and plated on Murashige and Skoog growth medium without sucrose. The plates were kept at 4°C in the dark for 4 d and then transferred for 1 week to a growth chamber with an 8-h photoperiod of white light provided by fluorescent lamps (Philips TLD 15W/54; 150 $\mu\text{moles m}^{-2} \text{s}^{-1}$) at 22°C. Seedlings with long hypocotyls were isolated and crossed to Col wild-type plants. F2 seedlings of a cross between the wild type and *csa1* (originally identified as T3) segregated 3:1 basta resistant:basta sensitive, suggesting that there was one locus containing the T-DNA. Three other mutants were identified in this screening, T1, T2, and T4. One was an allele of *elf3*, and the identity of the other mutants is still unknown because the T-DNA insertions do not cosegregate with the mutant phenotype.

Growth Conditions and Phenotypic Characterization

Seeds were sown in clear plastic boxes on 0.8% agar-water, incubated in darkness at 4°C for 3 d, given 2 h of white light at 22°C, and transferred to darkness to induce homogeneous germination and then to different light or dark conditions for 3 d (22°C). Alternatively, the seedlings were transplanted to pots and transferred to short days (8 h white light/16 h darkness). Petiole length and leaf angle between the petiole and a line perpendicular to the soil surface were measured for the second fully

expanded leaf of these plants. The number of leaves in the rosette was counted when the tip of the flowering primordia reached 1-cm height. Physiological data are the mean \pm SE of 4 to 20 replicates from at least two independent experiments.

Light Treatments

White light was provided by fluorescent tubes (Philips TLD 15W/54). EOD far red was provided by incandescent lamps combined with a water filter and an RG9 filter. Continuous far red was provided by incandescent lamps in combination with a water filter, a red acetate filter, and six 2-mm-thick blue acrylic filters (Paolini 2031; La Casa del Acetato). Red light was provided by light-emitting diodes, and blue light was provided by fluorescent white light tubes filtered through a blue acrylic filter. The red:far-red ratio was measured with a Skye meter (SKR 100, remote probe SKR 110; Skye Instruments).

Cloning of the Mutant Locus

The flanking genomic sequence at the T-DNA insertion site was recovered using the thermal asymmetric interlaced-PCR protocol (Liu et al., 1995). The T-DNA insertion was confirmed by PCR with specific primers. Perfect cosegregation (in 220 chromosomes analyzed) between the T-DNA insertion and the mutant phenotype was observed in F2 seedlings derived from a cross between the *csa1* mutant and wild-type Col.

Mutant Alleles and Genotyping

Plants of each mutant line were genotyped using two PCR reactions, one for the mutant allele with the T-DNA insertion and one for the wild-type allele. The *csa1-1* mutant allele was genotyped by PCR with primers TDNACSA1 (5'-TAATAACGCTGCGGACATCTAC-3') and WTA (5'-CAACATCTGGAGAGCTAGTG-3'). To amplify the wild-type allele, we used primer WTA in combination with primer WTB (5'-GCTGGTGAAGTCTACTGACA-3'). The other T-DNA mutant alleles used in this study were obtained from the ABRC. The *csa1-2* line (SALK_023219) has an insertion in exon 4. The T-DNA insertion in *RPS4* (SALK_012799) has an insertion in exon 2. The T-DNA insertion in At5g44870 (SALK_087262) has an insertion in exon 2. For *csa1-2*, the wild-type allele was amplified with primers 023219LP (5'-CATCGCATCGTTTAAAGCGCAC-3') and 023219RP (5'-TGT-TGTTTCATTCGCCAGGAT-3'), and the mutant allele was amplified with primers LBFH (5'-CGCTTGCTGCAACTCTCTCAGG-3') and 023219RP.

For the T-DNA insertion in *RPS4*, the wild-type allele was amplified with primers 012799LP (5'-TGCCAGCTTTCTAAGCACCAAT-3') and 012799RP (5'-GAGTTGGATCGCTTGCCCTCAA-3'), and the mutant allele was amplified with primers LBFH and 012799RP. For the T-DNA insertion in At5g44870, the wild-type allele was amplified with primers 087262LP (5'-CATCCTCCACCAACTAGGA-3') and 087262RP (5'-GCAAGTTCCTTGCGAATGTG-3') and the mutant allele with primers LBFH and 087262RP.

Recapitulation of the *csa1* Mutant Phenotype

The Δ CSA1 construct was generated using primers that amplified the At5g17880 locus from the ATG start codon (5'-GACGGATCCATGACAAGCTCCTCCTCTG-3'), adding a *Bam*HI site to the site of insertion of the T-DNA within the second exon (5'-GTAGTCGACTTACTTAATTCGAGCTCGATTC-3'), adding a TAA stop codon and the restriction site *Sal*I. The PCR fragment amplified with the above primers was introduced into the binary vector CHF3 (Staiger et al., 2003) between the 35S promoter and the *rbcs* terminator. The resulting plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101 and used to transform *Arabidopsis* plants of the Col ecotype. Fourteen transgenic lines were

generated, and two lines with the strongest phenotype were used for phenotypic characterization.

Complementation of the *csa1* Mutant Phenotype with *RPS4*

RPS4-Col driven by the CaMV 35S promoter was obtained by cloning a 6-kb genomic *RPS4* fragment containing 0.5 kb of 5' upstream and 1.5 kb of 3' downstream sequence into vector pMD1, a derivative of vector pBI121 (CLONTECH) with a synthetic polylinker replacing the β -glucuronidase reporter gene (courtesy of M. Dixon, Sainsbury Laboratory, Norwich, UK). This construct was introduced into *Agrobacterium* strain GV3101 and used to transform *csa1* mutant plants.

RT-PCR Analysis

Total RNA was extracted with the RNeasy miniprep kit (Qiagen). One microgram was used for the RT reaction with ImProm-II reverse transcriptase (Promega). Amplification of genomic DNA was undetectable in non-retrotranscribed controls. PCR products were detected in DNA gel blots using standard methodology in the exponential range of amplification. Primer sequences will be provided upon request.

Protein Blots

Extracts were prepared according to Martínez-García et al. (1999) and subjected to SDS-PAGE in 1.5-mm-thick, 4.5/7.5% stacking/resolving gels. Proteins were electroblotted to nitrocellulose, and PHYB was detected using a monoclonal antibody kindly provided by Peter Quail (University of California, Berkeley, and USDA Plant Gene Expression Center, Albany, CA).

Bacterial Infection

The bacterial growth assay was performed as described by Tornero and Dangl (2001). The bacterial strain used was *Pseudomonas syringae* pv tomato DC3000, containing the avirulence gene *avrRpt2*.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM_121794 (At5g17880) and NM_123893 (At5g45250).

Supplemental Data

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

- Supplemental Figure 1.** The *csa1* Mutation Is Semidominant.
- Supplemental Figure 2.** Cellular Localization of PHYB:GFP in *csa1* Mutant Plants.
- Supplemental Figure 3.** The Long Hypocotyl Phenotype of *csa1* Seedlings Is Sustained under High Fluences of Red Light.
- Supplemental Figure 4.** *RPS4* Overexpression Rescues the *csa1* Mutant Phenotype.
- Supplemental Figure 5.** Red Light Inhibition of Hypocotyl Negative Gravitropism.

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A Constitutive Shade-Avoidance Mutant Implicates TIR-NBS-LRR Proteins in Arabidopsis Photomorphogenic Development

Ana Faigón-Soverna, Franklin G. Harmon, Leonardo Storani, Elizabeth Karayekov, Roberto J. Staneloni, Walter Gassmann, Paloma Más, Jorge J. Casal, Steve A. Kay and Marcelo J. Yanovsky
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