An Arabidopsis Mutant Hypersensitive to Red and Far-Red Light Signals

Thierry Genoud, Andrew J. Millar, Naoko Nishizawa, Steve A. Kay, Eberhard Schäfer, Akira Nagatani, and Nam-Hai Chua

A new mutant called psi2 (for phytochrome signaling) was isolated by screening for elevated activity of a chlorophyll a/b binding protein–luciferase (CAB2–LUC) transgene in Arabidopsis. This mutant exhibited hypersensitive induction of CAB1, CAB2, and the small subunit of ribulose-1,5-bisphosphate carboxylase (RBCS) promoters in the very low fluence range of red light and a hypersensitive response in hypocotyl growth in continuous red light of higher fluences. In addition, at high- but not low-light fluence rates, the mutant showed light-dependent superinduction of the pathogen-related protein gene PR-1a and developed spontaneous necrotic lesions in the absence of any pathogen. Expression of genes responding to various hormone and environmental stress pathways in the mutant was not significantly different from that of the wild type. Analysis of double mutants demonstrated that the effects of the psi2 mutation are dependent on both phytochromes phyA and phyB. The mutation is recessive and maps to the bottom of chromosome 5. Together, our results suggest that PSI2 specifically and negatively regulates both phyA and phyB phototransduction pathways. The induction of cell death by deregulated signaling pathways observed in psi2 is reminiscent of retinal degenerative diseases in animals and humans.

INTRODUCTION

Because light provides the energy source for photosynthesis, plants must adapt to changes in ambient light quantity and quality to optimize the efficiency of this important process. The results of this adaptation are apparent: almost every step of plant development, from seed germination to fruit maturation, is dependent on light. Moreover, light regulates the circadian rhythm, the adaptive size modification of tissue and organs, and the expression of photosynthetic genes and many other known and unknown genes (Terzaghi and Cashmore, 1995).

To date, what we know about light information processing and signal transduction concerns the nature and function of photoreceptors. A family of photoreceptors called phytochromes has been shown to be involved in the perception of red and far-red light. Their biochemical and molecular properties and physiological roles are now well described (reviewed in Quail et al., 1995; Smith, 1995). In addition, a long-postulated UV and blue light receptor (cryptochrome) was recently characterized by Ahmad and Cashmore (1993). Mutants lacking one or several of these receptors have provided useful information concerning the role of individual photoreceptors and their interaction in transducing light signals (reviewed in Barnes et al., 1997).

Much less is known concerning molecules that link light perception to gene activation. The CONSTITUTIVE PHOTO-MORPHOGENIC/DEETIOLATED/FUSCA (COP/DET/FUS) class of proteins has been suggested to play an important role in regulating the repression of light-inducible genes and the maintenance of skotomorphogenesis (Chory et al., 1989). For some of these proteins, the genes have been cloned and include COP1 (Deng et al., 1992), DET1 (Pepper et al., 1994), COP9 (Wei et al., 1994), and FUS6 (Castle and Meinke, 1994). Mutations in one of these genes induce a characteristic phenotype in dark-grown Arabidopsis plants. The morphology of mutant plants in the dark resembles a light-grown wild-type plant in many respects. Proteins encoded by the COP/DET/FUS gene group were initially thought to be important for the repression of light-responsive gene expression only. Recent evidence, however, indicates that they also repress the expression of several other gene sets (Mayer et
al., 1996). Mutants impaired in far-red light perception but not in phytochrome A levels (fhy1 and fhy3; Whitelam et al., 1993) seem to lack functional proteins involved in regulating secondary branches of the phyA signal transduction pathway. In addition, the cue1 mutation of Arabidopsis (Li et al., 1995) has been implicated in the regulation of plastid development and light-responsive gene expression, and the DOC gene product is hypothesized to be a repressor of chlorophyll a/b binding protein (CAB) genes in the dark (Li et al., 1994). Recently, Wagner et al. (1997) described a putative signal transduction mutant, red1, that appears to be specific to the phyB pathway.

Other approaches have elucidated some steps involved in phytochrome signal transduction. For example, by microinjecting various compounds directly into tomato cells lacking functional phytochromes and treating plant cells with specific inhibitors, Neuhaus et al. (1993) and Bowler et al. (1994a, 1994b) have been able either to trigger chloroplast differentiation and anthocyanin accumulation or to block certain branches of the putative phyA signaling pathway. A G protein, calcium, calmodulin, and cGMP have been implicated in the transduction and amplification of the phyA signal within cells. Proteins involved in the regulation of these intermediates, however, still remain to be identified.

To search for new components of the phytochrome-dependent signal transduction pathway, we introduced a cab2, Ω, and luciferase (cab2::Ω::luc) reporter gene into Arabidopsis (Millar et al., 1995). Seeds from a transgenic line carrying this construct were mutagenized by treatment with ethyl methanesulfonate (EMS), and the M2 seedlings were screened for altered reporter gene expression after various light irradiations. We assumed that the phytochrome phototransduction pathway is regulated not only by positively acting components but also by negatively acting components, for example, those that desensitize or terminate the light signal. A mutation modifying a negatively acting component would lead to a significant amplification of light signal responses and/or suppress a desensitization of the pathway. These modifications of a plant’s responses to light could be easily recognized by scoring the photon emission of mutagenized transgenic seedlings before (dark image) and after very low fluence red light pulses. Among the postulated kinetic modifications, we expect to find hypersensitive responses (i.e., higher than the wild-type response at low light intensity but with a wild-type amplitude at high light intensity) and amplified responses (i.e., with a higher response at all light intensities). Here, we describe the isolation and characterization of a novel mutant, psi2 (for phytochrome signaling), that is hypersensitive to light.

RESULTS

In an attempt to identify new elements of phytochrome signaling pathways, we introduced a chimeric construct (cab2::Ω::luc) into Arabidopsis C24. This reporter gene is composed of a light-responsive region of the CAB2 gene promoter fused to the luc coding sequence (Millar et al., 1995). Transgenic seeds were mutagenized with EMS and screened for mutations that would modify the reporter gene expression after short red light pulses.

We used a low-fluence red light pulse that did not induce detectable luciferase gene expression in transgenic cab2::Ω::luc seedlings. After screening >80,000 seedlings, we isolated different groups of putative mutants with high luciferase activity 20 hr after the light treatment. The next generation of the surviving seedlings was screened under the same conditions, and putative mutants with high luciferase activity were backcrossed three times with the wild-type cab2::Ω::luc transgenic line before further investigation. Twelve putative mutants exhibiting amplified luciferase response to a short red light pulse were monitored by camera and identified (data not shown). In this study, we focused on a group of three mutants showing a similar light-dependent phenotype and called them psi2.

Genetic Analysis

From two unrelated M1 pools of mutagenized seeds, we isolated three mutants with the same morphological and molecular phenotypes. Each of these mutants was crossed with the transgenic wild type, and F2 seedlings were examined for the visible psi2 traits. The complete cosegregation of these traits with the luciferase phenotype (data not shown) suggested that each psi2 mutant contains a monogenic and recessive mutation (Table 1). Complementation analysis showed that the three mutants are allelic (Table 2). From this group of three alleles, only psi2-1 was used in the following experiments.

After segregation of psi2 from the cab2::Ω::luc transgene in the original C24 background, we continued to obtain the

<table>
<thead>
<tr>
<th>Table 1. psi2 Is a Recessive Mutation</th>
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<tr>
<td>Cross</td>
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</tr>
<tr>
<td>cab2::Ω::luc × psi2-1</td>
</tr>
<tr>
<td>cab2::Ω::luc × psi2-2</td>
</tr>
<tr>
<td>cab2::Ω::luc × psi2-3</td>
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* Psi2 corresponds to three psi2 traits: overexpression of the reporter transgene after a pulse of red light (1 μmol m⁻² sec⁻¹), short hypocotyl in dim white light, and necrotic lesions on leaves at high white light intensity.
A transgenic plant containing a plastocyanin promoter–ciferase enzyme uses these compounds for the photon transduction could interfere with luciferase activity because the transgene. Variations in cellular ATP or coenzyme A concentrations forming spontaneous lesions mapped to this region. Reduced light pulses. This time point corresponds to the maximum in luciferase activity in the transgenic wild-type seedlings and is 9.5 centimorgans from nga129. For this mapping, 42 plants were derived from the same M1 pool and may present the same allele.

Table 2. Complementation Analysis

<table>
<thead>
<tr>
<th>Cross</th>
<th>No. of F1 Plants</th>
<th>Psi2 in F1 Plants</th>
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<tbody>
<tr>
<td>psi2-1 × psi2-2</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td>psi2-1 × psi2-3</td>
<td>61</td>
<td>61</td>
</tr>
<tr>
<td>psi2-2 × psi2-1</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>psi2-2 × psi2-3</td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td>psi2-3 × psi2-1</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>psi2-3 × psi2-2</td>
<td>8</td>
<td>8</td>
</tr>
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</table>

*See footnote in Table 1. psi2-2 and psi2-3 were derived from the same M1 pool and may present the same allele.

The psi2 allele was mapped by simple sequence length polymorphism (Bell and Ecker, 1994) and cleaved amplified polymorphic sequence methods (Konieczny and Ausubel, 1993). The allele is located at the bottom of chromosome 5 and is 9.5 ± 2.9 centimorgans from LEAFY3 (LFY3) and 19.5 ± 4.8 centimorgans from nga129. For this mapping, 42 plants (84 chromatids) were examined. No phy, det, cop, fus, or mutants forming spontaneous lesions mapped to this region.

**Light Response of the cab2::luc Transgene in psi2**

Luciferase activity in the transgenic wild-type seedlings and the psi2 mutants was analyzed using a video-imaging system (Millar et al., 1992a). Figure 1A shows that exposure to a short red light pulse (in a fluence range that would elicit very low fluence responses) induced an amplification in luciferase expression in the psi2 seedlings, whereas in the wild-type seedlings, no expression was induced. In this experiment, the reporter activity was monitored 20 hr after the red light pulses. This time point corresponds to the maximum for CAB expression (Millar and Kay, 1991). Exposure to far-red light pulses under the same conditions (Figure 2) also induced an amplification of luciferase activity in the psi2 mutant. This amplification was dependent on phytochrome

A typical effect of light on plant growth is the reduction of seedling hypocotyl length. Table 3 shows that in the dark, the hypocotyl lengths of psi2 and wild-type seedlings are practically indistinguishable. However, exposure to red or far-red light induced a greater reduction in hypocotyl length in psi2 compared with the wild type, and this effect was more
Figure 1. The psi2 Mutation Increases Light-Inducible Gene Expression in Etiolated Seedlings Exposed to Red Light Pulse in the Very Low Fluence Range.

Plants were grown for 7 days in darkness and subjected to a red light pulse of different fluence rates before being returned to the dark. After 20 hr, seedlings were harvested for RNA extraction, and gene expression levels were determined by RNA gel blot hybridization. The luciferase activity of seedlings exposed to similar conditions was measured as described in Methods.

(A) Activity of the CAB2–luc reporter gene 20 hr after a red light pulse of fluences; bars indicate standard deviation. Each point represents the average of 150 to 200 seedlings. wt, cab2::V::luc transgenic seedlings.

(B) RNA gel blot analysis of light-responsive gene expression under the same conditions as given in (A). The nonspecific CAB probe was derived from a CAB1 coding region fragment. Each lane contains 30 μg of RNA.

(C) RNA expression levels of some light-responsive genes induced by different fluences of red light. Symbols in graphs are the same as those used in (A).

Autoradiograms from (B) and other experiments were measured using a PhosphorImager. Specific probes were used to distinguish CAB1 and CAB2 expression. The 18S rRNA was used as a loading control.
pronounced at low fluence rates. In blue light, on the other hand, psi2 showed a slightly greater reduction in hypocotyl length under high rather than low fluence conditions, relative to the wild type. To investigate the light sensitivity in more detail, we grew psi2 and wild-type seedlings under low- or high-fluence red light for 4 days. Under these conditions, there was clearly an increased sensitivity of psi2 hypocotyl response to red light of increasing fluences, and this hypersensitivity was not affected by the absence of sucrose in the medium (data not shown). Because the phytochrome B pathway is thought to control hypocotyl elongation (Smith, 1995), our results suggest that psi2 modifies this pathway. This type of enhanced sensitivity is similar to that observed in transgenic plants overexpressing either phytochrome A or B (Nagatani et al., 1991; Wagner et al., 1991).

Plastid and Leaf Structures of psi2

psi2 etioplast and chloroplast structures were examined by using light and electron microscopy and were similar to those of their cab2::Ω::luc counterparts (Figures 5A to 5D). When grown at high light fluences, the chlorophyll and anthocyanin contents were slightly higher in the mutant when compared with the wild type (psi2, 2.6 ± 0.28 mg of chlorophyll per mg of protein and 2.5 ± 0.36 arbitrary units of anthocyanin per mg of protein; wild type, 2.3 ± 0.07 mg of chlorophyll per mg of protein and 2.2 ± 0.06 arbitrary units of anthocyanin per mg of protein). No differences in starch accumulation or membrane ultrastructure were visible.

General leaf anatomy was not modified in the psi2 mutant, even after exposure to high light fluence rates (Figures 5E and 5F). Tissues from the epidermis and mesophyll and vascular bundles were correctly developed. However, both cell

Figure 2. The psi2 Mutation Increases Phytochrome A Signal Transduction under Low Far-Red, High-Irradiance Conditions.

Plants were grown for 7 days in darkness and subjected to a far-red pulse of different fluence rates before being returned to darkness. After 20 hr, luciferase activity of seedlings (seedl.) was measured as described in Methods. Bars indicate standard deviation. Each point represents the average of 150 to 200 seedlings. WT, cab2::Ω::luc transgenic seedlings.

Figure 3. Light-Responsive Gene Expression in the psi2 Mutant Is Normal at Saturating Intensity of Red or Far-Red Light.

(A) Etiolated seedlings (4 days old) grown on agarose medium in aseptic conditions were illuminated with continuous red light for 60 hr. Seedlings were harvested at the indicated time points (0 to 60 hr), and RNA gel blot analyses were performed as described in Methods. Each lane contains 30 μg of RNA.

(B) The same as given in (A), except the seedlings were illuminated with continuous far-red light for 60 hr.
Table 3. psi2 Hypersensitivity of Hypocotyl Response to Red and Far-Red Light Is Phytochrome Dependent

<table>
<thead>
<tr>
<th>Planta</th>
<th>Hypocotyl Length (mm) in Continuous Treatmentb</th>
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<tbody>
<tr>
<td></td>
<td>Dark</td>
</tr>
<tr>
<td>psi2::luc</td>
<td>12.4 (1.0)</td>
</tr>
<tr>
<td>psi2</td>
<td>12.5 (1.0)</td>
</tr>
<tr>
<td>phyA</td>
<td>11.8 (0.8)</td>
</tr>
<tr>
<td>phyB</td>
<td>12.5 (0.7)</td>
</tr>
<tr>
<td>hy2</td>
<td>12.0 (0.8)</td>
</tr>
<tr>
<td>fhy1</td>
<td>12.6 (1.1)</td>
</tr>
<tr>
<td>psi2 phyA</td>
<td>12.2 (0.9)</td>
</tr>
<tr>
<td>psi2 phyB</td>
<td>11.9 (0.7)</td>
</tr>
<tr>
<td>psi2 hy2</td>
<td>12.0 (0.8)</td>
</tr>
<tr>
<td>psi2 fhy1</td>
<td>12.3 (1.0)</td>
</tr>
</tbody>
</table>

aPlants were grown for 4 days after germination under 1 μmol m⁻² sec⁻¹ red light (low red), 40 μmol m⁻² sec⁻¹ red light (high red), 0.4 μmol m⁻² sec⁻¹ far-red light (low far-red), 13 μmol m⁻² sec⁻¹ far-red light (high far-red), 2 μmol m⁻² sec⁻¹ blue light (low blue), or 38 μmol m⁻² sec⁻¹ blue light (high blue).
bNumbers in parentheses indicate standard deviation.

size and intercellular space dimensions were obviously reduced in psi2 plants exposed to high light fluence rates. Because the number of cells per leaf was only slightly lower in the psi2 mutant, significant tissue modification induced by the mutation could be excluded. We also examined the dimensions of the cuticle layer and found no difference between the psi2 mutant and the wild type.

Light-Inducible Lesions

We found that leaves of the psi2 mutant developed necrotic spots when the plants were grown under continuous white light at fluence rates >40 μmol m⁻² sec⁻¹. This light-dependent phenotype was observed for each of the three psi2 alleles. The formation and the nature of these lesions were studied in some detail. Figure 6A shows psi2 plants grown in continuous white light. Microscopic analysis of the necrotic spots indicated that the lesions mimicked defensive cell death occurring during the hypersensitive response (HR) to a pathogen (see Figure 6B). In the cells surrounding the lesions, features normally seen in cell death induced by pathogen attack or elicitors were clearly visible. Symptoms included callose deposition, formation of vesicles containing electron-opaque material, ribosome aggregation, and chloroplast degradation (Meyer and Heath, 1988). This spontaneous formation of necroses in the absence of any pathogen was observed in several Arabidopsis mutants (Greenberg and Ausubel, 1993; Dietrich et al., 1994; Greenberg et al., 1994; Weymann et al., 1995) as well as in maize (for example, Hoisington et al., 1982) and barley (reviewed in Jorgensen, 1992). Compounds typically involved in these responses, such as polyphenols and callose, accumulated in the cell wall of the psi2 lesions (Figures 6C and 6D).

Molecular markers were used to follow the light-dependent appearance of necrotic spots. PR-1a (encoding a protein of unknown function), PR-4 (encoding an endocellular chitinase), and PR-5 (encoding a thaumatin-like protein) are examples of pathogenesis-related protein genes induced during the HR in Arabidopsis (Uknes et al., 1992). Figure 7A shows that PR-1a was induced by light (fluence rate <40 μmol m⁻² sec⁻¹) in the psi2 mutant before any visible lesions appeared. However, PR-4 was induced only slightly by light after a significant reduction in its expression by the dark-to-light transition. PR-5 expression was not modified by light under the conditions used.

To further analyze the induction of PR-1a and to demonstrate its dependence on light perception, we compared the psi2 mutant and the cab2::luc transformant after continuous light treatment with different wavelengths and intensities (Figure 7B). As controls, we analyzed CAB expression with a CAB1 coding region probe as well as the expression of KIN2, an abscisic acid-inducible gene encoding a protein expressed during drought or cold shocks; PR-4 and GST (encoding a glutathione S-transferase inducible by pathogen attack); γ-TIP, a gibberellin-inducible gene (Phillips and Hutty, 1994); SAUR-AC1, an auxin-inducible gene (Gil et al., 1994); LOX1, a lipoxigenase gene inducible by methyl jasmonate (Bell and Mullet, 1993); ADH, an alcohol dehydrogenase gene inducible by hypoxia (Dolferus et al., 1994); and an albumin (ALB) 2S gene that is strongly expressed during late embryogenesis (da Silva Conceição and Krebbers, 1994).
From these results, we conclude that PR-1a is indeed induced by light of high fluences, in particular by continuous red light. The latter result excludes the influence of UV-B light, another factor known to stimulate PR-1 expression (Green and Fluhr, 1995). Blue light could also activate the PR-1a gene in the mutant, but with less efficiency than red light, whereas far-red light was ineffective. Thus, we observed an amplification of a light-inducible response in the psi2 mutant. The expression of PR-4 and GST, both related to the HR, was more pronounced in the mutant but only at high white light intensity (Figure 7B). Their negative regulation, occurring after light exposure, was not wavelength specific. An increase in white light resulted in downregulation of the CAB gene (Figure 7B). This reduction was not modified in the psi2 mutant, suggesting that a different pathway controls high light-induced negative regulation. No significant difference in the expression of ADH, γ-TIP, KIN2, SAUR-A1, ALB25, and LOX1 was observed. Our results indicate that PR-1a overinduction in psi2 is specific and due to a hypersensitivity to light perception.

The fluence rate necessary for necrotic lesion formation was determined by exposing plants to white light of increasing fluence rates. Figure 8A shows plants with typical light-induced lesions. In this experiment, plants were first grown under low fluence rates of white light (8 μmol m⁻² sec⁻¹) for 2 weeks: 1 week under aseptic conditions followed by another week in soil. At 15 days, the plants were transferred to white light of different fluence rates for 7 days before photographs were taken. The severity of the phenotype clearly depended on the fluence rate. Some leaves formed lesions at a fluence rate of ∼45 μmol m⁻² sec⁻¹, whereas at 100 μmol m⁻² sec⁻¹, the whole plant died after a few days.

Red light alone was also able to induce HR-like necrotic lesions on psi2 mutant cotyledons (Figure 8B) and leaves (data not shown) when plants were exposed to a fluence rate >45 μmol m⁻² sec⁻¹ for 4 days. This latter observation suggested that phytochrome pathway deregulation likely triggered lesion formation in mutant green tissues exposed to high light intensity.

Role of Phytochrome

To determine whether a specific phytochrome pathway was modified by the psi2 mutation, we constructed double mutants by crossing psi2 with three phytochrome-deficient mutants: the phyB-1 mutant (Somers et al., 1991), which lacks the PhyB apoprotein, the phyA-201 mutant, which lacks phytochrome A (Nagatani et al., 1993), and hy2, a mutant in the chromophore of any phytochrome type (Koornneef et al., 1980). A double mutant was also constructed between psi2 and fhy1, a mutant that is impaired in a branch of the phytochrome A signaling pathway (Whitelam et al., 1993; Barnes et al., 1996). In the four cases, we isolated F₃ lines homozygous for the luciferase and the long-hypocotyl phenotype.

The hypocotyl response to light as well as the formation of necrotic spots on leaves of the double mutants exposed to high white light were tested. Tables 3 and 4 show the results, obtained with 12 different F₃ lines of psi2 phyB-1, nine lines of psi2 hy2, and six lines of psi2 phyA-201 and psi2 fhy1. Each line is represented by 15 to 20 plants. Table 3 demonstrates the influence of phytochrome on the light amplification of the hypocotyl response. The lack of phytochrome B

Figure 4. Phytochrome Content in psi2.
(A) Phytochromes A and B in darkness and white light. Extracts of 7-day-old psi2 and cab2::T::luc (wild type) seedlings grown in the dark or continuous strong white light (75 μmol m⁻² sec⁻¹) were analyzed by protein gel blotting using monoclonal antibodies raised against phyA and phyB, as described in Methods. Lanes 1 and 3 contain cab2::T::luc; lanes 2 and 4, psi2.
(B) Destruction kinetics of light-labile phytochrome in red light. Phytochrome content of 5-day-old etiolated seedlings (50 to 100 mg fresh weight) was measured by difference spectrophotometry after transfer to continuous red light. Absolute Δ(ΔA) values are the average of triplicates. Bars indicate standard deviation. Open circles, cab2::T::luc; closed circles, psi2; Rc, continuous red light.
Figure 5. Structural Comparison of Leaf and Plastids in psi2 and cab2::fp::luc Plants.

(A) A chloroplast of a cab2::fp::luc plant. Plants were grown under continuous white light (50 μmol m$^{-2}$ sec$^{-1}$) on Murashige and Skoog (MS) plates for 2 weeks. Bar = 1 μm.

(B) A chloroplast of a psi2 plant. Plants were grown as given in (A). Bar = 1 μm.

(C) An etioplast of a cab2::fp::luc plant. Cotyledons of 7-day-old etiolated plants were used. Bar = 1 μm.

(D) An etioplast of a psi2 plant. Cotyledons of 7-day-old etiolated plants were used. Bar = 1 μm.

(E) Cross-section of a cab2::fp::luc leaf. Plants were grown as given in (A). Bar = 10 μm.

(F) Cross-section of a psi2 leaf. Plants were grown as given in (A). Bar = 10 μm.
and the reduction of all functional phytochromes suppressed the psi2 mutation effect. Hypocotyl length was not reduced in the psi2 phyB-1 mutant exposed to continuous red light; rather, the double mutant plant was longer than the cab2::Ω::luc transformant, thus resembling the phyB-1 mutant. Similarly, under high fluence of blue light, psi2 phyB-1 was also longer than the cab2::Ω::luc transformant. In the psi2 phyA-201 and psi2 fhy1 double mutants, psi2-induced reduction of hypocotyl length under far-red light was almost abolished. Plants lacking phyB showed little or no response to blue light and behaved similarly to a hy2 mutant that exhibited a low sensitivity to blue light. The absence of phytochrome A (phyA) or a lesion in phyA signaling (e.g., fhy1) had no significant effect on blue light perception in these experiments because the double mutants psi2 phyA and psi2 fhy1 responded like the psi2 mutant. The perception of blue light seems to be dependent on the presence of phytochrome, especially phyB, at least for hypocotyl elongation.

Table 4 provides data on lesion formation when plants were grown in continuous white light of high fluence rates. The number of leaves showing visible necrotic lesions was reported for double mutant and control plants grown under inducing conditions (50 μmol m⁻² sec⁻¹ of continuous light in soil). The cab2::Ω::luc transformants and phytochrome-deficient plants were not totally free of lesions, which sometimes appeared at leaf borders shortly after transfer from plate to soil. However, these lesions were clearly different in nature, localization, size, frequency, and times of appearance from those that developed on psi2 and double mutant leaves. The lack of phytochrome B, or any phytochrome, clearly reduced the number of leaves displaying necrotic spots; however, when phytochrome A was absent, we obtained only a small reduction in the number of necrotic leaves, indicating that this phytochrome was less important than phytochrome B. Interestingly, the reductions due to deficiencies of phyA and phyB together were approximately equal to the difference in the frequency of necrotic lesions between psi2 and the cab2::Ω::luc transformants. This observation lends further support to the notion that both phytochrome A and phytochrome B signaling pathways contribute to necrotic formation.

Figure 6. Light-Dependent Necrotic Lesion Formation in psi2.

(A) Leaves from cab2::Ω::luc (left) and psi2 (right) plants are shown. Plants were grown for 3 weeks in soil under dim white light (8 μmol m⁻² sec⁻¹) and transferred to strong white light (80 μmol m⁻² sec⁻¹) for an additional 4 days. Leaves of mutant and control plants (second pair of true leaves) were detached and photographed on a dark field. Note lesions on the psi2 leaf. Bar = 1 mm.

(B) Electron microscopy of a psi2 mesophyll cell undergoing necrosis. The black arrow indicates reinforcement of the cell wall (probably callose). The white arrow indicates vesicles containing electron-opaque material also seen in the normal HR to pathogen attack (Meyer and Heath, 1988). The chloroplast thylakoids have lost their typical structure. Bar = 1 μm.

(C) Aniline blue staining of a light-induced necrotic spot on a psi2 leaf. The UV-fluorescent material corresponds to a calloselike polymer. Bar = 100 μm.

(D) Autofluorescence of a lesion similar to the one shown in (C). In this case, the yellow fluorescence of the cell wall suggests an accumulation of ligninlike polyphenolic compounds. Bar = 250 μm.
DISCUSSION

Mutants of Increased Specificity

In this work, we have taken two measures in an attempt to recover mutants of increased specificity for phytochrome signal transduction pathways. First, we used a light-responsive CAB2 promoter fused to a luc reporter gene so that mutants with altered promoter activity, rather than altered morphology, could be visualized. Second, we screened for mutants with a modified dynamic response to red light with respect to CAB2 promoter activity (Figure 1). Our screen resulted in several mutants. One is called psi2 and is characterized in some detail here.

PSI2 Negatively Regulates Both phyA and phyB Pathways

We have compared hypocotyl growth responses and expression levels of phytochrome-responsive genes (CHS, CAB, and RBCS) between psi2 and the wild type under different light fluences. Results from these two sets of assays indicate that psi2 is hypersensitive to red as well as far-red light (Figures 1 to 3 and Table 3). Compared with the wild type, psi2 showed a greater amplitude of response at low fluences but a comparable amplitude of response at high fluences at both wavelengths of light.

Because far-red and red light are known to activate primarily phyA and phyB, respectively, our results suggest that psi2 may act downstream of these two photoreceptors. This notion is supported by experiments with psi2 phyA and psi2 phyB double mutants. In the case of psi2 phyA, the hypersensitivity to far-red light was abolished, whereas red light hypersensitivity was suppressed in psi2 phyB (Table 4). Taken together, these results demonstrate that PSI2 regulates both the phyA and phyB signaling pathways.

Analyses of the psi2 fhy1 double mutant provided further evidence of the involvement of psi2 in phyA signaling. We found that the effect of psi2 on hypocotyl length reduction under far-red light, but not red light, was abolished by the fhy1 mutation (Table 3). The latter has been shown to specifically control signal transduction downstream of phyA (Whitelam et al., 1993; Barnes et al., 1996).

Figure 7. PR-1a Expression Is Overinduced in psi2.

Seedlings were grown in aseptic conditions under various light intensities and wavelengths before RNA extraction. Gene expression levels were determined by RNA gel blot analysis.

(A) PR gene expression in plants irradiated with continuous white light of increasing fluence rates for 4 days after germination. Fluence rates in micromoles per square meter per second are indicated at the top.

(B) Expression of pathogenesis-inducible genes and control genes under various wavelengths of light. Plants germinated in darkness (1 day) were grown in continuous light of various wavelengths for 5 days before RNA extraction and gel blot analysis. Red light and strong white light overinduced PR-1a in the psi2 mutant but did not similarly modify expression of control genes. The fluence rate of red light (R) was 25 μmol m^{-2} sec^{-1}; far-red light (FR), 8.5 μmol m^{-2} sec^{-1}; blue light (B), 30 μmol m^{-2} sec^{-1}; dim white light (DW), 5 μmol m^{-2} sec^{-1}; and strong white light (HW), 85 μmol m^{-2} sec^{-1}. D, dark; wt, cab2::Ω::luc.

In (A) and (B), each lane contains 30 μg of RNA. The 18S rRNA was used as a loading control.
interactions between phytochrome and blue light receptor signaling pathways have previously been proposed (Casal and Boccalandro, 1995; Ahmad and Cashmore, 1997).

The site of action of PSI2 is not known; however, it probably acts near the top of phototransduction pathways because both phytochrome-responsive gene expression and hypocotyl elongation are affected. Because psi2 is recessive and the mutation results in hypersensitivity to red and far-red light, we suggest that its wild-type allele, PSI2, encodes a negative regulator that is likely to be involved in a reaction that attenuates or terminates the phyA or phyB signal. For example, PSI2 might act as a kinase that could desensitize phyA and phyB by phosphorylation. Desensitization of receptors by phosphorylation has been described in other signaling systems (Wang et al., 1993; Goodman et al., 1996; Zhang et al., 1997).

Light-responsive genes are constitutively (i.e., irrespective of the presence of light) derepressed by cop/det/fus mutations, which also affect the expression of several other gene sets (Mayer et al., 1996). In contrast, the psi2 phenotype is strictly dependent on red or far-red light. This observation emphasizes the specific involvement of PSI2 in the negative regulation of phytochrome pathways.

Whereas the hypocotyl response of psi2 to light is reminiscent of the hp mutant of tomato (Peters et al., 1989, 1992), there are differences between the two mutants. hp shows an increased reduction of hypocotyl length at all light intensities tested; by contrast, the reduction of hypocotyl elongation in psi2 is proportionately more at low light intensity rather than high light intensity, as compared with the wild type (Table 3). Thus, hp is likely a response amplification or an amplitude mutant rather than a light-hypersensitive mutant. Moreover, hp accumulates anthocyanin in leaves, stems, and fruits and also overaccumulates chlorophyll in fruits (Peters et al., 1992). These features were not seen in psi2.

Spontaneous Lesions on psi2 at High Light Intensity

An interesting trait of the psi2 mutant is the appearance of spontaneous lesions on leaves at high light intensities. These lesions have been shown to exhibit many characteristics of the HR normally induced by pathogen attack. Such formation of spontaneous necroses has already been described in two groups of Arabidopsis mutants, namely, the LESIONS SIMULATING DISEASE (lsd) and ACCELERATED CELL DEATH (acd) classes (Greenberg and Ausubel, 1993; Dietrich et al., 1994; Greenberg et al., 1994). The LSD1 gene codes for a zinc finger protein that negatively regulates a cell death pathway (Dietrich et al., 1997). In the case of the psi2 mutant, we assumed that the lesions resulted from the deregulation of phytochrome signaling pathways for three main reasons. (1) The lesions were clearly dependent on light intensity because no lesions were observed, even microscopically, on plants exposed to low light intensities.
(Figure 8). (2) Light-dependent lesion formation was seen in all of the three psi2 alleles similar to extents. (3) More importantly, the induction of lesions by light was blocked by the absence of the phyB photoreceptor, indicating that active phyB is needed for lesion formation at high light (Table 4). Because the phyA mutation also slightly reduced formation of the necrotic lesions at high intensity of white light, phyA signaling most likely contributes to this phenotype as well, albeit in a minor way (Table 3).

The mechanisms for the induction of cell death in psi2 at high light intensities are not known at present. Because transgenic plants overexpressing phyA or phyB do not develop necrotic lesions at high light intensities, we suggest that PSI2 may be the limiting factor in this light-dependent process. Levine et al. (1994) provided evidence of a role for peroxide in the HR, and in a recent study, J abs et al. (1996) showed that superoxide may be important in HR propagation. Therefore, one might expect to find an increased production of active oxygen species in psi2, at least enough to initiate lesions at high light intensities. However, at this time, experimental evidence supporting this explanation is still lacking.

Although the precise mechanism for induction of necrotic spots in the psi2 mutant is not known, it is strikingly reminiscent of various cases of retinal degeneration in both vertebrates and invertebrates. In Drosophila, the absence of arrestin, a protein that negatively regulates photoreceptor activity, could induce a light-dependent retinal degeneration (Dolph et al., 1993). In human retinitis pigmentosa diseases, deregulation of rhodopsin signaling leading to an accumulation of signal intermediates (e.g., cGMP) can cause cell degeneration and induce programmed cell death (Farrar et al., 1991; McLaughlin et al., 1993; Dryja et al., 1995). Similar mechanisms may apply here.

In conclusion, we have isolated a mutant that is specific for phytochrome signaling. The PSI2 gene product negatively regulates phototransduction pathways downstream of both phyA and phyB. Further genetic and biochemical characterization of PSI2 is likely to lead to a better understanding of how phytochrome signaling is regulated.

METHODS

Plant Maintenance and Light Treatment

Arabidopsis thaliana seeds of the various genotypes were routinely soaked and sterilized once with 70% hypochlorite solution containing 0.1% Tween 20 for 1 min and then with 100% hypochlorite solution containing 0.1% Tween 20 for 10 min, finally washed with three changes of sterile water, and sown on Murashige and Skoog (MS) J RH Biosciences, Lenexa, KS) solid medium containing 3% sucrose. Plates were kept in the dark at 4°C for 4 to 5 days before germination. To synchronize seed germination, plates were exposed to white light (25 \( \mu \text{mol} \text{ m}^{-2} \text{ sec}^{-1} \)) for 20 to 30 min after imbibition in darkness.

For red light pulse tests, the plants were first grown in the dark at 22°C for 7 days (germination took 2 days). Plates were covered with eight layers of 3MM filter paper (Whatman, Maidstone, UK) and then exposed to pulses of red light from cadmium-coated red light bulbs (model F20T12R; General Electric Co., Cleveland, OH). After red light pulses, the plates were wrapped in four layers of aluminum foil and incubated in the dark for 20 hr at 20°C before luciferase imaging or RNA extraction. For measurement of luciferase activity, the plates were exposed to various fluences for 25 min and imaged 20 hr later in the same order. For RNA extraction, each treatment was separated by 5 min, which was the time needed for harvesting. Either before or after the pulses, the plates were kept in complete darkness to avoid any interference from the green safelight. For RNA extraction, the seedlings were harvested with a sterile microscope glass, and the tissues were immediately frozen in liquid nitrogen.

Wide-spectrum fluorescent bulbs (model GRO-LUX F96T12/GRO; Sylvania Co., Danvers, MA) were used for continuous light treatment. The fluence rate was decreased by covering the plates with a combination of paper filters. The usual high white fluence rate is 50 to 80 \( \mu \text{mol} \text{ m}^{-2} \text{ sec}^{-1} \), and dim white light is 5 to 10 \( \mu \text{mol} \text{ m}^{-2} \text{ sec}^{-1} \). Continuous red light treatment was provided by red light bulbs (model 660 F48T12/2364/VHO, SR No. 5954; Sylvania Co.) filtered through one layer of Plexiglas (No. 2793; Atohaas North America Inc., Philadelphia, PA), with a fluence rate of 25 \( \mu \text{mol} \text{ m}^{-2} \text{ sec}^{-1} \) or as indicated; continuous far-red light (output of light bulbs: model F48T12/232/VHO, SR No. 5976; Sylvania Co.) was filtered through one layer of Plexiglas (No. 067894; West Lake Plastics, Lenni, PA) and had a fluence rate of 8.5 \( \mu \text{mol} \text{ m}^{-2} \text{ sec}^{-1} \). Continuous blue light was provided by F20T12 8 fluorescent bulbs (General Electric Inc.) and filtered through two layers of Plexiglas (No. 2424; Dayton Plastics, Columbus, OH), the fluence rate under the filter was 30 \( \mu \text{mol} \text{ m}^{-2} \text{ sec}^{-1} \). Fluence rates were measured using an Li1 895A quantum sensor (Li-COR Inc., Lincoln, NE). Light treatments were given at a temperature of 20 to 22°C, with the humidity kept at 80% in a tissue culture room.

### Table 4. Light-Inducible Lesions in psi2 Are Phytochrome Dependent

<table>
<thead>
<tr>
<th>Plant(^a)</th>
<th>Percentage of Necrotic Leaves in Continuous White Light (±SD)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cab2::zf::luc</td>
<td>7.7 (5.1)</td>
</tr>
<tr>
<td>psi2</td>
<td>75.0 (13.3)</td>
</tr>
<tr>
<td>hy2</td>
<td>4.0 (3.3)</td>
</tr>
<tr>
<td>phyA-201</td>
<td>ND(^c)</td>
</tr>
<tr>
<td>phyB-1</td>
<td>3.5 (2.5)</td>
</tr>
<tr>
<td>psi2 hy2</td>
<td>13.6 (10.1)</td>
</tr>
<tr>
<td>psi2 phyA-201</td>
<td>55.2 (8.7)</td>
</tr>
<tr>
<td>psi phyB-1</td>
<td>23.2 (8.3)</td>
</tr>
</tbody>
</table>

\(^a\)Plants were first grown for 7 days on plates under 10 \( \mu \text{mol} \text{ m}^{-2} \text{ sec}^{-1} \) white light, then transferred to soil and exposed for 3 days to 12 \( \mu \text{mol} \text{ m}^{-2} \text{ sec}^{-1} \) white light, and finally placed under a fluence rate of ~75 \( \mu \text{mol} \text{ m}^{-2} \text{ sec}^{-1} \) white light for 10 days.

\(^b\)Six plants per F\(_1\) double mutant line (~60 leaves) and 10 plants for psi2 and the other mutants or control cab2::zf::luc (~120 leaves) were examined, and the number of leaves showing necrotic lesions was scored. Numbers in parentheses indicate standard deviation.

\(^c\)ND, not determined.
Plants in soil were grown at 20°C with 80% constant humidity in a growth chamber (Conviron, Asheville, NC). Continuous white light of various fluence rates was provided by fluorescent bulbs (model GRO-LUX F96T12/GRO; Sylvania Co.).

Transgenic Plants and Luciferase Activity Imaging

Plants containing a chlorophyll a/b binding protein, Ω, and luciferase (cab2::Ω::luc) reporter transgene were generated as described by Millar et al. (1992a, 1992b). Plants containing the plastocyanin::luc reporter transgene, which was mapped to chromosome 2, were kindly provided by S. Smeekens (Utrecht University, Utrecht, The Netherlands). Sterile-filtered luciferin was sprayed onto seedlings, as described by Millar et al. (1992b). We used a video imaging (VIM)-intensified CCD camera and Argus-50 photon-counting image processor from Hamamatsu Photonic Systems (Bridgewater, NJ) for luciferase imaging.

Screening of Mutants

Mutants were screened on MS plates containing 3% sucrose. Each plate contained 400 to 500 cab2::Ω::luc ethyl methanesulfonate (EMS)-treated M2 seeds. Growth conditions are mentioned above. Seven days after transfer to darkness, the seedlings were pre-sprayed with luciferin solution to eliminate preaccumulated luciferase activity (Millar et al., 1992b). After an additional 24 hr in the dark, a first image of the plant-emitted light was taken for 25 min (i.e., the dark image). Directly after imaging, a pulse of red light (0.1 μmol m^{-2} sec^{-1}) was given to the plants (see light treatment), and the plates were returned to darkness for 20 hr before a second image was taken. Both images were compared, and plants with amplified luciferase activity were isolated. Candidate mutants were grown for seeds in a growth chamber. The responses of the M₂ generation were scored again after a red light pulse of 0.1 μmol m^{-2} sec^{-1}. Amplified plants were isolated on two equivalent plates. The activity of ~50 seedlings was monitored together, and an average value was calculated. For a finer response study, we proceeded as described above under red light pulse treatment.

RNA Gel Blot Analysis

RNA was prepared from leaves kept at ~80°C using the aurintricarboxylic acid method (Barnes et al., 1996). RNA (30 μg per lane) was blotted onto nylon filters (Stratagene, La Jolla, CA) and hybridized with random-primed probes prepared according to the manufacturer's recommendations (Amersham). Membranes were routinely stripped for reprobing. The CAB probe was a 537-bp fragment from the coding sequence of the CAB1 gene (Millar and Kay, 1991). The chickene synthase (CHS) probe was a 0.4-kb XbaI-HindIII fragment from the coding sequence of the CHS gene contained within the plasmid pCH53.9 (Feinbaum and Ausbel, 1988). The ribulose-1,5-bisphosphate carboxylase (RBCC) small subunit probe was obtained from clone ATTS0375 (Arabidopsis cDNA expressed sequence tag sequencing project; gift of J. Giraudat, Centre National de la Recherche Scientifique, Gil-Sur-Yvette, France). The pathogenesis-related protein PR-1A probe and PR-4 probe were Xhol-EcoRI fragments of the respective coding sequences cloned into pBluescript SK+ (Stratagene), and the PR-5 probe was a BamHI-KpnI fragment of the coding sequence cloned in the same vector (Uknes et al., 1992). The vectors containing the PR probes were a gift from the laboratory of J. Ryals (Paradigm Genetics, Inc., Research Triangle Park, NC).

For the lipoygenase (LOX1) gene and the glutathione S-transferase (GST) gene probe preparation, see Mayer et al. (1996). The probe for the tonoplast intrinsic protein (γ-TIP) gene is an EcoRI fragment of plasmid AI2122 (Phillips and Huttly, 1994), and the small-auxin-upregulated SAUR-AC1 probe is a 0.3-kb BglII-BamHI fragment of the plasmid p1013 open reading frame (Gil et al., 1994). The alcohol dehydrogenase (ADH) gene probe is a 0.6-kb SalI-NotI fragment of the 194E117T7 expressed sequence tag clone, and the albumin (ALB) 25 gene probe is a 0.7-kb SalI-NotI fragment of the 107I17T7 expressed sequence tag clone. As loading control for total RNA, we used an 18S rDNA fragment cloned from Arabidopsis (Takahashi et al., 1995).

The CAB2-specific probe is a 22-base oligonucleotide from the specific RNA leader of the CAB2 transcript (Leutwiler et al., 1986), 5′-ATGACTAAGTTGGAAGAAC-3′. The CAB1 probe is a 42-base oligonucleotide from the CAB1 mRNA leader region, 5′-CAGCACCCAGGTTAAGATTGTTGTAAGCCAA-3′. The absic acid-inducible KIN2-specific probe is a 67-base oligonucleotide of the KIN2 gene (Kurkeila and Berg-Franck, 1992; 5′-CATTAAAAACATTACACTAACTAAACAAAACAGTCTATACGAGAATCT-3′). Oligonucleotide probes were labeled with poly nucleotide kinase (Boehringer Mannheim) and purified as described by Sambrook et al. (1989). Hybridization signals were quantified using a PhosphorImager (model 400E; Molecular Dynamics, Sunnyvale, CA).

Immunochromic Detection of Phytochromes A and B

Crude extracts were prepared from 7-day-old etiolated and light-grown seedlings, as described by Nagatani et al. (1993). Proteins were separated by 7.5% acrylamide SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were probed with either a monoclonal antibody against Arabidopsis phyA (mAA2) or a monoclonal antibody against Arabidopsis phyB (mA32) or a monoclonal antibody against Arabidopsis phyB (mA32) or a monoclonal antibody against Arabidopsis phyB (mA32) or a monoclonal antibody against Arabidopsis phyB (mA32) or a monoclonal antibody against Arabidopsis phyB (mA32). The membranes were stained with ProtoBlot AP System (Promega, Madison, WI), according to the manufacturer's instructions. A prestained kit for the molecular weight range 27,000 to 180,000 (Sigma) was used for markers. Phytochrome content of seedlings (50 to 100 μg) was measured by spectrophotometry, as described by Schäfer et al. (1976).

Genetic Analysis

Complementation and segregation analyses were performed as described by Koornneef and Stam (1992). The double mutant plants were selected in the F₂ generation for their overexpression of luciferase against a background of less luminous plants. The plants with the longest hypocotyls in either white light, for psi2 hy2 and psi2 phyB-1 double mutant selection, or continuous far-red light, for psi2 phyA-201 and psi2 phy1 double mutant selection, were grown and crossed with their parents. Fifteen to 20 F₂ seedlings of those backcrosses were examined and compared with their parents for the long-hypocotyl phenotype or amplification of luciferase activity. The mapping was conducted as described by Bell and Ecker (1994) for the microsatellite nga229 and by Konieczny and Ausbel (1993) for the cleaved amplified polymorphic sequence (CAPS) technique. The
polymerase chain reaction primers for CAPS were purchased from Research Genetics (Huntsville, AL). Map distances were calculated as described by Koumene and Stam (1992).

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