

Phototropins 1 and 2: versatile plant blue-light receptors

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Blue and ultraviolet-A light regulate a wide range of responses in plants, including phototropism, chloroplast migration and stomatal opening. However, the photoreceptors for these light responses have been identified only recently. The phototropins (phot1 and phot2) represent a new class of receptor kinases that appear to be exclusive to plants. Recent genetic analysis has shown that phot1 and phot2 exhibit partially overlapping functions in mediating phototropism, chloroplast migration, and stomatal opening in *Arabidopsis*. Although significant progress has been made in understanding the early photochemical and biochemical events that follow phototropin excitation, the details of how this excitation activates such different responses remain to be elucidated.

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Blue (390–500 nm) and ultraviolet-A (UV-A; 320–390 nm) light elicit a variety of physiological responses in plants. Of these, there are four that maximize photosynthetic potential in weak light and prevent damage to the photosynthetic apparatus in excess light. These include PHOTOTROPISM (bending of plant organs towards or away from a source of incident light, see Glossary) [1], light-induced opening of STOMATA [2,3], chloroplast migration in response to changes in light intensity [4] and solar tracking by leaves of certain plant species [1]. All four of these responses have been known for a century or more. As early as 1887, phototropism was known to be induced by blue, but not by red, light [5]; by 1933, blue light had been shown to be more effective than red in inducing stomatal opening [6]; and, by 1934, it had been shown to be the most effective light quality for inducing chloroplast displacement in the moss *Funaria hygrometrica* [7].

Although these early experiments indicating the importance of blue light were crude by modern standards, they have nonetheless stood the test of time. The ACTION SPECTRA for phototropism [8–10], stomatal opening [11,12], and the chloroplast ACCUMULATION and AVOIDANCE responses [13,14] are all similar (Fig. 1). They show a band with one major and two minor absorption peaks in the blue region of the spectrum and a broad absorption band in the UV-A. These spectral properties are characteristic of a flavoprotein photoreceptor.

Phototropins 1 (phot1) and 2 (phot2) [15], the most recently characterized blue-light receptors in plants, have spectral properties that closely match these action spectra (Fig. 1). Both phot1 and phot2 mediate not only phototropism, after which they were named, but also blue-light-induced chloroplast migration [16–18]

and blue-light-induced stomatal opening [19]. In addition, the rapid inhibition of stem growth [20–23] by blue light is probably mediated by phot1 [24]. phot1 also plays a role in blue-light-mediated calcium uptake [25] and might have a minor role in blue-light-induced membrane depolarization [24].

Here, we briefly review the photochemical and biochemical properties of the phototropins, and present evidence for their participation in these diverse blue-light responses. Recent reviews of phototropism [26,27], chloroplast migration [28], stomatal responses [29] and the rapid inhibition of stem growth [30] provide a more detailed discussion of these blue-light responses. A more general review of blue-light receptors in higher plants [31] is also available.

phot1: light-activated, FMN-binding receptor kinase
The initial observation that blue light activates the phosphorylation of a plasma-membrane protein in growing regions from etiolated seedlings [32] led to the extensive photochemical and biochemical characterization of the phosphorylation reaction.

Glossary

Accumulation

Movement of chloroplasts within a cell to minimize mutual shading (induced by dim light).

Action spectra

Relative effectiveness of different wavelengths of light to induce a biological response.

Avoidance

Movement of chloroplasts to maximize mutual shading (induced by bright light).

Flash photolysis study

Investigation of extremely rapid spectral changes in response to a short (nanosecond or even picosecond) flash of light.

Isosbestic points

Wavelengths at which the substrate and the product of a reaction have exactly the same absorbance.

Phototropism

Growth- or turgor-driven movement of a plant organ toward or away from a light source.

Stomata

Small pores in the leaf and stem epidermis that regulate gas exchange by opening or closing in response to various environmental stimuli.

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It also led to correlative physiological studies indicating that the phosphorylation reaction was in some way associated with phototropism [33,34]. The demonstration that an *Arabidopsis* mutant impaired in its phototropic response was also impaired in light-activated phosphorylation [35] added powerful genetic evidence that the reaction plays a role in phototropism. However, efforts to purify the phosphorylated protein in a form that retained photosensitivity using conventional biochemical methods met with repeated failure. It was eventually the use of *Arabidopsis* mutants with an impaired phototropic response (designated *nph* for non-phototropic hypocotyl) [36] that led to the cloning and characterization of the first phototropin gene. Of the four loci identified (*NPH1-NPH4*), all mutant alleles at the *NPH1* locus either lacked or showed a greatly diminished capacity for light-activated phosphorylation.

Light-activated protein phosphorylation requires three components: a substrate, a kinase and a photoreceptor. In theory, these could all be separate entities. It took the ingenious technique of amplified fragment-length polymorphism (AFLP) to clone and characterize the *NPH1* gene and its encoded protein [37] (Fig. 2), and to analyze its domain structure. The C-terminal half of the *nph1* protein is a classic serine/threonine kinase [38], indicating that at least substrate and kinase constitute a single entity. All *nph1* mutant alleles sequenced had lesions in their kinase domains, suggesting that the kinase activity is essential for the protein's function. The N-terminal half of the protein contains two repeated domains of ~100 amino acids [37], with ~40% amino acid sequence identity [39]. These domains also show sequence similarity to domains found in a range of signaling proteins in organisms ranging from Archaea to mammals. Because all these proteins are regulated by light, oxygen or voltage, the *nph1* domains were assigned the acronym LOV [37]. LOV domains constitute a subset of the PAS-domain superfamily, which is known to mediate both ligand binding and protein-protein interactions [40].

Further work using recombinant *Baculovirus* successfully expressed the *NPH1* gene in insect cells and showed that, in the absence of additional plant proteins, the recombinant protein retained the capacity to undergo autophosphorylation in response to light [41]. The protein was also found to bind flavin mononucleotide (FMN). In addition, the fluorescence excitation spectrum of the undenatured recombinant protein closely resembled the action spectrum of phototropism. Therefore, the authors concluded that *nph1* undergoes autophosphorylation and functions as a photoreceptor for phototropism. As a result, they named the photoreceptor phototropin [39] [subsequently renamed phototropin 1 (phot1) [15]] instead of *nph1*.

The *Arabidopsis* phototropin investigated in the above studies was one of three LOV-domain-containing

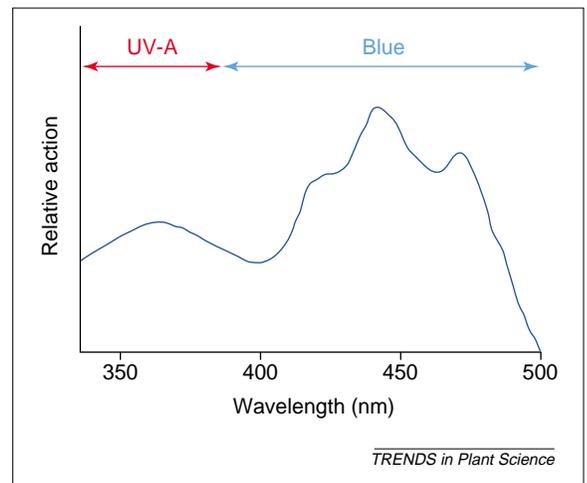


Fig. 1. Stylized action spectrum typically observed for phototropin-mediated responses. Notice the presence of a major peak at 450 nm, a shoulder at 425 nm and a minor peak at 470 nm in the blue region of the spectrum. This fine structure is not observed in the broad absorption band at 365 nm in the ultraviolet region of the spectrum.

proteins known to bind a flavin cofactor. The other two were Aer, the oxygen sensor mediating aerotaxis in *E. coli* [42], and NIFL, an oxygen-sensing protein found in *Azotobacter* and other free-living nitrogen-fixing bacteria [43]. Because all three proteins possess a LOV domain, it seemed logical that this domain might function to bind the flavin moiety. The LOV1 and LOV2 domains from several phototropin proteins were therefore produced either singly or in tandem in *E. coli* [39] to determine whether these short peptides bound a flavin. All LOV-domain fusion proteins were found to bind FMN. Moreover, FMN binding was stoichiometric: peptides containing one LOV domain bind one molecule of FMN, whereas peptides containing both LOV1 and LOV2 bind two. Hence, phototropin is a dual chromophoric photoreceptor in which both chromophores are FMN.

LOV domains of phot1 are light sensors

When isolated LOV domains are irradiated with blue light, they undergo a complex spectral change, characterized by a loss of absorption in the blue region of the spectrum combined with the appearance of a new peak near 390 nm (Fig. 3). These light-induced absorbance changes result in the formation

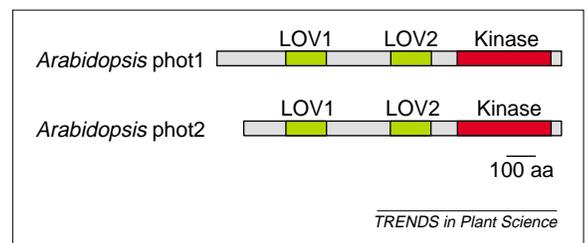


Fig. 2. Protein structures of the *Arabidopsis* blue-light receptors, phot1 and phot2 (996 and 915 amino acids, respectively). Light, oxygen or voltage (LOV) domains are shown in green. The kinase domains, which catalyse the phosphorylation of proteins on specific amino acid residues (threonine and serine in this case), are shown in red.

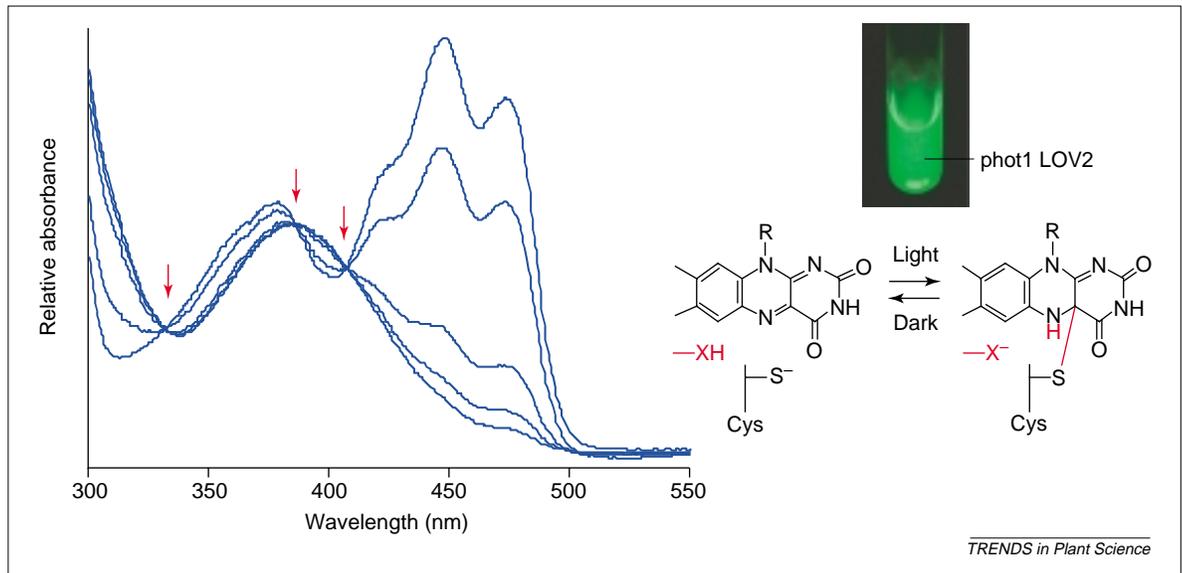


Fig. 3. Photochemistry observed for the phototropin light, oxygen or voltage (LOV) domains (modified from Ref. [44]). A sample of purified *Arabidopsis* phot1 LOV2 is shown in the upper right. Purified LOV domains bind the chromophore FMN (flavin mononucleotide) and exhibit green fluorescence when viewed under ultraviolet light. The graph illustrates the light-induced absorbance changes observed for purified phot1 LOV2 over several seconds. The absorption spectrum that is uppermost at 450 nm represents the protein sample in the dark state. The sample is then irradiated with blue light and spectra recorded at 1-s intervals. The following light-induced absorbance changes result in the formation of three isosbestic points, indicated by the red arrows, which are characteristic of the formation of a flavin-cysteinyll adduct. Proposed light-induced formation of the covalent adduct between the FMN chromophore and a conserved cysteine residue within the LOV domain is shown in the lower right. Recent studies [50] indicate that this reaction involves an, as yet unidentified, proton donor-acceptor (designated X).

of ISOSBESTIC POINTS at 330 nm, 375 nm and 410 nm [44] (Fig. 3, arrows). The observed light-induced spectral changes are fully reversible in darkness, recovering over a matter of seconds or minutes depending on the chromopeptide. These spectral changes are not typical of a simple reduction [45] but resemble those observed for the formation of an adduct between a cysteine residue and the C(4a) carbon of the flavin [46]. Indeed, the mutation of a highly conserved cysteine in LOV1 and LOV2 to alanine or serine completely abolishes their photochemical reactivity. Thus, cysteinyl-adduct formation probably represents the primary mechanism underlying light detection by the phototropins [44].

The crystal structure of the LOV2 domain from a chimeric photoreceptor from the fern *Adiantum capillus-veneris*, phy3, has recently been solved [47]. The structure shows that the side chain of the conserved cysteine is 4.2 Å from the C(4a) carbon of the FMN, which is consistent with the light-induced formation of a flavin-C(4a) cysteinyl adduct (Fig. 3). More recent X-ray crystallography has now shown that, on adduct formation, the cysteine moves to within ~2 Å of the C(4a) carbon of the FMN [48]. Recent nuclear magnetic resonance studies using heavy-isotope-labeled FMN [49] have confirmed this mechanism of the light reaction and suggest a

light-activated protein conformational change for the LOV2 domain of oat phot1.

FLASH PHOTOLYSIS STUDIES [50] indicate that the first detectable photoproduct generated (within 30 ns) is a flavin triplet. This product decays within 4 μs to a long-lived species with a spectrum consistent with the proposed flavin-C(4a) cysteinyl adduct. Fluorescence studies at various pHs indicate that the cysteine exists as a thiolate anion in the dark state. Circular dichroism studies also show that the formation of the stable product leads to a major distortion of the FMN chromophore [44], which in turn probably leads to a protein conformational change that activates the C-terminal kinase and initiates signaling.

phot2: a second phototropin

A second phototropin-like gene from *Arabidopsis* has now been cloned and sequenced [51] (Fig. 2). The encoded protein was subsequently designated phototropin 2 (phot2) [15]. Like phot1, phot2 has two LOV domains, a kinase domain and undergoes light-activated autophosphorylation [17]. Studies have shown that the LOV domains of phot2 also bind FMN and exhibit photochemical reactivity similar to that observed for the phot1 LOV domains [17].

Phototropism

Two main problems have hindered the identification of blue-light receptors in the past. First, the action spectra cited above suggest that a flavoprotein is the photoreceptor for phototropism, chloroplast migration and stomatal opening. However, plants contain many diverse flavoproteins. Furthermore, free flavins and flavoproteins can be photochemically active under certain conditions. Thus, biochemical approaches to identifying putative flavoprotein photoreceptors have low odds of success. Second, it now appears that there is functional redundancy between phot1 and phot2 in regulating the physiological responses mentioned above. Hence, experiments with single-photoreceptor mutants are

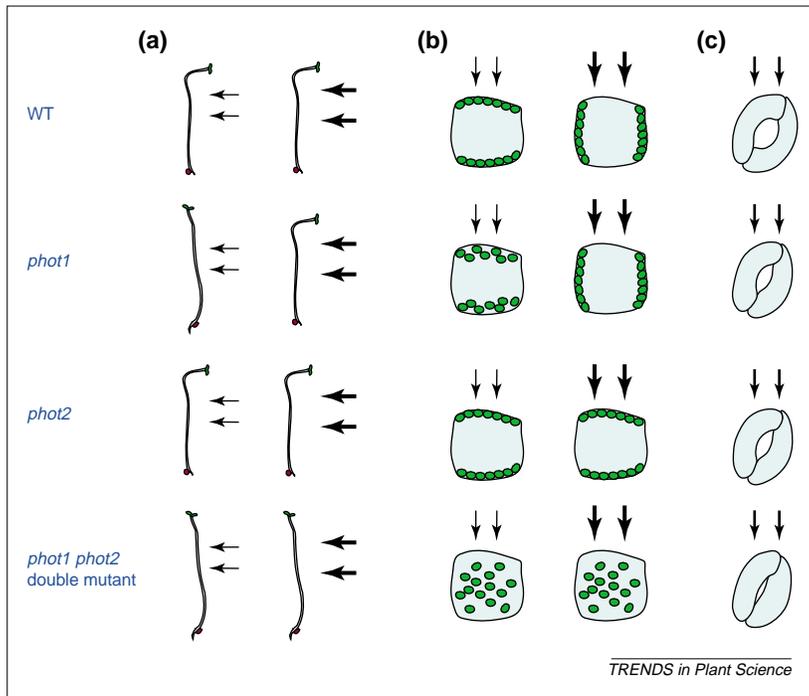


Fig. 4. Blue-light-induced phototropic responses, chloroplast migration and stomatal opening observed for wild-type (WT) *Arabidopsis* plants and single and double phototropin-deficient mutants (*phot1*, *phot2* and *phot1 phot2*). (a) The phototropic response to low- and high-intensity unilateral blue light. (b) A side view of the chloroplast migration responses in leaf mesophyll cells. (c) The opening response of stomatal guard cells. The size of the black arrows shown for all three responses corresponds to the relative intensity of the blue-light treatment.

less than conclusive. It was only when mutants lacking both *phot1* and *phot2* became available that the situation could be clarified for all three responses.

Phototropism provides an excellent example of the redundancy problem. When the *nph1* (*phot1*) mutants were originally described [36], the authors reported a lack of both hypocotyl and root phototropism in response to low-intensity blue light. However, it was subsequently observed that *phot1* mutants retain phototropic responsiveness to high-intensity blue light [17]. Clearly, a second photoreceptor can mediate phototropism but only at intensities higher than those used in the earlier study [36]. Only in the *phot1 phot2* double mutant was the response at these higher light intensities severely impaired [17]. Thus, both *phot1* and *phot2* mediate phototropism. However, *phot2* appears to function only at high light intensities. The phototropic responses of wild-type seedlings and those of single and double phototropin mutants of *Arabidopsis* to low and high intensity unilateral blue light are illustrated in Fig. 4. In spite of an earlier report suggesting a role for the cryptochromes (*cry1* and *cry2*) as photoreceptors for phototropism [52], subsequent studies showed that *cry1 cry2* double mutants retain both normal phototropism over a wide range of light treatments and normal light-activated autophosphorylation of *phot1* [53].

Blue-light-activated chloroplast migration

Chloroplasts are distributed in low light in a manner that presumably maximizes light capture for

photosynthesis (accumulation response). By contrast, they are redistributed in high light in a manner that minimizes light interception, presumably to reduce photodamage (avoidance response) (Fig. 4). As with phototropism, *phot1* and *phot2* exhibit partially overlapping functions for light-induced chloroplast migration. Two research groups [16,18] have independently described the impaired chloroplast-migration responses for *phot2* single mutants of *Arabidopsis*. Each study showed that the avoidance response is absent and that the accumulation response can occur even at the highest light levels tested. Evidently, *phot2* activated at high fluence rates counteracts the action of another photoreceptor that mediates the accumulation response. Both studies also found that *PHOT2* gene expression is upregulated by light, indicating that *phot2* probably predominates under high light conditions.

It had been previously reported that, although the high-light avoidance response is normal in the *phot1* mutant, the accumulation response shows a somewhat higher light threshold requirement than in the wild type [54]. This result indicates that *phot1* somehow contributes to the accumulation response but is not the only player. It was only when chloroplast migration in a *phot1 phot2* double mutant was investigated [17] that the situation was clarified. In this mutant, chloroplasts show neither an accumulation nor an avoidance response, regardless of the light treatment given. Hence, both *phot1* and *phot2* can mediate the accumulation response of chloroplasts to low-intensity blue light, although *phot2* is somewhat less sensitive. Only *phot2* mediates the avoidance response and it counteracts *phot1* action under high light conditions. The various chloroplast responses in wild-type *Arabidopsis* plants, as well as single and double phototropin mutants, are shown in Fig. 4.

Blue-light-induced stomatal opening

The redundancy problem has also plagued efforts to identify the photoreceptor mediating blue-light-induced stomatal opening. It was clear, even from the earliest studies, that both red and blue light could induce stomatal opening in dark-adapted leaves. A review in 1962 [55] concluded that there was not yet sufficient evidence to attribute light-activated stomatal opening to anything but photosynthesis, a view reached independently two years later [56]. Later studies strengthened the case for a blue-light-activated mechanism that was separate from photosynthesis [57,58]. It was only when the effects of blue light on stomatal aperture were measured against a background of red light that was sufficient to saturate photosynthesis that a role for a separate blue-light photoreceptor system was firmly established [59]. Blue light has also been shown to induce the swelling of individual stomatal guard-cell protoplasts isolated from onion epidermal tissue [60]. This study thus localized a blue-light receptor to the guard cells themselves.

Although the available action spectra [11, 12] support a role for a flavoprotein as the photoreceptor for stomatal opening, there is also evidence that carotenoids might be involved. The stomata of the *Arabidopsis npq1* mutant, which is blocked in light-induced formation of the carotenoid zeaxanthin, showed reduced opening in response to blue light [61]. More detailed studies [62] confirmed the lack of blue-light sensitivity in this mutant. It was subsequently shown that stomatal opening induced by blue light can be counteracted by a pulse of green light if given sufficiently soon after the blue-light treatment [63]. The action spectrum for this reversal extends from 480 nm to 600 nm, red-shifted far beyond the expected absorption range of either a flavoprotein or a carotenoid. The authors speculated that this action spectrum might reflect the blue-light-induced formation of a zeaxanthin isomer with absorption at these longer wavelengths. However, other workers [64] later reported identical blue-light-induced increases in transpiration (as a measure of stomatal function) in both wild-type *Arabidopsis* plants and the *npq1* mutant. The reason for this discrepancy is currently unknown.

However, once again, photoreceptor redundancy had led investigators astray. The photoreceptor question was finally clarified by investigations of the stomatal responses, not only of *phot1* and *phot2* single mutants, but also of the *phot1 phot2* double mutant [19]. Whereas both single mutants respond to blue light with an increase in stomatal aperture, they are slightly less sensitive than the wild type. By contrast, the double mutant shows no stomatal response to blue light. Therefore, as for phototropism and the chloroplast accumulation response, both *phot1* and *phot2* are sufficient to mediate stomatal opening, although they might differ in their relative photosensitivity. The stomatal responses of wild-type *Arabidopsis*, as well as of the single and double phototropin mutants, are shown in Fig. 4.

Blue light is also known to activate proton secretion by stomatal guard cells [65]. Indeed, blue light leads to the phosphorylation and consequent activation of two guard-cell proton ATPases [66]. Whether phosphorylation of the proton ATPases is directly regulated by *phot1* and *phot2* remains to be determined. Blue-light-induced proton secretion is also lacking in the *phot1 phot2* double mutant [19]. Unlike blue-light-induced proton secretion, proton secretion induced by the fungal toxin fusicoccin is unaltered in either single or double phototropin mutants. Thus, failure of stomata to open in the double mutant does not arise from an indirect effect on ATPase activity.

phot1 and rapid inhibition of stem growth by blue light
Transfer of dark-grown seedlings to blue light causes an inhibition of stem growth. In *Arabidopsis* (and other dark-grown seedlings), blue light inhibition of stem growth can be separated into

two phases: a rapid, transient inhibition occurring within a few minutes of a blue-light pulse and a slower inhibition that persists for many hours after the blue-light treatment [67]. The rapid phase was recently found to be absent in a *phot1* single mutant, indicating that *phot1* mediates the rapid inhibition of stem growth by blue light [24]. The slow, persistent phase of stem growth inhibition is controlled by the cryptochrome blue-light receptors and involves blue-light-activated membrane depolarization [67]. However, cryptochrome-mediated stem-growth inhibition is delayed in mutants lacking *phot1* [24]. Similarly, *phot1* mutants show somewhat impaired blue-light-activated membrane depolarization. Therefore, *phot1* also appears to modulate the cryptochrome-mediated phase of growth inhibition. It would be interesting to test *phot2* single mutants and *phot1 phot2* double mutants over a range of light intensities to see whether, as in the responses described above, there are functional redundancy and different light sensitivities between *phot1* and *phot2* in mediating blue-light-induced inhibition of stem growth and blue-light-induced membrane depolarization.

Another recent study has examined blue-light-induced calcium uptake in the *phot1* mutant [25]. In contrast with cryptochrome-deficient mutants, mutants lacking *phot1* show a strong reduction in blue-light-activated calcium import. The residual calcium uptake in these experiments might well be caused by excitation of *phot2* and therefore the response might be lacking in the double mutant. Further studies are required to determine whether blue-light-activated membrane depolarization and calcium uptake are related to the physiological responses described above and, if so, how.

Is the list of plant photoreceptors complete?

The answer to this question is 'No'. For instance, other LOV-domain-containing proteins have been identified in *Arabidopsis*. Their LOV domains contain all the conserved flavin-binding residues found in the phototropin LOV domains, including the cysteine involved in light-activated adduct formation [47]. These proteins might also act as photoreceptors. Somewhere in the plant lurks at least one UV-B receptor [68]. Although it has been known since 1938 [69] that solar tracking by leaves is a blue-light response, the photoreceptor(s) responsible has not been identified. Because this response involves light-driven turgor changes in cell volume, as does stomatal opening, the phototropins might be involved. The counteracting effect of green light on stomatal opening after a blue-light pulse clearly implicates a photoreceptor with spectral properties different from those of either the cryptochromes or the phototropins. The role of zeaxanthin in stomatal opening also remains to be resolved. Although the results with the *phot1 phot2* double mutant [19] make it highly unlikely that

zeaxanthin plays a direct role as a photoreceptor for stomatal opening, it might have some modulating role.

Identification of the phototropins as blue-light receptors and knowledge of their photochemistry and biochemistry represents a major advance. However, much remains to be done to elucidate the downstream events following phototropin excitation. For example,

phototropism requires cell-cell communication [27], whereas chloroplast migration [70] and stomatal opening [60] are strictly cell autonomous. Given that the initial photochemical and biochemical events are similar for phot1 and phot2, it will be fascinating to discover where and how their signaling pathways diverge to bring about these different blue-light responses.

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Priming in plant–pathogen interactions

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Plants can acquire enhanced resistance to pathogens after treatment with necrotizing attackers, nonpathogenic root-colonizing pseudomonads, salicylic acid, β -aminobutyric acid and many other natural or synthetic compounds. The induced resistance is often associated with an enhanced capacity to mobilize infection-induced cellular defence responses – a process called ‘priming’. Although the phenomenon has been known for years, most progress in our understanding of priming has been made only recently. These studies show that priming often depends on the induced disease resistance key regulator NPR1 (also known as NIM1 or SAI1) and that priming has a major effect on the regulation of cellular plant defence responses.

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In addition to constitutive barriers, plants have evolved distinct inducible defence mechanisms to protect themselves against pathogen attack. For example, upon inoculation with NECROSIS-inducing pathogens (see Glossary) [1,2] or various nonpathogenic root-colonizing pseudomonads [3], or treatment with SALICYLIC ACID (SA) [1,2], β -AMINOBTYRIC ACID (BABA) [4] or various other natural and synthetic compounds [5], plants acquire enhanced resistance to a broad spectrum of pathogens. The induced resistance occurs not only at the site of the initial treatment but also in distal, untreated plant parts. The various induced resistance phenomena are all associated with an enhanced capacity for the rapid and effective activation of cellular defence responses, which are induced only after contact with a (challenging) pathogen [4,6–10]. These responses include the HYPERSENSITIVE RESPONSE (HR) [11], cell-wall strengthening [12–14], the OXIDATIVE BURST [15] and the expression of various defence-related genes [1,2].

By analogy with a phenotypically similar phenomenon in mammalian monocytes and macrophages, the augmented capacity to mobilize cellular defence responses has been called the ‘PRIMED’ [16] (or ‘sensitized’ [6]) state of the plant. Although the priming phenomenon has been known for years as a part of induced-resistance phenomena [6,13,14], it has mostly been overlooked in studies dealing with induced disease resistance of plants, because it only becomes apparent after challenge of the primed tissue. Therefore, the molecular mechanism(s) and genetic basis of priming and its role in induced disease resistance have remained largely unclear. This article reviews recent findings supporting a crucial role for priming in induced plant disease resistance.

Priming and systemic acquired resistance

The systemic resistance response activated upon infection of plants with necrotizing pathogens is called systemic-acquired resistance (SAR) [1,2], but SAR can also be induced by exogenous application of salicylic acid or its functional analogues 2,6-DICHLOROISONICOTINIC ACID (INA) and BENZOTHIADIAZOLE (BTH) [1,2,17]. Establishment of SAR requires an endogenous increase in salicylic acid levels [1,2,17] and its onset is associated with the expression of *SAR* genes [1], some of which encode PATHOGENESIS-RELATED (PR) PROTEINS [1,2,17]. Some PR proteins display antimicrobial activity *in vivo* [18] but their actual role in SAR remains uncertain. Unfortunately, the availability of tools and markers for monitoring other cellular plant defence responses such as the HR or local cell-wall strengthening is limited. Therefore, it is important to

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