

Shining Light on the Function of NPH3/RPT2-Like Proteins in Phototropin Signaling¹[CC-BY]

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Plasma membrane receptors play fundamental roles in shaping plant growth and development. A large proportion of these are autophosphorylating Ser/Thr kinases (De Smet et al., 2009). Some function as dual-specificity kinases, autophosphorylating additionally on Tyr residues, one of which is the extensively studied steroid receptor BRASSINOSTEROID-INSENSITIVE1 (BRI1; Oh et al., 2009). Light regulation of plant growth also is mediated by plasma membrane-bound Ser/Thr kinases known as the phototropins (phot; Fankhauser and Christie, 2015). Seed plants contain two phot (phot1 and phot2) that have important roles in regulating leaf positioning and expansion, chloroplast photorelocation movement, stomatal opening, and phototropism, all of which serve to optimize photosynthetic efficiency (Christie et al., 2015). Phot are members of the AGCVIII kinase family (Rademacher and Offringa, 2012) but are distinct from transmembrane receptor kinases such as BRI1, as they are hydrophilic and bind to the intracellular side of the plasma membrane (Kong et al., 2013) to initiate signaling (Preuten et al., 2015). Although the ability to associate with the plasma membrane is conserved in algal phot (Sullivan et al., 2016a), the mechanism underlying this attachment is still not known, but it is thought to involve some form of lipid binding/modification.

Kinase-inactive versions of phot1 and phot2 are non-functional, highlighting the importance of receptor autophosphorylation in phot signaling (Inoue et al., 2008a, 2011). Autophosphorylation occurs predominantly on multiple Ser residues. At least 21 and 29 phosphorylation sites have been identified for *Arabidopsis thaliana* phot1 and phot2, respectively (Christie et al., 2015). Most of these sites are found in the N terminus of the protein, which contains two light-sensing modules

known as LIGHT, OXYGEN, OR VOLTAGE SENSING (LOV) domains known as LOV1 and LOV2 (Christie et al., 2012). Autophosphorylation of Ser-350, Ser-376, and Ser-410 within the LOV-linker region promotes the binding of 14-3-3 regulatory proteins to phot1 (Inoue et al., 2008a; Sullivan et al., 2009). However, the biological significance of this interaction is still not known. The occurrence of some these phosphorylation sites also is fluence rate dependent (Salomon et al., 2003) and is thought to play a role in receptor desensitization (Christie and Murphy, 2013). By contrast, the autophosphorylation of two conserved Ser residues within the activation loop of the C-terminal kinase domain (Ser-849 and Ser-851 in phot1 and Ser-761 or Ser-763 in phot2) is necessary for receptor signaling (Inoue et al., 2008a, 2011). Mutation of these sites to Ala impairs phot1 function in *Arabidopsis*, whereas phosphomimetic substitutions to Asp are without effect (Inoue et al., 2008a).

ADVANCES

- Phototropin (phot) blue-light receptors regulate phototropism, chloroplast accumulation movement, leaf positioning and leaf flattening in *Arabidopsis* through the coordinated action of specific NRL family members.
- NPH3 is essential for establishing lateral auxin gradients underpinning phototropic curvatures and possibly leaf positioning and expansion.
- RPT2 modulates phototropic responsiveness through alterations in the localization and phosphorylation status of NPH3.
- NCH1 and RPT2 function redundantly to mediate chloroplast accumulation movement independently of auxin.
- NPH3 and NCH1 exhibit biochemical features of CRL3 substrate adaptors.
- Functional divergence of phot-NRL signaling has occurred during land plant evolution.

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Despite considerable efforts over the last two decades, the signaling events that follow receptor autophosphorylation remain poorly understood for most phot-mediated responses. Yet, it is becoming increasingly apparent that members of the NPH3/RPT2-Like family (NRL) are required to elicit several phot responses. Moreover, accumulating evidence suggests that these plant-specific proteins act in concert with different AGCVIII kinases to regulate various aspects of auxin transport and signaling. In this review, we summarize our current understanding of the biochemical properties of the NRL family in Arabidopsis and how these proteins function to coordinate different aspects of phot signaling. We also discuss to what extent these signaling processes are conserved in land plants and algae.

STRUCTURE AND FUNCTION OF ARABIDOPSIS NRL PROTEINS

The NRL family is named after its two founding members, NONPHOTOTROPIC HYPOCOTYL3 (NPH3) and ROOT PHOTOTROPISM2 (RPT2; Liscum et al., 2014), which were first identified from genetic screens for Arabidopsis mutants impaired in hypocotyl and root phototropism (Okada and Shimura, 1992; Liscum and Briggs, 1995; Sakai et al., 2000). The primary amino acid structure of NRL proteins can be separated into three main parts based on sequence conservation: a BTB (bric-a-brac, tramtrack, and broad complex) domain at the N terminus, followed by an NPH3 domain and a C-terminal coiled-coil domain (Liscum et al., 2014). In addition to NPH3 and RPT2, Arabidopsis contains another 31 NPH3/RPT2-Like proteins (NRL1–NRL31), all of which are defined by the presence of the NPH3 domain (Pedmale et al., 2010). Ten of these members lack the C-terminal coiled-coil domain, whereas two are devoid of the BTB domain (Pedmale et al., 2010). The reasons for these differences in domain structure are not clear at present, because many of these NRL proteins have yet to be ascribed a biological function.

Functions for 10 out of the 33 Arabidopsis NRL proteins have been identified so far. Phylogenetic analysis has shown that NRL proteins can be classified into seven distinct clades in land plants (Suetsugu et al., 2016). However, in angiosperms such as Arabidopsis, only six of these clades are present (Fig. 1). NRL proteins from three of these clade classifications (Suetsugu et al., 2016) are known to interact with the phots. These include NPH3 (Motchoulski and Liscum, 1999), RPT2 (Inada et al., 2004), NRL PROTEIN FOR CHLOROPLAST MOVEMENT1 (NCH1/NRL31; Suetsugu et al., 2016), and NRL2 (Sullivan et al., 2009). NRL2 was identified as a phot1-interacting protein through yeast-two hybrid screening (Sullivan et al., 2009). However, its functional role in phot signaling remains undetermined. NPH3 and RPT2 are required for several auxin-mediated growth processes, including phototropism, leaf positioning, and leaf expansion (Christie et al.,

2015). A central role for coordinating auxin distribution also has been linked to other members of the NRL family, which function independently from the phots. For instance, DEFECTIVELY ORGANIZED TRIBUTARIES3/NRL23 is associated with vascular development and leaf vein patterning (Petricka et al., 2008), whereas NRL proteins (NRL6, NRL7, NRL20, NRL21, and NRL30) known as NAKED PINS IN YUCCA (NPY; Fig. 1) function redundantly to regulate auxin movements required for organogenesis and root gravitropism in concert with AGCVIII kinases other than the phots (Cheng et al., 2008; Li et al., 2011). NRL8 (otherwise known as SETH6) is involved in pollen tube growth (Lalanne et al., 2004), which also requires AGC kinases activity (Zhang et al., 2008). Consequently, NRL proteins are proposed to function as AGC kinase modules that regulate various aspects of auxin trafficking and signaling. However, as discussed below, new evidence for the function of NRL proteins in phot signaling has begun to break down the paradigm that these proteins are solely mediators of auxin-dependent processes.

NRL-DEPENDENT AND -INDEPENDENT PHOT RESPONSES

Phots regulate a range of physiological responses in Arabidopsis. These are listed in Table I and can be separated into two categories depending on the involvement of NRL proteins (NRL dependent and NRL independent). Phototropism (Motchoulski and Liscum, 1999; Sakai et al., 2000), petiole positioning and leaf expansion (Inoue et al., 2008b), and chloroplast accumulation (Suetsugu et al., 2016) are examples of NRL-dependent responses, whereas chloroplast-avoidance movement (Kong and Wada, 2014) and stomatal opening (Inoue and Kinoshita, 2017) are NRL independent. While several of these responses can be described as NRL independent, it is worth remembering that functions for 23 members of the NRL family are still lacking. Whether these processes are truly devoid of NRL involvement awaits further genetic characterization of these remaining family members. In the following sections, we summarize progress made to date in uncovering the role of NRL proteins in phot signaling.

NPH3 AND ITS ROLE IN PHOTOTROPISM

A central role for NPH3 in phototropism has been firmly established for some time now. Arabidopsis mutants lacking NPH3 fail to exhibit phototropism under a variety of different light conditions (Liscum et al., 2014; Fankhauser and Christie, 2015). A detailed discussion of phototropism is beyond the scope of this review, and readers are directed to other articles that provide a comprehensive overview of the different types of photophysiological responses involved (Sakai and Haga, 2012; Christie and Murphy, 2013). While the

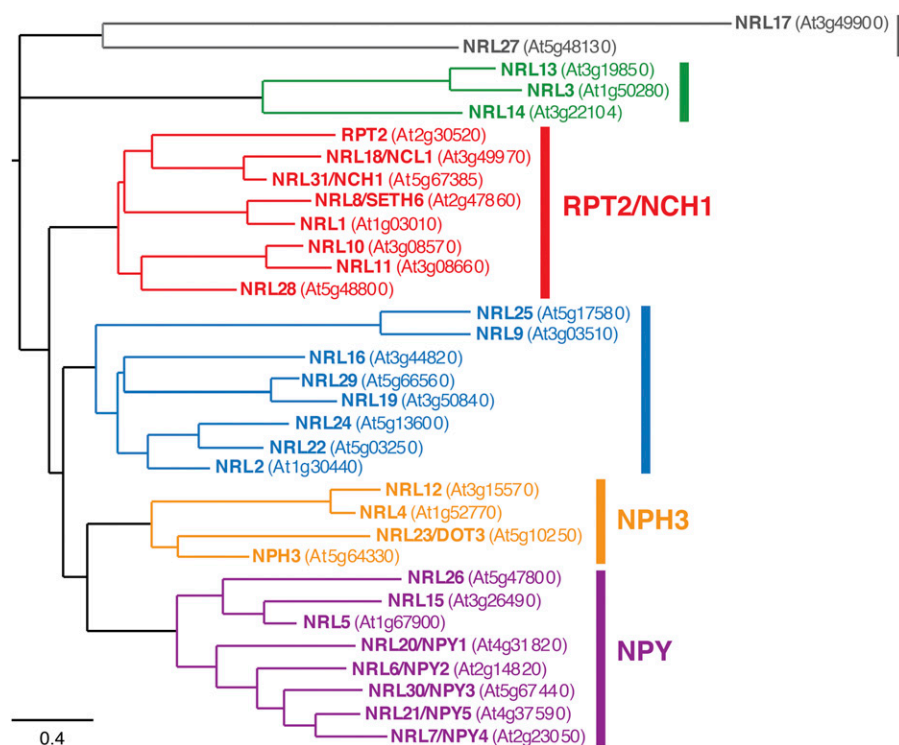


Figure 1. Bayesian phylogenetic tree of the NRL protein family in Arabidopsis. The tree was modified from that of Suetsugu et al. (2016) with Archaeopteryx software. Six of the seven major clades of land plant NRL proteins are color coded. RPT2/NCH1, NPH3, and NPY reside in different clades.

biochemical function of NPH3 remains poorly understood, it appears to be instrumental for establishing lateral auxin gradients required for this differential growth response (Haga et al., 2015). NPH3 is localized to the plasma membrane and has been shown to interact directly with phot1 via its C-terminal region (Motchoulski and Liscum, 1999). Like the phot1s, NPH3 contains no obvious transmembrane-spanning domain, so how it associates with the plasma membrane is still not known. However, truncation and transient expression analysis of *NPH3-GFP* in stomatal guard cells of *Vicia faba* suggests that the C terminus, including the coiled-coil domain, is important for localization to the plasma membrane (Inoue et al., 2008b).

A recent *in vivo* coimmunoprecipitation analysis indicates that phot1-NPH3 interactions at the plasma membrane are transiently disrupted upon irradiation (Haga et al., 2015). Light not only impacts the ability of NPH3 to interact with phot1 but also leads to dynamic changes in its subcellular localization. The expression of *YFP-NPH3* under the control of the constitutive cauliflower mosaic virus 35S promoter restores hypocotyl phototropism in the *nph3* mutant (Haga et al., 2015). *YFP-NPH3* is localized to the plasma membrane in darkness but is rapidly (within minutes) internalized into microdomain aggregates upon phot1 activation by blue light (Haga et al., 2015). The appearance of these microdomains is diminished in periods of prolonged irradiation or when seedlings are returned to darkness, suggesting that their formation is reversible (Haga et al., 2015). The biological significance and the role of these microdomains in phot signaling are currently

unknown. However, biochemical fractionation experiments indicate that these NPH3 aggregates accumulate in the cytosol (Haga et al., 2015). These findings agree with observations that cytoskeleton inhibitors or inhibitors of vesicle trafficking fail to affect NPH3 microdomain formation (Haga et al., 2015).

The above phot1-driven changes in NPH3 localization correlate well with alterations in NPH3 phosphorylation status. NPH3 is phosphorylated in darkness and becomes rapidly (within minutes) dephosphorylated following phot1 activation (Pedmale and Liscum, 2007). This dephosphorylation process is tissue, and most likely cell, autonomous (Sullivan et al., 2016b) and does not appear to be initiated by phot2 (Inada et al., 2004; Pedmale and Liscum, 2007; Tsuchida-Mayama et al., 2008; Haga et al., 2015). As found for *YFP-NPH3* microdomain formation, NPH3 dephosphorylation is recoverable either in darkness (Pedmale and Liscum, 2007) or over periods of prolonged irradiation (Haga et al., 2015). The kinetics for NPH3 dephosphorylation and rephosphorylation correlate closely with the time scale described for the changes in *YFP-NPH3* localization (Haga et al., 2015). This has led to the proposal that the phosphorylation of NPH3 is necessary for it to form an active signaling complex with phot1 at the plasma membrane (Fig. 2). Phot1-NPH3 interactions are disrupted following NPH3 dephosphorylation and its release from the plasma membrane (Haga et al., 2015), leading to a cessation of receptor signaling. Thus, reassembly of this complex would be necessary to reestablish and sustain phototropic signaling, especially under continuous irradiation (Fig. 2).

Table 1. *NRL-dependent and -independent phot-mediated responses in Arabidopsis*

Phot Response	NRL Dependent	NRL Independent	References
Phototropism	Requires NPH3 and RPT2		Motchoulski and Liscum (1999); Sakai et al. (2000)
Petiole positioning	Requires NPH3 and RPT2		Inoue et al. (2008); Harada et al. (2013)
Leaf expansion	Requires NPH3 and RPT2		Inoue et al. (2008); Harada et al. (2013)
Chloroplast accumulation	Requires RPT2 and NCH1 (NRL31)		Suetsugu et al. (2016)
Chloroplast avoidance		Does not require NPH3, RPT2, or NCH1 (NRL31)	Suetsugu et al. (2016)
Nuclear avoidance		Does not require NPH3	Higa et al. (2014)
Stomatal opening		Does not require NPH3, RPT2, or NCH1 (NRL31)	Suetsugu et al. (2016)
Destabilization of <i>Lhcb</i> mRNA	Requires NPH3		Folta and Kaufman (2003)
Inhibition of hypocotyl elongation		Does not require NPH3	Folta and Spalding (2001)
Circadian control of PSII photosynthetic efficiency		Does not require NPH3	Litthauer et al. (2015)

The identity of the kinase(s) responsible for phosphorylating NPH3 is still not known. Moreover, studies have yet to examine whether NPH3 is a substrate for phot1 kinase activity. However, pharmacological studies have implicated a role for a type 1 protein phosphatase in dephosphorylating NPH3 (Pedmale and Liscum, 2007). To date, 18 phosphorylation sites have been identified within NPH3 through global phosphoproteomic approaches (Heazlewood et al., 2008). The contribution of some of these sites to phototropic signaling has been investigated through structure-function analysis. Ser-212, Ser-222, and Ser-236 downstream of the BTB domain are phosphorylated in darkness and become dephosphorylated following phot1 activation (Tsuchida-Mayama et al., 2008). Collective mutation of these sites to Ala or deletion of this region ($\Delta 212-238$) does not adversely affect the ability of NPH3 to mediate hypocotyl phototropism in *Arabidopsis* (Tsuchida-Mayama et al., 2008). Therefore, phosphorylation sites other than Ser-212, Ser-222, and Ser-236 are clearly important (Haga et al., 2015). Identifying these sites and dissecting how changes in their phosphorylation status impact NPH3 function present a formidable challenge for future research. However, recent work has now demonstrated that the second founding member of the NRL family, RPT2, has a role in modulating the phosphorylation status and localization dynamics of NPH3 in *Arabidopsis*.

CONTRIBUTION OF RPT2 TO PHOT SIGNALING

RPT2 was identified originally from a genetic screen for *Arabidopsis* mutants impaired in root phototropism (Okada and Shimura, 1992; Sakai et al., 2000) and has been shown to interact directly with both NPH3 (Inada et al., 2004) and phot1 (Inada et al., 2004; Sullivan et al., 2009). The transposon insertion mutant *rpt2-2* lacks the

RPT2 protein (Inada et al., 2004) and is impaired in hypocotyl phototropism at blue light intensities of $0.17 \mu\text{mol m}^{-2} \text{s}^{-1}$ or greater (Haga et al., 2015). By contrast, the phototropic response of *rpt2-2* mutants is indistinguishable from that of wild-type seedlings at blue light intensities of $0.017 \mu\text{mol m}^{-2} \text{s}^{-1}$ or less (Haga et al., 2015). A requirement for RPT2 in regulating phototropism to higher light correlates well with its expression profile. The expression of *RPT2* is barely detectable in etiolated seedlings but increases in response to red and blue light (Inada et al., 2004; Tsuchida-Mayama et al., 2008; Haga et al., 2015) in a fluence rate-dependent manner (Sakai et al., 2000). As indicated above, reconstitution of a phot1-NPH3 complex is considered necessary to sustain effective phototropic signaling under continuous irradiation (Fig. 2). The presence of RPT2 is necessary to facilitate this process at high light intensities (Haga et al., 2015). Based on these findings, RPT2 is proposed to contribute to photosensory adaption and promote efficient phototropism under brighter light conditions (Fig. 2).

Mutants deficient in either NPH3 or RPT2 exhibit leaf positioning and leaf expansion phenotypes that resemble those of the *phot1 phot2* double mutant (Inoue et al., 2008b; Harada et al., 2013). These findings demonstrate that NPH3 and RPT2 have roles in phot signaling besides phototropism. Leaf positioning and leaf expansion both require differential growth and most likely arise from alterations in auxin distribution. The severity of the *rpt2* phenotype for these responses (Harada et al., 2013) is reduced dramatically under moderate light conditions when crossed with the *phot1* single mutant. This phenotype of the *phot1 rpt2* double mutant has uncovered a level of complexity regarding the contribution of RPT2 to phot signaling. RPT2 acts positively in conjunction with phot1 to mediate leaf positioning and leaf expansion. In contrast, RPT2 is not required for phot2 signaling. Instead, RPT2 appears to have some role in suppressing the ability of phot1 to

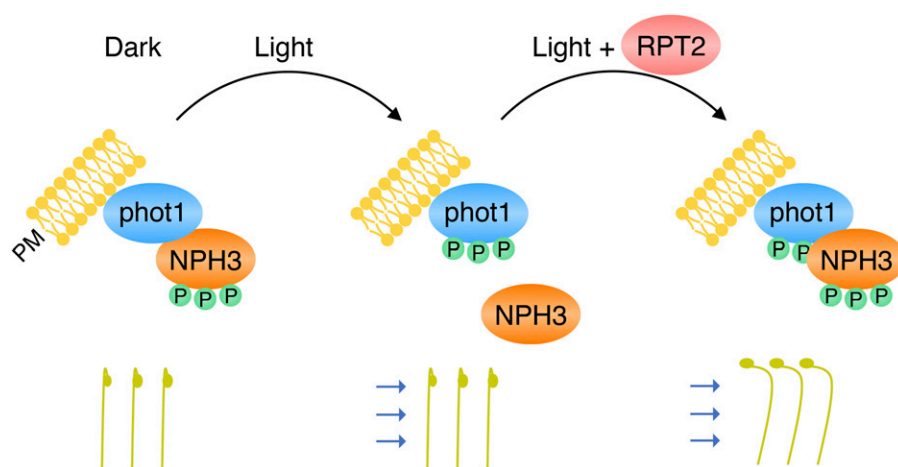


Figure 2. Roles of NPH3 and RPT2 in mediating phototropic signaling in Arabidopsis. The model was modified from Haga et al. (2015), where phot1 and NPH3 form a plasma membrane (PM) complex in darkness. Phot1-NPH3 interactions are diminished upon phot1 activation and dephosphorylation of NPH3. However, efficient signaling under continuous blue light conditions requires the reconstitution of a phot1-NPH3 complex that involves RPT2 at higher blue light intensities.

inhibit phot2 signaling for these responses. In this model (Fig. 3), both phot1 and phot2 signaling pathways would be impaired in the *rpt2* mutant. The phot1 pathway would be inactive, since its signaling is dependent on RPT2, whereas phot2 signaling is impaired, owing to the suppressive action of phot1. Likewise, both phot1 and phot2 signaling pathways would be inactive in a *phot2 rpt2* double mutant. However, phot2 signaling for leaf positioning and leaf expansion still would be viable in this model for the *phot1 rpt2* double mutant consistent with the phenotype observed, since the inhibitory action of phot1 is removed (Fig. 3).

Additional evidence suggests that this complex interplay between phot1 and phot2 signaling may occur for other phot responses. The *rpt2-1* mutant shows impaired hypocotyl phototropism under low and high fluence rates of unilateral blue light (Inada et al., 2004). Phototropic responsiveness was restored at higher fluence rates in the *phot1-101 rpt2-1* double mutant, indicative of functional phot2 signaling. Similarities can be drawn between these results and those observed for the leaf positioning and leaf expansion phenotypes of the *phot1 rpt2* mutant (Harada et al., 2013). However, some caution should be exercised when comparing these findings, given the difference in ecotypes used as well as the apparent leakiness of the *rpt2-1* mutant (Haga et al., 2015).

PHYTOCHROME KINASE SUBSTRATE PROTEINS

NPH3 and RPT2 are not the only mediators of phototropism, leaf positioning, and leaf expansion in Arabidopsis. PHYTOCHROME KINASE SUBSTRATE proteins (PKS1–PKS4) also play a role in establishing these processes. PKS1 was identified originally as a kinase substrate for phytochrome A (phyA; Fankhauser et al., 1999). Whether phyA is bona fide protein kinase is still debated, although recent work has offered renewed support for its role as a protein kinase in regulating photomorphogenesis (Shin et al., 2016). While PKS proteins are required for phytochrome signaling

(Lariguet et al., 2003; Schepens et al., 2008), some of the phenotypes associated with *pks* mutants resemble those of *nph3*, *rpt2*, and phot-deficient mutants. For instance, the *pks1 pks2 pks4* triple mutant exhibits reduced phototropic curvature (Lariguet et al., 2006; Kami et al., 2014). PKS1 and PKS2 also act in concert with NPH3 to establish leaf positioning and leaf expansion (de Carbonnel et al., 2010). Their role in phot signaling appears to be restricted to these processes, since *pks* mutants are not impaired in blue light-induced stomatal opening and chloroplast photorelocation movement (de Carbonnel et al., 2010). Consistent with their involvement in phot signaling, PKS proteins are known to localize to the plasma membrane, where they are known to associate with phot1, phot2, and NPH3 (Lariguet et al., 2006; de Carbonnel et al., 2010; Demarsy et al., 2012). Furthermore, studies have shown that PKS4 is a substrate target for phot1 kinase activity (Demarsy et al., 2012). Together, these data clearly demonstrate that PKS proteins are integral components of the phot signaling pathways associated with NPH3 and RPT2. Resolving the biochemical functions of these proteins and how they integrate with NPH3 and RPT2 will be important to fully understand how phot1 initiate signaling from the plasma membrane. Like NPH3, PKS proteins are suggested to modulate auxin transport or signaling (de Carbonnel et al., 2010; Kami et al., 2014). Yet, their mechanism of action in this regard remains to be elucidated.

NCH1 AND RPT2 ARE MEDIATORS OF CHLOROPLAST ACCUMULATION MOVEMENT

Phot-induced chloroplast accumulation movement was shown recently to involve the coaction of two NRL proteins. At low blue light intensities, phot1 and phot2 overlap in function to direct chloroplast relocation to the upper and lower cell surfaces to maximize light capture for photosynthesis (Sakai et al., 2001). Chloroplast-avoidance movement is mediated solely by phot2 (Kagawa et al., 2001) and serves to prevent

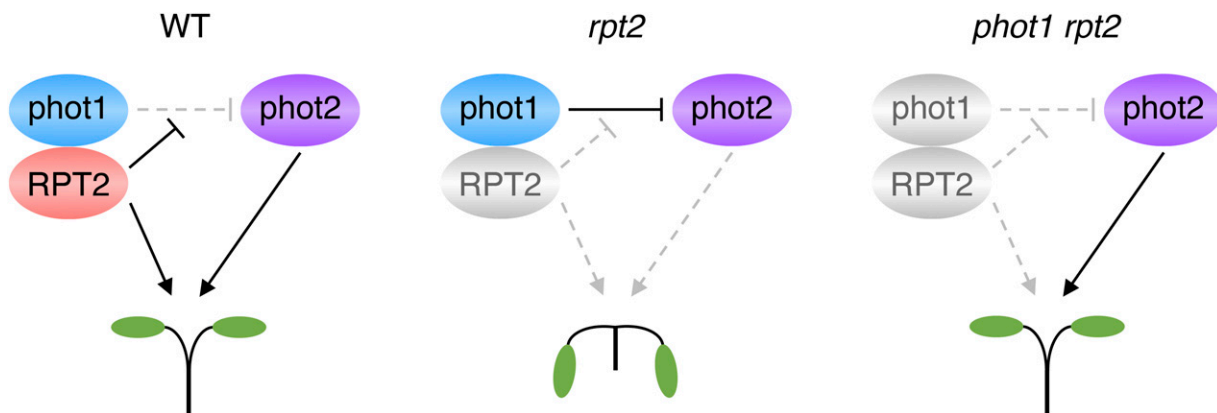


Figure 3. Functional complexity for RPT2 in controlling phot-mediated leaf positioning in Arabidopsis. The model was adapted from Harada et al. (2013). Solid black lines/arrows illustrate the signaling pathways operational in wild-type (WT) plants and mutant backgrounds. Nonoperational pathways in each of the genotypes are grayed out for clarity.

photodamage of the photosynthetic apparatus in excess light (Kasahara et al., 2002). Chloroplast movement is achieved by the formation of chloroplast actin (cp-actin) filaments that polymerize on the leading edge of the chloroplast (Kadota et al., 2009). Polymerization of cp-actin requires the action of CHLOROPLAST UNUSUAL POSITIONING1 (CHUP1; Oikawa et al., 2003) and KINESIN-LIKE PROTEIN FOR ACTIN-BASED CHLOROPLAST MOVEMENT (KAC) proteins (Suetsugu et al., 2010). PLASTID MOVEMENT IMPAIRED1 (PMI1) also is required to stabilize cp-actin filaments (Suetsugu et al., 2015). Consequently, cp-actin formation, and thus chloroplast accumulation and avoidance movement, are impaired in *chup1*, *pmi1*, and *kac1 kac2* mutants.

How light perception by phot1 and phot2 is coupled to the activity of CHUP1, PMI1, and KAC proteins is not known. At least for chloroplast accumulation movement, the NRL proteins NCH1 (NRL31) and RPT2 have been shown to function as key mediators of this response (Suetsugu et al., 2016). NCH1 and RPT2 reside within the same clade of the NRL family tree in land plants (Fig. 1). Both *nch1* and *rpt2* mutants exhibit a weaker chloroplast accumulation response compared with wild-type plants (Suetsugu et al., 2016). However, this process is abolished completely in the *rpt2 nch1* double mutant, indicating that NCH1 functions redundantly with RPT2 to mediate chloroplast accumulation movement (Suetsugu et al., 2016). Moreover, chloroplast-avoidance movement is unaltered in the *rpt2 nch1* double mutant, demonstrating that NCH1 and RPT2 are only necessary for the accumulation response. While these findings have uncovered a new function for NRL proteins in phot signaling, they also demonstrate that RPT2 plays a central role in establishing several different phot responses. RPT2 could possibly mediate additional aspects of phot signaling in conjunction with other members of the NRL family. Further genetic analysis of the NRL family in combination with the *rpt2* mutant will be required to shed light on this possibility.

The discovery of NCH1 and its role in chloroplast accumulation movement has now changed our view of how different NRL proteins contribute to phot1 and AGCVIII kinase signaling. Chloroplast photorelocation movement is cell autonomous and occurs independently of gene expression (Kong and Wada, 2016; Wada, 2016). Thus, auxin trafficking and auxin-mediated transcription are not required for this processes, in contrast to multicellular responses like phototropism, leaf positioning, and leaf expansion. To our knowledge, these findings are the first to demonstrate that NRL proteins can function with AGCVIII kinases to elicit responses independently of auxin.

BIOCHEMICAL FUNCTION OF NRL PROTEINS

Molecular genetic analysis in Arabidopsis has been successful in identifying physiological functions for almost one-third of the NRL family. Nevertheless, it is still not clear how these proteins operate at the biochemical level. The majority of NRL proteins contain a BTB domain that can function as a substrate adaptor to recruit specific proteins for ubiquitination and degradation (Hua and Vierstra, 2011). Indeed, NPH3 has been reported to interact with CULLIN3a (CUL3a) when coexpressed in insect cells and may form part of the CUL3 RING E3 UBIQUITIN LIGASE (CRL3) complex that directs the ubiquitination of signaling targets (Roberts et al., 2011). Biochemical and mass spectrometry analyses have shown that phot1 becomes ubiquitinated by a CRL3^{NPH3} complex following blue light activation (Deng et al., 2014). CRL3^{NPH3}-mediated polyubiquitination of phot1 is associated with its degradation in response to prolonged irradiation, whereas monoubiquitination is proposed to stimulate the rapid trafficking of phot1 from the plasma membrane in response to low light intensities (Roberts et al., 2011). Whether this

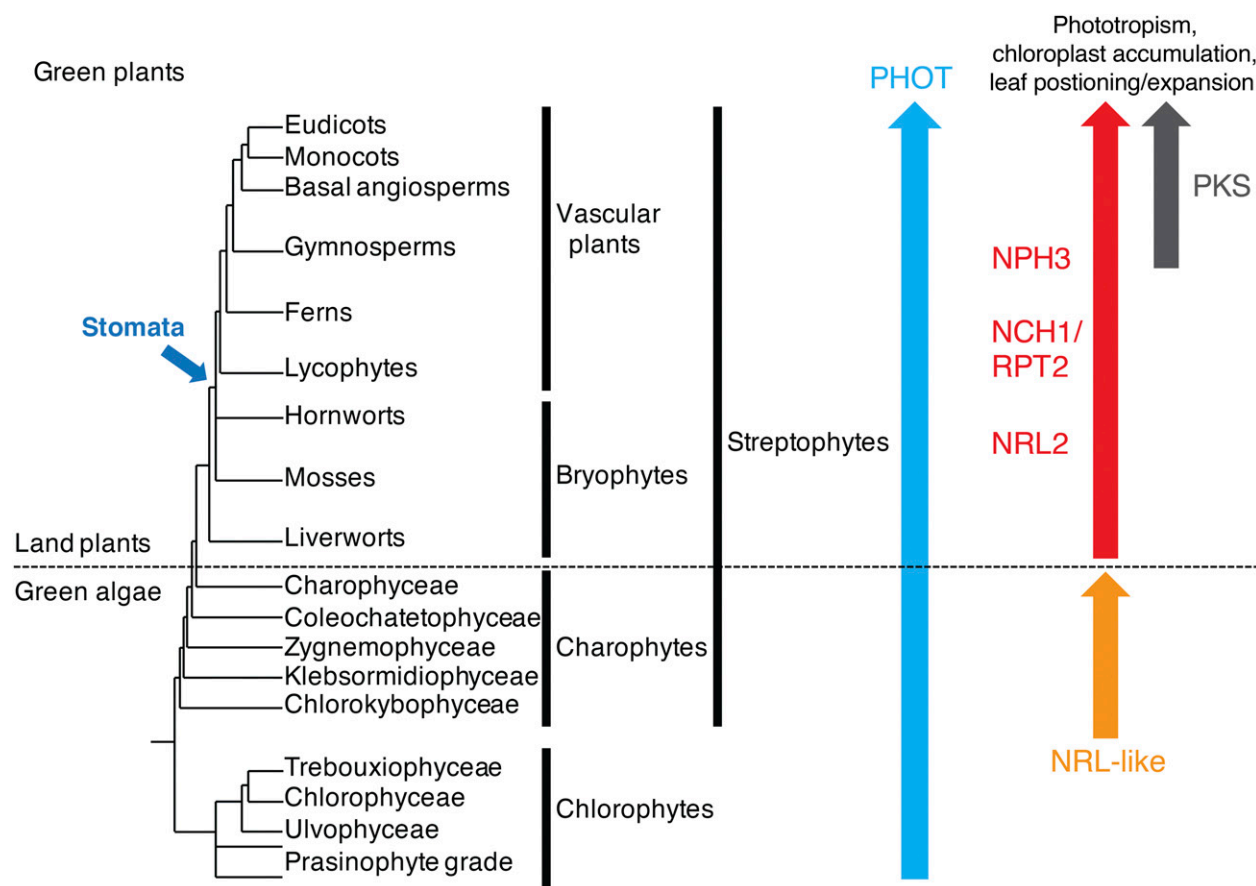


Figure 4. Schematic representation depicting the evolution of phot signaling components in the green plant lineage. The topology of the lineages is derived from Bowman et al. (2007). Arrows indicate the lineages in which orthologs of PHOT, NRL, and PKS proteins were identified from genomic and/or transcriptomic data. Stomata evolved after hornwort diversification.

light-activated change in subcellular localization plays a role in phot1 signaling is still open to question (Liscum, 2016). Recent approaches aimed at tethering phot1 to the plasma membrane by myristoylation or farnesylation suggest this not to be the case, as incorporation of these modifications severely diminishes the light-induced internalization of phot1 without impacting its functionality in *Arabidopsis* (Preuten et al., 2015). So far, no other targets besides phot1 have been identified for the CRL3^{NPH3} complex. Yet, it is worth noting that phot1-driven changes in subcellular trafficking of the auxin efflux carrier PIN2 are dependent on NPH3 (Wan et al., 2012). Since dynamic adjustments in PIN2 distribution and turnover have been linked to the ubiquitination of Lys-63 (Leitner et al., 2012), it is tempting to speculate that auxin transporters could be CRL3^{NPH3} targets. That said, there is currently no published evidence indicating that NPH3 can interact physically with auxin transport proteins.

NPH3 is not the only NRL protein to exhibit features of a CRL3 substrate adaptor. NCH1 (NRL31) was identified previously as AtSR1 INTERACTING PROTEIN1 (SR1IP1; Zhang et al., 2014). SR1IP1 and CUL3a interact

when transiently coexpressed in *Nicotiana benthamiana*. SR1IP1 is responsible for ubiquitination and subsequent degradation of the *Arabidopsis* Ca²⁺/calmodulin-binding transcription factor SIGNAL RESPONSIVE1 (AtSR1), which functions to suppress plant defense responses to bacterial pathogens. Consequently, *sr1ip1* mutants are more susceptible to *Pseudomonas syringae* infection, indicating that SR1IP1 serves as a positive regulator of plant immunity (Zhang et al., 2014). By contrast, virus-induced gene silencing of SR1IP1 orthologs in *N. benthamiana* reduces susceptibility to *Phytophthora infestans* infection, suggesting that SR1IP1 also can act as a negative regulator of plant defense (Yang et al., 2016). Clearly, more work is needed to elucidate the role of SR1IP1 (NCH1) in plant immunity and how this function integrates with its role in regulating chloroplast accumulation movement (Suetsugu et al., 2016). Chloroplasts can aid the transport of pro-defense signals to the nucleus via highly dynamic connections known as stromules (Caplan et al., 2015). Therefore, it will be of interest to examine whether NRL proteins such as NCH1 are involved, especially since light is known to impact stromule formation (Gray et al., 2012; Brunkard et al., 2015).

OUTSTANDING QUESTIONS

- How do NPH3 and RPT2 coordinate auxin fluxes for phototropism?
- How do the dynamic changes in NPH3 localization and phosphorylation contribute to its action?
- Do NRL proteins besides NPH3 and NCH1 exhibit E3 ubiquitin ligase activity and what are their substrates?
- How do PKS proteins integrate with NPH3 and RPT2 to mediate phot signaling?
- How do NCH1 and RPT2 drive cp-actin polymerization required for chloroplast accumulation movement?
- What is the function of the phot1-interacting protein NRL2?

EVOLUTIONARY CONSERVATION OF PHOT SIGNALING

Phots are restricted to the green plant lineage from photosynthetic algae to flowering plants (Fig. 3), but they are absent from red algae (Li et al., 2015). In contrast to land plants, green algae typically contain one phot (Huang et al., 2002; Prochnik et al., 2010; Sullivan et al., 2016), although two phots have been identified in Zygnematales including *Mougeotia scalaris*, both of which might function to mediate chloroplast photorelocation movement in this alga (Suetsugu et al., 2005; Li et al., 2015). Several functions have been ascribed to phot from *Chlamydomonas reinhardtii*. These include the light regulation of various aspects of the sexual life cycle (Huang et al., 2002; Huang and Beck, 2003; Ermilova et al., 2004), the control of eyespot development and phototactic behavior (Trippens et al., 2012), and the transcriptional regulation of enzymes involved in chlorophyll and carotenoid biosynthesis (Im et al., 2006). More recently, *C. reinhardtii* phot was shown to be pivotal for modulating the abundance of key molecular effectors required for photoprotection of the photosynthetic machinery. As a result, the phot-deficient mutants of *C. reinhardtii* displays reduced fitness under excessive light conditions (Petroutsos et al., 2016).

Despite its divergent functions, *C. reinhardtii* phot can successfully restore phototropism, chloroplast photorelocation movement, and stomatal opening when expressed in the *phot1 phot2* double mutant of Arabidopsis (Onodera et al., 2005). These findings suggest that the mode of action between plant and algal phots is highly conserved. However, recent studies centered on phot from the marine picoalga *Ostreococcus tauri* are not consistent with this conclusion. While *O. tauri* phot is capable of mediating chloroplast accumulation movement, stomatal opening, as well as leaf positioning and

leaf expansion when expressed in the *phot1 phot2* double mutant of Arabidopsis, it fails to restore phototropism and chloroplast-avoidance movement (Sullivan et al., 2016a). At least for phototropism, this lack of functionality correlates with the inability of *O. tauri* phot to complex with NPH3 (Sullivan et al., 2016a). These findings fall more in line with what is known regarding the evolutionary conservation of NPH3 and other NRL proteins involved in phot signaling. NPH3, RPT2, and NCH1 are prevalent in land plants, including liverworts and mosses (Suetsugu et al., 2016), but they have not been identified in green algae (Fig. 4). The presence of these NRLs in land plants would coincide with their cooption to modulate multicellular (e.g. phototropism) as well as cell-autonomous (e.g. chloroplast movement and stomatal opening) responses.

Chloroplast photorelocation movement is prevalent in both land plants and green algae. The liverwort *Marchantia polymorpha* contains seven NRL proteins, one protein for each of the seven NRL clades (Suetsugu et al., 2016). The ortholog of NCH1 is essential for chloroplast accumulation movement in *M. polymorpha* but not for the avoidance response, indicating that the signaling events underlying chloroplast photorelocation movements are conserved in land plants (Suetsugu et al., 2016). In contrast to Arabidopsis, *M. polymorpha* contains one representative ortholog for the NPH3 clade and one for the NCH1/RPT2 clade, suggesting that the divergence of these NRL proteins occurred early in land plant evolution (Suetsugu et al., 2016). PKS proteins, by comparison, appear to have evolved later than NPH3 and NCH1/RPT2 in the land plant lineage (Fig. 4). CHUP1, KAC, PMI1, and NRL proteins are present in Streptophytes (including land plants and charophytes) and are essential factors for driving chloroplast photorelocation movement (Suetsugu and Wada, 2016). However, no orthologs of these genes have been found in chlorophytes (including *C. reinhardtii* and *O. tauri*). Thus, NRL-mediated chloroplast movement may have been acquired during evolution of the streptophyte lineage. Yet, the charophyte alga *Klebsormidium flaccidum* (charophyte) does contain two NRL-like proteins (Suetsugu et al., 2016). It is possible that these proteins mediate chloroplast photorelocation in this organism. Clearly, more work is needed to determine how light-induced chloroplast movement is achieved in photosynthetic algae.

CONCLUSION AND FUTURE PERSPECTIVES

Molecular genetic analysis in Arabidopsis has been instrumental in shedding light on the biological functions of NRL family members. Work so far has coordinated their mode of action with AGCVIII kinases and auxin signaling. Studies have now shown that NRL proteins can mediate signaling independently of auxin, at least in the case of chloroplast accumulation movement. A major challenge for future research will be to elucidate how NRL proteins function at the biochemical

level and how changes in their localization and phosphorylation status can impact their activity. At least 24 out of the 33 NRL members are known to be phosphorylated (Heazlewood et al., 2008). Unlike NPH3 and NCH1, RPT2 does not exhibit E3 ubiquitin ligase activity *in vitro* (Roberts et al., 2011). Nor does its plasma membrane localization change in response to blue light treatment (Haga et al., 2015). Hence, the biochemical and localization properties for specific NRL family members are clearly different. Resolving the biochemical functions of NPH3 and RPT2 and their role in phot signaling will be essential if we are to understand how auxin gradients are established to promote responses such as phototropism, leaf positioning, and leaf expansion. NRL2 is known to interact with phot1 (Sullivan et al., 2009), but its biological role in phot signaling awaits additional molecular and genetic characterization. Further functional dissection of the Arabidopsis NRL family will be a demanding task, given the large size of this protein family and the redundancy between members. The reduced size and complexity of the NRL family in *M. polymorpha* now provides a tractable alternative to assigning functions to each of seven NRL clade classifications found in land plants.

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