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PKS2: A LINK BETWEEN PHOTOTROPIN SIGNALLING AND AUXIN TRANSPORT
-
A STUDY ON HOW PLANTS SENSE AND RESPOND TO LIGHT

Thèse de doctorat ès sciences de la vie (PhD)

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pour Le Doyen
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SUMMARY

The blue light photoreceptors phototropins (phot1 and phot2 in *Arabidopsis thaliana* (L.)) carry out various light responses of great adaptive value that optimize plant growth. These processes include phototropism (the bending of an organ induced by unequal light distribution), chloroplast movements, stomatal opening, leaf flattening and solar tracking. The biochemical pathways controlling these important blue light responses are just starting to be elucidated. The PHYTOCHROME KINASE SUBSTRATE (PKS1-4) proteins – the subject of this research – have recently been identified as novel phototropism signalling components. PKS1 (the founding member of this family) interacts in a same complex *in vivo* with phot1 and the important phot1 signalling element NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3). This suggested that the PKS may act as early components of phot signalling. This work further investigates the role of this protein family during phototropin signalling

Genetic experiments clearly showed that the PKS do not control chloroplast movements or stomatal opening. However, PKS2 plays a critical role with NPH3 during leaf flattening and solar tracking. Epistasis data indicated that both proteins act in phot1 and phot2 pathways, which is consistent with their *in vivo* interaction with both phototropins. Because phototropism, leaf flattening and solar tracking are developmental processes regulated by the hormone auxin, the role of PKS2 and NPH3 during auxin homeostasis was also investigated. Interestingly, *PKS2* loss-of-function restores leaf flattening in the auxin transporter mutant *aux1*. Moreover, PKS2 and NPH3 are found in a same complex with AUX1 *in vivo*.

Taken together, these results suggest that PKS2 may act with NPH3 as a connecting point between phot signalling and auxin transport. Further experiments were performed to explore the molecular mode of action of PKS2 and NPH3 in this process. The significance of these results is discussed.

RESUME

Les plantes possèdent des photorécepteurs à la lumière bleue appelés phototropines (phot1 et phot2 chez *Arabidopsis thaliana* (L.)). Ces phototropines contrôlent de nombreux procédés qui permettent à la plante d'optimiser son développement dans un environnement lumineux défavorable. Ces réponses incluent le phototropisme, le mouvement des chloroplastes, l'ouverture des stomates, l'aplanissement et l'héliotropisme des feuilles. Les voies de signalisation qui régulent ces procédés en réponse à la lumière bleue sont encore peu connues. Récemment nous avons identifié les protéines PHYTOCHROME KINASE SUBSTRATE (PKS1-4 dans *Arabidopsis*) comme de nouveaux éléments participant au contrôle du phototropisme. En particulier, PKS1 forme un complexe *in vivo* avec phot1 et NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3), un composant de la voie phot1. Les PKS agiraient précocement dans les voies de signalisation des phototropines. Le but de ce travail de recherche est d'analyser l'implication des membres de cette famille dans la signalisation des phototropines.

Les expériences génétiques ont montré que les PKS ne sont impliquées ni dans le mouvement des chloroplastes ni dans l'ouverture des stomates. En revanche, PKS2 joue un rôle critique avec NPH3 pour diriger l'aplanissement et l'héliotropisme des feuilles. Les données d'épistasie indiquent que ces deux gènes agissent dans les voies de signalisation de phot1 et phot2. Ceci est corrélé au fait que PKS2 et NPH3 sont associées avec phot1 et phot2 *in vivo*. Étant donné que l'hormone auxine est un régulateur central du phototropisme, de l'aplanissement et de l'héliotropisme des feuilles, nous avons recherché s'il existait un lien entre PKS2 et cette hormone. PKS2 et NPH3 font partis d'un complexe avec le transporteur d'auxine AUX1 in vivo. Alors qu'un mutant *aux1* présente des feuilles courbées, l'introduction de la mutation *pks2* dans ce mutant rétablit le phénotype sauvage, ce qui laisse à penser que PKS2 contribuerait au développement de la feuille à travers le transport d'auxine.

L'ensemble de ces données suggère donc que PKS2 ferait le lien entre les phototropines et le transport d'auxine. Des analyses moléculaires et biochimiques pour décortiquer le mode de fonctionnement de PKS2 ont été initiées et les résultats préliminaires sont présentés.

Projet de thèse de:
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Laboratoire du prof. Christian Fankhauser

Titre:
La protéine végétale PKS2 est un régulateur important de l'orientation des feuilles en réponse à la lumière.

Résumé simplifié:

Chacun d'entre nous sait qu'une plante placée à proximité d'une fenêtre redirige sa croissance vers la source lumineuse. Cette réponse est appelée photo-tropisme (du Grec « photo » - lumière, et « tropisme » - tourner). Les plantes utilisent cette stratégie pour orienter leurs feuilles afin d'optimiser la photosynthèse (mécanisme générant de l'énergie biologique à partir de la lumière).

Le phototropisme peut être séparé en deux grandes étapes: d'une part la perception de la direction de la lumière, d'autre part la courbure de la tige. La première étape est accomplie par des senseurs (ou photo-récepteurs) appelés phototropines. Leur rôle est de convertir l'information lumineuse en signal biologique. Les signaux émis par les phototropines agissent sur le transport au niveau de la tige d'une hormone très importante appelée auxine. Cette dernière coordonne ensuite la croissance asymétrique des cellules ce qui induit mécaniquement une courbure de la tige. Plusieurs décennies de recherche ont permis d'obtenir une description moléculaire détaillée du mode de fonctionnement des phototropines et de l'auxine. En revanche, il n'existe que peu d'informations sur les relais de signalisation intermédiaires.

Notre laboratoire étudie une petite famille de protéines : les PKS (Phytochrome Kinase Substrate). Nous avons récemment montré que certains PKS sont des messagers importants de l'information transmise par les phototropines lors du phototropisme de la tige. Dans ce travail de thèse, nous avons découvert que la protéine PKS2 est aussi un élément de signalisation lors de l'aplanissement et du phototropisme des feuilles (deux autres procédés contrôlés par les phototropines). De plus, nous avons montré que PKS2 influence le transport de l'auxine. Les PKS représentent donc un nouveau lien entre les phototropines et l'auxine. Etant donné la position centrale des PKS dans la régulation du phototropisme en général, une future caractérisation détaillée de ces protéines contribuera grandement à la compréhension moléculaire de ce phénomène important pour la survie des plantes.

FOREWORD

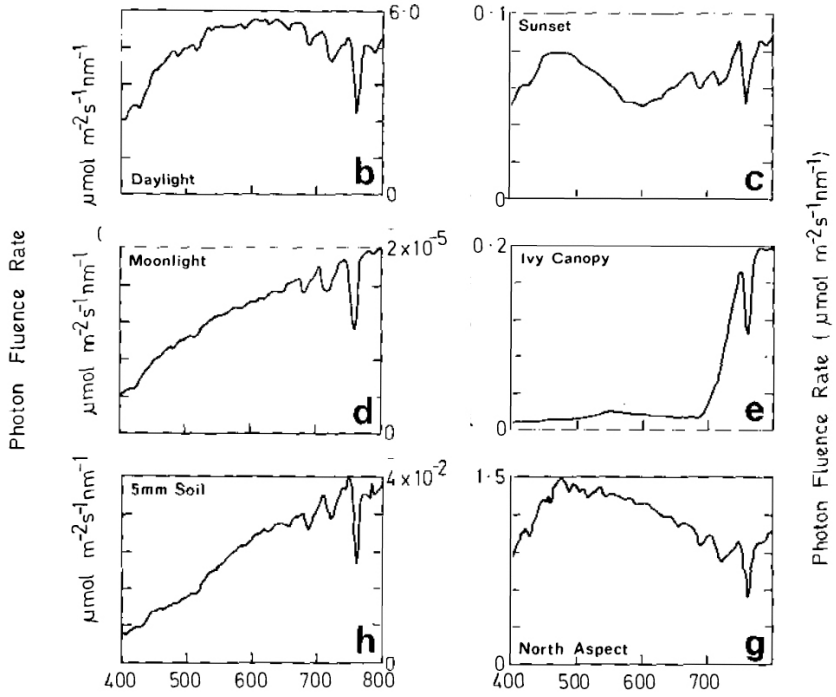
Humans have always been fascinated by the capacity plants have to adapt and survive in a hostile and continuously changing environment. To compensate for their sessile lifestyle, plants have evolved many strategies that allow them to adapt to environmental changes. For instance, plants display extraordinary plasticity in shape and behaviour, as well as strong potential of regeneration after damage.

Light is a particularly important environmental cue because plants directly depend on solar radiation for growth and reproduction. “How light influences plant shape “– for example during phototropism (the bending of an organ away or towards light) – is a question which already at the time of the Greeks has interested the human mind. In these early days, poets, philosophers and herbalists viewed plants as passive organisms on which light solely had a magical or physical effect. However, with the advent of the renaissance, the notion of plant sensitivity emerged and scientists started to ponder on the inductive nature of the response. In other words, “how do plants perceive light” (Whippo and Hangarter, 2006).

During their studies on plant movements, Charles Darwin and his son Francis made a discovery which may be seen today as the origin of photomorphogenesis: the connection between light perception and light response. During phototropism experiments on the canary grass stem, they found that the site of photoperception at the shoot tip was separable from the location of curvature in lower regions of the stem. From this observation, the Darwins proposed that a transmissible substance produced at the tip “influenced” curvature in the lower part of the stem (Darwin and Darwin, 1880). This insightful discovery eventually led to the discovery of the first plant hormone, auxin.

Over the last century - and particularly since the use of the model organism *Arabidopsis* as a powerful tool for genetic and molecular research – molecular mechanisms have emerged and are starting to explain at a mechanistic level how plants sense and respond to light. The present work belongs to this general theme.

GENERAL INTRODUCTION



	µmol $m^{-2}s^{-1}$	R:FR
Daylight	1900	1.19
Sunset	26.5	0.96
Moonlight	0.005	0.94
North aspect	480	1.3
Ivy canopy	17.7	0.13
5 mm soil	8.6	0.88

Figure 1: Aspects of the natural light environment illustrated by spectroradiometer scans of the 400-800 nm waveband. (adapted from Harry Smith (1982)).

Light irradiance (i.e. photon flux density, or photon fluence rate) is expressed as micromoles of photons per square meter per second ($\mu mol.m^{-2}.s^{-1}$).

Upper graphs show the measured irradiance corresponding to different wavelengths of photons. Wavelengths between 400 and 500 nm include blue light (BL) while wavelengths between 600 and 750 nm comprise red (RL) and far-red (FRL) light. A description of each graph is in the text.

Lower table shows (i) total fluence rate over the whole 400-800 nm waveband scanned by spectroradiometer ($\mu mol.m^{-2}.s^{-1}$) and (ii) the ratio of R to FR fluence rates (R:FR).

1. Plants continuously monitor the light in their environment

1.1. Light is a crucial source of information for the plant

Sunlight is filtered by the atmosphere before reaching the Earth's surface. High-energy short-wave radiation is absorbed by ozone, while long-wave radiation is stopped by water vapor. (Gates, 1966). The terrestrial photon flux ("light intensity") is greatest between 400nm and 800nm and corresponds to a waveband that induces most biological responses to light. A typical spectral distribution of daylight under a clear midsummer sky is shown in Figure 1b (Smith, 1982). The quality of light can change enormously depending on the environment a plant is located in. For instance, a plant growing under a canopy or a germinating seed under a few millimeters of soil will be exposed to light strongly depleted in blue light (BL) and highly enriched in far-red light (FRL) (Figures 1e and 1h). On the other hand, under sunset and in regions that do not receive direct solar radiation (scattered skylight on a northern hillside for example – "north aspect"), plants will receive much more BL (Figure 1c and 1g). To acquire information on their environment, plants can sense these different wavelengths of light and their relative intensities (Smith, 1982). The ratio of red light (RL) to FRL photon flux is a particularly good example of the extreme differences in light a plant may receive (Figure 1, lower table). Finally, plants are also exposed to photoperiodism and have evolved strategies to use and respond to this other variable. This is particularly visible in the seasonality of flowering and bud dormancy (Smith, 1982). Thus, plants are exposed to a tremendous diversity of light environments during their lifetime. This important environmental cue is a source of information that is used by the plant as an indicator of time and place. To do so, plants possess several types of photo-receptors that confer them the capacity to perceive light wavelength, intensity and diurnal fluctuations (Lin, 2000; Neff et al., 2000; Christie and Briggs, 2001; Chen et al., 2004; Folta and Maruhnich, 2007; Jenkins, 2009).

1.2. Plants use photo-receptors to sense and respond to light

In classical photobiology experiments, investigators have used coloured glass and coloured solutions to measure the action spectra of different light responses (i.e. the magnitude of the plant response as a function of wavelength). By these means, phototropism was shown to be induced by the blue region of the spectrum (Sachs, 1887). Germination of lettuce seeds was shown to be stimulated by RL and inhibited by FRL (Flint, 1936; Borthwick et al., 1952). These early photobiology experiments inferred the existence of different sets of light sensors, and provided first descriptions of important biological properties of these photoreceptors (Sage, 1992). The observation that different light responses followed similar action spectra indicated that similar photoreceptors could control different types of processes. For instance phototropism, chloroplast relocations (chloroplasts in leaf cells move to enhance light capture or avoid excessive light) and stomatal opening (pores that control gas exchanges between the plant and the atmosphere) are BL-induced processes that were believed to be controlled by a common BL photoreceptor (Gabrys and Walczak, 1980; Short, 1994; Quinones et al., 1996). Similary, several different processes were shown to be controlled by common photoreceptors sensing RL and FRL (Parks, 2003).

The first photoreceptor discovered was the protein pigment phytochrome that absorbs RL and FRL most strongly, but also BL. Decades of photobiology experiments progressively characterized the biological properties of phytochrome (Sage, 1992). The protein was eventually isolated biochemically from plant extracts and *in vitro* studies confirmed that it was indeed phytochrome (Butler et al., 1959). A candidate BL light photoreceptor was also identified using a combination of photochemical, biochemical and physiological experiments but the molecular identity of the photoreceptors still remained elusive until the use of the genetic model *Arabidopsis thaliana* (L.) (Gallagher et al., 1988; Reymond et al., 1992; Palmer et al., 1993) .

Arabidopsis has proved an extremely powerful tool for the genetic and molecular study of plant physiology. Forward genetic screens have led to the isolation of mutants impaired in specific light responses and have uncovered many genetic loci controlling associated processes (Huala et al., 1997; Weigel and Glazebrook, 2002). Map-based positioning of these mutations has enabled the identification and cloning of many genes underlying the light responses (Khurana and Poff, 1989; Liscum and Hangarter, 1991; Liscum, 1994; Briggs and Liscum, 1997). Subsequent molecular and biochemical studies on the corresponding proteins have characterized several photoreceptors, including the cryptochromes and the phototropins (Banerjee and Batschauer, 2005).

Photoreceptors in *Arabidopsis* are classified into different families: the R and FR sensing phytochromes (phyA-E) (Sharrock and Quail, 1989; Franklin et al., 2005), and the UV-A/BL sensing phototropins (phot1-2) (Briggs et al., 2001; Christie, 2007), cryptochromes (cry1-3) (Lin and Shalitin, 2003) and ZTL/ADO (Schultz, 2005) family. Phots, crys and ZTL/ADO proteins all bind flavin-type chromophores (pigments) that confer them absorption properties in the UV-A/BL range of the spectrum (320-500nm) (Christie et al., 1999; Imaizumi et al., 2003). Crys also bind pterin or deazaflavin chromophores (Sancar, 2003). Phys covalently bind phytochromobilin (a linear tetrapyrrole molecule) which absorbs light mainly in the R/FR region of the light spectrum (600-750 nm) (Quail, 1997). The polypeptide component of the photoreceptor is called the apoprotein and is written in caps (e.g. PHOT1). The apoprotein bound to its chromophore is called holoprotein and is written in small letters (e.g. phot1). Hereafter I refer to the holoprotein.

Light excitation of the chromophore induces chemical reactions between the pigment and the reactive sensory domain of the protein (signal input). These reactions lead to conformational changes of the protein and activation of biochemical cascades *via* output signaling domains (signal output). Photoreceptor proteins also contain many regulatory domains that modulate this process. Interestingly, absorption spectra of photoreceptors can change upon light excitation by light of a specific wavelength. For instance, phys exist in two interconvertable conformers: an R-absorbing Pr form and a FR-absorbing Pfr (Quail, 1997). Photoconversion between Pr and Pfr occurs upon

FRL and RL absorption, respectively. Crys also undergo photoconversion induced by blue and green light (Bouly et al., 2007). These photochemical switches confer one type of photoreceptor the ability to induce different physiological responses according to the relative intensities of two specific light wavelengths. The best example is the perception of R:FR ratio by phys which is particularly variable and provides essential environmental information to the plant (Figure 1 lower table). The detection of these subtle changes of light composition by a single photoreceptor may be regarded as color vision by the plant.

The physiological responses mediated by these different photoreceptor families are diverse. Phys, crys and zeitlupe-like photoreceptors appear to control mainly plant morphogenesis processes such as germination, seedling establishment and flowering (Neff et al., 2000; Chen et al., 2004; Kim et al., 2007). Phototropins are rather specialized in rapid adaptive movement responses of the plant such as phototropism, chloroplast relocation and stomatal opening (Briggs and Christie, 2002). The shade avoidance response is also an important adaptive process controlled by the phys (Ballare, 1999).

Other flavin-binding proteins occur in *Arabdidopsis*, suggesting that as-yet uncharacterized BL receptors exist (Crosson et al., 2003; Demarsy and Fankhauser, 2009). UV-B (280-320 nm) also triggers developmental responses such as inhibition of hypocotyl elongation and transcriptional regulation and this is probably mediated by a UV-B photoreceptor (e.g. UVR8) (Jenkins, 2009). Finally, green light has discrete effects on plant physiology that can be attributed to known and also putative uncharacterized sensory systems (Bouly et al., 2007; Folta and Maruhnich, 2007).

During my thesis I have focused on the UV-A/BL photoreceptors phototropins.

1.3. Photoreceptor signaling networks

Once a plant has acquired environmental information it must interpret it and coordinate the adequate physiological responses. The role of photoreceptors is to translate light information into biological information. Signals emitted by photoreceptors are transduced and integrated inside the cell by signaling components. One role of these signaling pathways is to regulate the activity of specific targets that in turn carry out the physiological response. Genetic and molecular approaches using *Arabidopsis* have uncovered many signaling elements, and molecular mechanisms underlying light signaling are being elucidated. (Deng and Quail, 1999; Lin, 2002; Gyula et al., 2003; Jiao et al., 2007). Our interests lie in phot and phy signaling.

Phys are synthesized in the dark as soluble cytoplasmic proteins. Upon light activation, phys are known to trigger cytosolic events such as ion conductance of plasma membrane channels and the regulation of actin-based cytoplasmic motility (Kim et al., 1993; Takagi et al., 2003). Phys also interact with cytoplasm-localized proteins such as NDPK2 and PKS1 to regulate light responses (Fankhauser et al., 1999; Ryu et al., 2005). Still, little is known about phy signaling mechanisms in the cytosol (Fankhauser and Bowler, 2006). Upon light activation a pool of phytochrome is translocated into the nucleus (Kevei et al., 2007), interacts with numerous transcriptional regulators, and influence their activities via post-translational modifications and proteolysis (Jiao et al., 2007; Bae and Choi, 2008; Henriques et al., 2009). Regulation of nuclear import represents a crucial step in phy signaling (Fankhauser and Chen, 2008). Overall, phy signaling in the nucleus is well characterized at the molecular level.

Phot signaling pathways, in contrast, do not directly control such important reorganization of the transcriptional program. This may be due to the fact that phots are tightly associated with the plasma membrane and cytosolic compartments, and do not localize in the nucleus (Sakamoto and Briggs, 2002; Ohgishi et al., 2004; Kong et al., 2006; Wan et al., 2008). These global differences may explain why phys predominantly control long-term morphogenesis responses while phots trigger mainly

rapid adaptive movement processes. As later described, initial signaling events at the level of the phot photoreceptor itself is well described (Tokutomi et al., 2008; Demarsy and Fankhauser, 2009). However, unlike for phys, the picture downstream of phot activation is less clear.

Ultimately, our knowledge on light signaling should provide insight into how plants sense and respond to light in natural environments. As previously introduced, natural light is composed of a whole spectrum of wavelengths that activate several families of photoreceptors at the same time. In fact most light responses are controlled by more than one photoreceptor (Casal, 2000; Chory and Wu, 2001). For instance, phototropism and chloroplast movements are primarily controlled by phots and crys but the amplitude of these responses is modulated by phytochromes (Hangarter, 1997; DeBlasio et al., 2003). The levels of these interactions are various. Photoreceptors can physically interact and presumably modulate each other's activities (Ahmad et al., 1998; Mas et al., 2000). Other points of convergence are signaling intermediates shared by different photoreceptors (Guo et al., 2001; Duek et al., 2004). Finally, the physiological output of distinct signals may be superimposed and complement or balance eachother to generate continuously fine-tuned responses, as during phototropism or shade avoidance (Vandenbussche et al., 2005; Iino, 2006). However, most of the interactions observed to date are not understood at a mechanistic level (Casal, 2000; Stowe-Evans et al., 2001; DeBlasio et al., 2003; Whippo and Hangarter, 2004; Stone et al., 2005; Lariguet et al., 2006). Thus, the different photoreceptor-mediated signals controlling light responses are only starting to be elucidated and interpreted at an ecological level.

The PKS1 protein – the subject of our research - illustrates this last point very well. Indeed, PKS1 is a plasma membrane-associated protein that can interact with phy and phot and which regulates both photoreceptors' signaling pathways (Lariguet et al., 2003; Lariguet et al., 2006; Boccalandro et al., 2008; Schepens et al., 2008). Through the characterization of PKS1 function at the molecular and physiological levels we hope to contribute to the understanding of how light signals are integrated. To do so, a first step is to determine the precise role of PKS1 in phy and phot signaling pathways individually. My project focuses on the role of PKS1 during phot signaling.

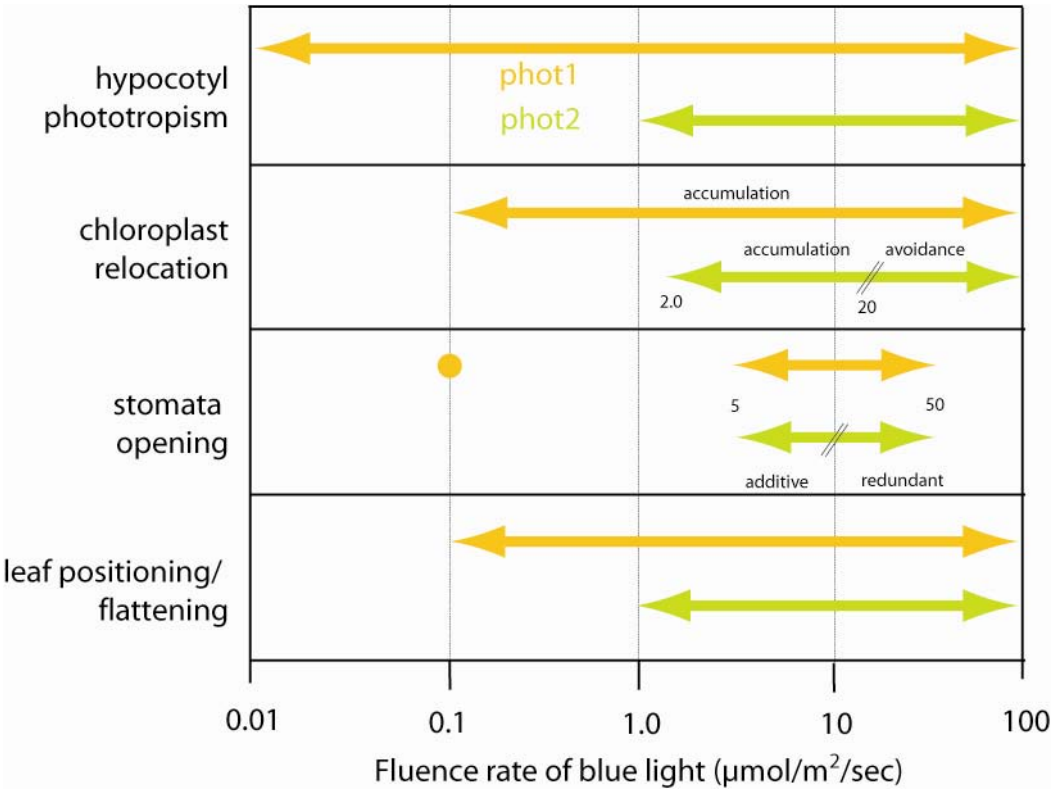


Figure 2: Different functional specificities phot1 and phot2 under various BL fluence rates.

The light fluence rates under which phot1 and phot2 are physiologically functional are represented in orange and green, respectively. For chloroplast movements, the mediation of accumulation and avoidance responses by phot1 and phot2 are noted on top of arrows. For stomatal opening, the additive or redundant functions of phots depending on the fluence rate of blue light are also specified.

Data for hypocotyl phototropism come from the work of Sakai *et al.* (2000) and Sakai *et al.* (2001); data for chloroplast relocation come from Kagawa *et al.* (2001) and Sakai *et al.* (2001); data for stomatal opening comes from Kinoshita *et al.* (2001), Doi *et al.* (2004) and Takemiya *et al.* (2005); and finally data for leaf flattening and positioning come from Takemiya *et al.* (2005) and Inoue *et al.* (2007).

2. Phototropins control numerous adaptive physiological processes

2.1. Two phototropins in Arabidopsis*: phot1 and phot2*

2.1.1. Sensors of light direction and intensity with specific and redundant roles

Early biochemical and physiological experiments on BL-induced phototropic bending in various plant species described in detail the properties of a putative BL photoreceptor involved in phototropism (Gallagher et al., 1988; Short and Briggs, 1990; Short et al., 1994). However, identification of the gene encoding that photoreceptor came only later with the use of the model plant *Arabidopsis* (Khurana and Poff, 1989; Liscum and Briggs, 1995; Huala et al., 1997). Photochemical and biochemical analysis of the isolated protein confirmed that it was indeed the BL photoreceptor controlling phototropism (Christie et al., 1998; Christie et al., 1999). This flavoprotein was named phot1 after its role in phototropism (Christie et al., 1999). A homologous phototropin (phot2) was later identified in *Arabidopsis* via independent genetic approaches during studies on chloroplast movements (Jarillo et al., 2001; Kagawa et al., 2001).

Early analysis of phot-deficient mutants in phototropism and chloroplast movements showed that phot1 and phot2 play partially redundant and also independent roles (Sakai et al., 2001). Subsequent studies of *phot1phot2* double mutants in BL responses showed that phots regulate many more processes such as stomatal opening, nuclear positioning, rapid inhibition of hypocotyl elongation, cotyledon expansion, leaf flattening, leaf positioning, negative root phototropism and destabilization of mRNA (Liscum and Briggs, 1995; Sakai et al., 2000; Folta and Spalding, 2001; Folta and Kaufman, 2003; Folta et al., 2003; Ohgishi et al., 2004; Takemiya et al., 2005; Iwabuchi et al., 2007; Inoue et al., 2008a). In addition, a possible role for phots in light-stimulated leaf movement in kidney bean has been reported (Inoue et al., 2005)

In most cases, phot1 and phot2 act redundantly, with phot1 being more sensitive to BL irradiance than phot2. However, this scenario does not apply for stomatal opening (Kinoshita et al., 2001). One particularly striking phenomenon is the role of phot2 during light-induced chloroplast relocation. Low BL causes chloroplasts to position along the periclinal cell wall relative to incident light (accumulation position) while high BL drives the plastids along the anticlinal cell walls (avoidance position) (Wada et al., 2003). Phot2 is able to switch function from chloroplast accumulation to avoidance under increasing intensities of BL. Phot1 in contrast constitutively induces the accumulation response even under extremely high light (Sakai et al., 2001). In the *phot2* mutant background this causes excess light capture and leaf photodamage (Kasahara et al., 2002). Figure 2 illustrates the functional photosensitivities of phot1 and phot2 in physiological processes that are of particular interest in this study.

2.1.2. Phots fine-tune plant photosynthesis

Phots appear to be particularly efficient in sensing light direction and light intensity and in triggering adaptive responses under these stimuli. For instance, hyopocotyl and leaf phototropism (or solar tracking) are induced by light direction while chloroplast relocation are movement processes induced by light intensity (Briggs and Christie, 2002). In fact, phots have been instrumental to demonstrate the adaptive advantages of several BL responses of the plant. For instance, *phot1* mutants are less resistant to drought supporting the notion that negative root phototropism and root growth away from the dry soil surface contribute to optimal water utilization (Galen et al., 2004; Galen et al., 2007). In addition, leaves of *phot2* mutants impaired in the avoidance response suffer photo-oxidative damage, demonstrating the importance of chloroplast movement for plant survival under extremely high light environments (Kasahara et al., 2002).

Likely functions of hypocotyl phototropism (e.g. under soil or leaf litter) and stem (e.g. under a canopy) are to allow the plant to orient its aerial parts towards the brightest source of light and optimize photosynthesis. Some plants may even track the sun efficiently by phototropism (Iino, 1990). Leaves also continuously adjust

their orienteation to track or avoid solar radiation depending on the light fluence rate (Wainwright, 1977; Lang and Begg, 1979). Different processes underlie such leaf movements, including reversible swelling of specialized cells (pluvini) and differential petiole elongation. These leaf movements are believed to have much adaptive value in the plant's life (Ehleringer and Werk, 1986; Niklas and Owens, 1989; Koller, 1990). Leaf size, shape (flatness for instance) and thickness are also strongly influenced by light and are proposed to fulfill various adaptive roles (Van Volkenburgh, 1999; Falster and Westoby, 2003). Phototropins are important regulators of leaf movements, leaf flattening and leaf positioning, suggesting that they play crucial roles in these adaptive processes (Sakamoto and Briggs, 2002; Inoue et al., 2005; Inoue et al., 2008a).

Two other important adaptive responses to light that occur in the leaf and are mediated by phots are chloroplast movements and stomatal opening, where stomata optimize photosynthetic CO_2 fixation and minimize transpirational water loss (Shimazaki et al., 2007). By comparing the selectively impaired *nph3* mutant with the *phot1* mutant (i.e. normal chloroplast movement and stomatal opening, but impaired leaf flattening), Ken-ichiro Shimazaki and colleagues showed that these two rapid adaptive responses significantly enhanced plant growth in epinastic plants grown under low light environments (Takemiya et al., 2005; Inoue et al., 2008a).

In summary, from quick chloroplast movements to long-term leaf shaping, phots control a large set of physiological and cellular processes of very different nature. A major physiological role of phots is the optimization of photosynthesis in unfavourable environments (damaging light or low light limiting photosynthesis). How solely two photoreceptors control such a wide set of processes is an important and fascinating question.

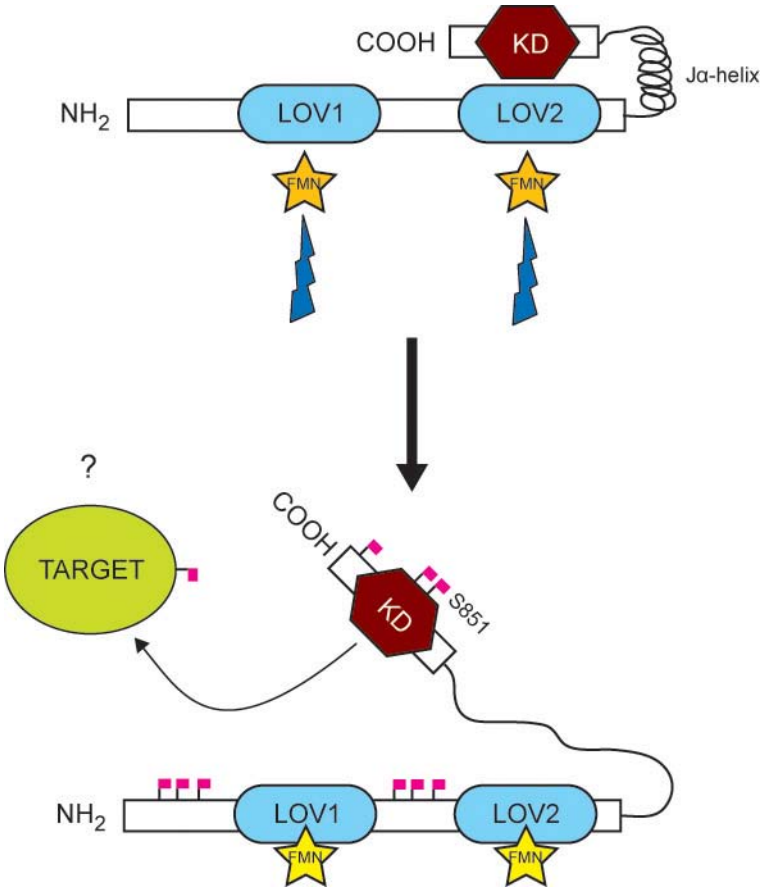


Figure 3: Phot domain organization and schematic illustration of light-induced phot1 kinase activity (based on Tokutomi et al., 2008 and Christie, 2006).

In darkness, LOV domains bind one FMN (flavin mono-nucleotide) chromophore by Van der Waals forces (orange stars). LOV2 is connected to the kinase domain (KD) via an amphipatic α-helix called Jα-helix. In the dark the Jα-helix is structured and LOV2 inhibits kinase activity. Upon irradiation (dark blue bolts), a covalent adduct between the C(4a) carbon of the chromophore and a conserved cysteine residue within the LOV domain is formed (yellow stars). This photochemical reaction disorders the helix and de-represses the kinase activity leading to autophosphorylation of the photoreceptor (purple flags). Autophosphorylation of phot1 enhances its kinase activity and triggers phosphorylation of yet-unidentified substrates. In darkness, the LOV2 domain returns back to its initial ground state within the order of tens to hundreds of seconds. This reversibility forms the basis of a characteristic light switch in phot activity.

2.2. Phototropin signalling: towards a mechanistic understanding of the physiological roles of phots

2.2.1. Early events in phot signalling

Phots are structured in two parts: a N-terminal photosensory moiety containing two flavin-binding LOV (Light, Oxgen and Voltage; LOV1 and LOV2) domains, and serine/threonine kinase domain the C terminus (Figure 3). LOV domains are part of a subfamily of the large and diverse PAS (Per, ARNT, Sim) superfamily of domains which associate with cofactors and mediate protein interactions (Taylor and Zhulin, 1999). Phots belong to the AGC-VIIIb subfamily of AGC kinases (cAMP-dependent protein kinase, cGMP-dependent protein kinase G and phospholipids-dependent protein kinase C) (Bogre et al., 2003; Galvan-Ampudia and Offringa, 2007).

A schematic diagram of light-induced phot activation is presented in Figure 3.

Autophosphorylation of phots have been known for a long time to be associated with phot activity (Christie, 2007). Recently, serine and threonine residues phosphorylated by BL in phot1 *in vivo* have been mapped. Several phosphorylation sites have been indentified, both in the photosensory and in the kinase domain (Cho et al., 2007; Inoue et al., 2008b). However, only one site (Ser851) in the kinase domain was shown to be required for phot1 to trigger its physiological responses, while other sites in the photosensory part of phot1 are not necessary (Sullivan et al., 2008; Inoue et al., 2008b) (Figure 3). This demonstrates that phosphorylation is an essential step of signaling. It also raises the question as to what the role of the other light-induced phosphorylation sites is. *In vitro* studies on phot1 showed that phosphorylation is proportional to the fluence rate of light applied to the photoreceptor (Salomon et al., 2003). Some residues phosphorylated under low BL may have a preparation role for signaling while other residues phosphorylated under HBL may have a desensitizing function (Christie, 2007). Alternatively, autophosphorylation may have less essential or yet-uncharacterized biochemical roles such as phot binding to signaling and target proteins (Emi et al., 2005; Inoue et al., 2008b).

It is not known yet whether early biochemical events in phot2 differ from those in phot1 because *in vivo* autophosphorylated residues of phot2 have not been mapped. However, phot1 and phot2 are known to display different photochemistries and localization patterns in the cell and in the plant. These distinct properties appear to confer phot1 and phot2 specific physiological roles.

2.2.2. Functional specificities of phot1 and phot2 underlying different physiological roles

Phot1 and phot2 proteins have overall 58% identity and 67% similarity. High similarity between both phototropins is found in the LOV domains and in the kinase domain (Jarillo et al., 2001). LOV1 and LOV2 have distinct properties and functions. LOV2 plays an essential role in the light-switch activation of the kinase domain and has a predominant role in light activation of phot signaling in both phot1 and phot2 (Christie et al., 2002; Cho et al., 2007). LOV1 is not essential for this process and instead may act as a dimerization domain (Salomon et al., 2004). t also appears to modulate the dark-recovery process of LOV2 as well as the light-activation of the photoreceptor (Christie et al., 2002). *In vitro*, phot1 and phot2 exhibit similar quantum efficiencies (i.e. similar proportions of protein undergo light-induced photochemistry when irradiated with similar fluences of light). However, tandem LOV domains of phot2 return to the dark state about ten times faster than in phot1, which is consistent with higher requirements of light for phot2 activity *in vivo* (Sakai et al., 2001; Christie et al., 2002; Kasahara et al., 2002). Support for this process occurring *in planta* is the fact that the N-terminal photosensory moiety determines the photosensitivity of each phot (Aihara et al., 2008). Differences in functional photosensitivities between phot1 and phot2 are also certainly due to irradiance-dependent induction of gene expression mediated by phytochromes. Indeed, high light induces *PHOT2* expression while long-term exposure of dark-grown seedlings to light results in decrease in *PHOT1* transcript levels (Tepperman et al., 2001; Elliott et al., 2004).

In the dark, both phot1 and phot2 are mainly associated with the plasma membrane via unknown mechanisms. A small fraction of phot2 is also found in the cytoplasm. Upon BL excitation, both phots undergo subcellular relocalization within a few minutes (Sakamoto and Briggs, 2002; Kong et al., 2006). A small portion of phot1 is released into a cytosolic soluble fraction which can be visualized by internal subcellular phot1-GFP patterns by confocal microscopy. However, it is not clear yet whether the intracellular pool of phot1 is present as a free soluble protein or is associated with extremely small vesicle (Knieb et al., 2004; Wan et al., 2008). Blue-light causes phot2 re-localisation into the Golgi apparatus, and treatment with the fungal toxin Brefeldin A (BFA) (an inhibitor of vesicle trafficking) leads to phot2 accumulation in BFA compartment even in the dark. These results indicate that phot2 can localize in different endomembrane compartments (vesicles and the Golgi) (Kong et al., 2006). Interestingly, Golgi association upon BL activation is independent of BFA suggesting that phot2 may cycle in the cell via different pathways. Although a small fraction of phot1 becomes soluble upon illumination, phot2 remains in the insoluble fraction suggesting a constitutive association of phot2 with membrane-derived compartments (Kong et al., 2006).

The significance of such intracellular movements during phot signalling is still unclear. In one case these movements may bring phots closer with signaling components (Geldner and Jurgens, 2006). In other cases, internalization may lead to degradation in the vacuole, or may be a withdrawal mechanism from the active site of signalling. These two latter possibilities may represent modulation and attenuation mechanisms of phot signalling (Han et al., 2008).

Structure-function studies are starting to elucidate the structural basis of phot1 and phot2 specificities. The N-terminal photosensory moiety was shown to confer phot photosensitivity (Aihara et al., 2008). The C-terminal kinase domain of phot2 associates the photoreceptor with the plasma membrane and the Golgi, and also triggers constitutive phot responses (chloroplast avoidance and stomata opening). On the other hand, the N-terminal region of phot2 is localized in the cytosol and cannot restore the chloroplast avoidance response (Kong et al., 2007). Interestingly, all chimeric combinations between phot1 and phot2 (swapping each-other's N- and C-terminal moieties) can induce the chloroplast avoidance response except for the phot1

full-length protein. This indicates that a full-length phot1 is required to inhibit the chloroplast avoidance response (Aihara et al., 2008). Thus, in addition to functional differences in their N- and C-termini, phots appear to possess distinct functions that involve whole-protein mechanisms.

Phot1 is found in cotyledon guard cells and abaxial epidermis, in mesophyll cells, and in the apical hook and elongation zone of the hypocotyl. However, it is much less expressed in the root (Knieb et al., 2004; Wan et al., 2008). High expression of *PHOT2* is found in cotyledons and leaves, but none in roots (Jarillo et al., 2001; Kong et al., 2006). Thus overall, phot1 and phot2 distribution is largely consistent with their physiological roles. As expected from their important function in leaf physiology, both phots are highly expressed in leaves.

Interestingly, different cell types and similar cell types at different stages of development show different subcellular patterns of phot1. For instance, phot1 is uniformly distributed in mesophyll cells but is limited to the anticlinal walls of the epidermal cells in cotyledons. Another interesting element is the fact that phot1 does not undergo relocalisation in stomata guard cells (Wan et al., 2008). In contrast, guard cells that constitutively express phot2-GFP forms display punctuate staining upon BL treatment (Kong et al., 2006). Finally, phot2 may localize around the chloroplasts (Suetsugu and Wada, 2007). Thus, functional differences between both phots may be due to a combination of differences in tissue and sub-cellular localization.

In summary, the distinct physiological roles accomplished by phot1 and phot2 may be explained by different photochemical and biological properties of both photoreceptors. As suggested by their dissimilar subcellular localisations, phot1 and phot2 may also utilise different sets of signalling components and regulate the activity of different targets.

2.2.3. Phototropin signalling pathways

2.2.3.1. Stomatal opening

BL-induced swelling of guard cells and opening of stomata is directly regulated by the activity of plasma membrane H^+-ATPase proteins (Roelfsema and Hedrich, 2005). This is indicated by the fact that proton extrusion is induced by blue light and is absent in the *phot1phot2* mutant (Kinoshita et al., 2001). The H^+-ATPase may thus be seen as an end-target of the phot signaling in guard cells. BL activation of the H^+-ATPase involves phosphorylation and subsequent binding of a 14-3-3 protein (Kinoshita and Shimazaki, 1999; Kinoshita and Shimazaki, 2002). phot1 can mediate binding of the 14-3-3 protein to the H^+-ATPase but this seems to be indirect because the H^+-ATPase phosphorylation and 14-3-3 binding can be induced by drugs in the absence of phots (Kinoshita and Shimazaki, 2001; Kinoshita et al., 2003; Ueno et al., 2005). A type 1 protein phosphatase associated with phot1 was shown to positively regulate BL-induced stomatal opening and thus, in association with a yet-unidentified protein kinase, may provide the link between phot1 activation and H^+-ATPase phosphorylation (Takemiya et al., 2006).

Two other proteins that interact with phot1, ROOT PHOTOTROPISM2 (RPT2) and a phot1-interacting protein from broad bean (VfPIP) are known regulators of BL-induced stomatal opening (Inada et al., 2004; Emi et al., 2005). However, their mode of action has not yet been characterized. Based on its homology with dyneins, VfPIP may control guard cell swelling by regulating cytoskeleton organisation. Finally, the intracellular messenger calcium does appear to act in phot signaling during stomatal opening (Harada and Shimazaki, 2009)

2.2.3.2. Chloroplast movements

As previously described, chloroplasts gather in areas irradiated with weak light to maximize photosynthesis (accumulation position along the periclinal cell walls).

They move away from high irradiation to minimize damage of the photosynthetic apparatus (avoidance position along the anticlinal cell walls). While the mechanism of chloroplast movement is not well understood, regulation of actin filaments is known to be important in this process (Suetsugu and Wada, 2007).

Chloroplast association with the anticlinal plasma membrane is a default position and is mediated by the coiled-coil region of CHLOROPLAST UNUSUAL POSITIONING1 (CHUP1) (Oikawa et al., 2008). Another domain of CHUP1 anchors the chloroplast to F-actin and mediates low BL-induced chloroplast movement towards the periclinal as well darkness-induced basal positioning in the cell. JAC1 (J-domain protein required for chloroplast accumulation response) is required for the accumulation response and contains a domain with high similarity with auxilin (known as a clathrin uncoating factor during endocytosis). Interestingly, chloroplasts in both *jac1* and *phot2* mutants move to the periclinal walls in the dark instead of the bottom of mesophyll cells (Suetsugu et al., 2005). This suggests that JAC1 may act downstream of phot2 in the accumulation response.

PLASTID MOVEMENT IMPAIRED (PMI) proteins are plant-specific proteins of unknown biochemical function that regulate BL-induced chloroplast movements. The role of these proteins in phot signalling during chloroplast photorelocation has not been yet characterized (DeBlasio et al., 2005; Luesse et al., 2006). Finally, calcium acts as a modulator of chloroplast motility rather than as a second messenger (Suetsugu and Wada, 2007). Thus, little is known about the signalling pathways regulating chloroplast movements.

2.2.3.3. Phototropism, leaf flattening and leaf positioning

Phototropism, leaf flattening and leaf positioning are physiological processes of different nature than stomatal opening and chloroplast movements. They involve whole-tissue growth regulation by cell division and cell expansion. Such regulation is performed by hormones such as auxin. As discussed later, many proteins related to hormone signaling are involved in these processes and are potential targets of phot signaling. Here I focus on the phot signal transduction cascade *per se*.

NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3), a protein homologous to RPT2, is required for normal phototropism under low BL (under conditions where only phot1 is activated). NPH3 interacts *in vitro* and forms a complex *in vivo* with phot1 at the plasma membrane (Motchoulski and Liscum, 1999; Lariguet et al., 2006). NPH3 is also required for phototropism under high BL but it is not known whether it can associate with phot2 (Motchoulski and Liscum, 1999). In the dark, NPH3 is phosphorylated and phot1 mediates its dephosphorylation upon BL illumination. This process is required for phototropic bending to occur and involves a type I protein phosphatase (PP1) (Pedmale and Liscum, 2007). Interestingly, NPH3 dephosphorylation is not required for phototropism under high BL (where phot2 acts redundantly with phot1) indicating that, if phot2 modulates NPH3 activity, then it is independent of de-phosphorylation events (Tsuchida-Mayama et al., 2008)

RPT2 is also required for phot1-mediated phototropism and interacts with phot1 *in vivo* and NPH3 *in vitro* (Inada et al., 2004). NPH3 and RPT2 are the founding members of the NPH3/RPT2-like (NRL) plant-specific protein family. They contain two protein-protein interaction domains: a coiled-coil domain and a BTB domain that can mediate interactions with CULLIN3 adaptors in (CUL3)-based E3 ligase complexes (Celaya and Liscum, 2005; Weber et al., 2005). Because of their protein-protein binding domains, NPH3 and RPT2 are believed to act as scaffolds during phot signalling.

NPH3 is the best studied phototropism signalling component to date and the following model was proposed: In the dark NPH3 is constitutively phosphorylated by a kinase. In the light, phot1 activates a regulator of PP1 that in turn activates a yet-uncharacterized PP1 that will dephosphorylate NPH3. NPH3 in its dephosphorylated state can possibly react with CUL3 and mediate the modification of downstream signalling elements by CUL3-based E3 ligases (Pedmale and Liscum, 2007).

PHYTOCHROME KINASE SUBSTRATE proteins form a small family in *Arabidopsis* (PKS1-4). Recently, PKS1, PKS2 and PKS4 were also shown to control phot1-mediated phototropism. PKS1 interacts with both NPH3 both phot1 *in vitro* and *in vivo*, and these three proteins form a complex at the plasma membrane

(Lariguet et al., 2006). Although these data indicate that PKS1 is likely to play an important signalling role during phototropism, its mode of action at the molecular level is currently unknown. Interestingly, because phyA controls *PKS1* expression under BL, PKS1 represents a molecular link between these two photoreceptor families during phototropism (Lariguet et al., 2006). Indeed, phys are well known modulators of the phototropic response (Iino, 2006). A role for PKS1 in high BL-induced phototropism has not been yet described.

NPH3 was identified in a mutational screen for plants impaired in normal BL-induced leaf positioning (Liscum and Briggs, 1996). Unlike during phototropism, NPH3 is required only for the low BL response (mediated by phot1) suggesting that other components mediate the high BL response (phot2 pathway) (Inoue et al., 2008a). Apart from NPH3, no other phot signalling components controlling leaf positioning or leaf flattening have been identified. The *pks* and *rpt2* mutants do not display obvious leaf flattening defects (Sakai et al., 2000; Inada et al., 2004; Lariguet et al., 2006).

Finally, it is interesting to note that BL-induced increase in cytosolic calcium in leaf cells is controlled by phot1 and phot2 in fluence-rate-dependent manners that correlate with phot1 and phot2 functional photosensitivities. Different cytosolic calcium waves appear to be the result of calcium release from different sources (extracellular or internal stores) (Harada and Shimazaki, 2007). These different Ca^{2+} "signatures" could result in different signaling processes that may regulate morphological processes in the leaf (Sanders et al., 1999; Sanders et al., 2002; Hepler, 2005). However, the direct implication of calcium on phot-mediated leaf positioning has not been shown yet. As for the hypocotyls, increase in cytoplasmic Ca^{2+} is required for phot1-dependent inhibition of hypocotyls elongation, but not for phototropism (Folta et al., 2003).

2.3. What is the structure of phot signaling?

2.3.1. General themes

Phot signaling pathways controlling the various phot-mediated BL responses are just starting to be elucidated. Only few components have been identified so far and their functions during phot signalling are still largely unclear.

Nonetheless, a few general properties of phot signaling can be outlined. For instance, protein phosphorylation and dephosphorylation appear important (Inoue et al., 2008b). These post-translational modifications are known to modulate the conformation, activity, localization and stability of substrate proteins (Farkas et al., 2007). Such signaling intermediates could act as "ON" and "OFF" switches for phot signals controlling the end physiological processes (DeLong, 2006). NPH3 is the best known example to date, although the downstream targets are not yet known (Pedmale and Liscum, 2007). PKS1, which is a known substrates for phytochrome kinase activity *in vitro*, may also be part of such phosphorelay during phot signaling (Fankhauser and Chory, 1999; Lariguet et al., 2006). 14-3-3 proteins also play a role in phot signaling. 14-3-3 proteins are well known to modulate protein-protein interactions (acting as scaffolds) in a phosphorylation-dependent manner and are important for plant development (Fulgosi et al., 2002; Roberts, 2003). Finally, regulated proteolysis may be one important step in phot signaling as suggested by the potential binding of NPH3 and RPT2 with CUL3-based E3 ligases (Inada et al., 2004; Pedmale and Liscum, 2007).

Several proteins that directly interact with phots have been identified so far (e.g. VfPIP, NPH3, PKS1). Presumably, these proteins act early in the phot signaling pathway. However, no direct substrates of phot kinase activity have been identified and the primary relay is still elusive. Forward genetic screens have identified many genes involved in phot-mediated processes. However, many of these gene products may be regarded as end-targets of phot signaling rather than signaling intermediates

(Holland et al., 2009). The best example is the numerous auxin signaling-related proteins involved in phototropism, as discussed later.

It is interesting to note that although being direct interactors of phot (*in vitro* and *in vivo*), some signalling elements mediate only a subset of phot responses. This suggests that signaling downstream of phots branches quickly. Branching may be explained by the existence of distinct phot-associated complexes both in the different cell compartments and in different tissues. Indeed, as described previously, phot1 and phot2 display different localisation patterns. In that sense, it will be particularly important to determine the sub-cellular localization of these signaling elements in order to better comprehend their function during phot signalling. As suggested by the dephosphorylation data obtained for NPH3, the biochemical relationship between a signaling protein and a phot may also be of different nature and this may provide functional specificity of a signalling protein during phot-mediated BL responses.

An important task is to discover more phot signaling elements to add more pieces to the puzzle. Experiments using low BL and high BL, in addition to epistasis studies, will allow to study their roles in both phot1 and phot2 pathways in high detail. Another task is to continue characterizing the biochemical activities of known signalling elements in order to understand their functions at a more mechanistic level. Our studies on the PKS family of proteins follow these aims.

2.3.2. The *Arabidopsi*s NRL family of proteins

The NRLs compose a 32-member family of plant-specific proteins. Based on *in silico* studies, NRLs appear to be highly modular with five regions ("NPH3 domains" DI-DIV) of primary amino acid sequence conservation. All NRLs contain the NPH3 domains. In addition, some members contain a coiled-coil domain, or a *broad complex, tamtrack, bric à brac* (BTB) domain, or both (Figure 4).

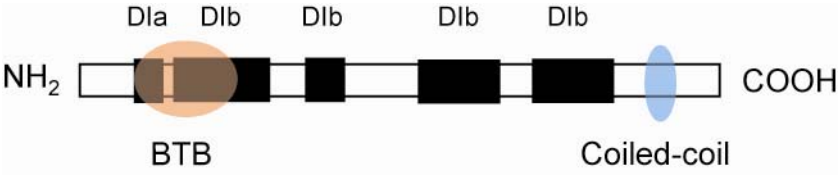


Figure 4: Domain organization of the NRL family of proteins (adapted from Celaya and Liscum, 2005).

Five regions of primary amino acid sequence conservation (black boxes – "NPH3-domains" designated DIa, DIb, DII, DIII and DIV) are separated by islands of nonconserved sequence (white stretches). The BTB (orange ellipse) and coiled-coil (blue ellipse) represent known protein-protein interaction motifs

To date, eight NRLs have been studied in detail: NPH3, RPT2, NAKED PINS IN YUC MUTANTS/ENHANCER OF PINOID/MACCHIBOU 4 (NPY/ENP/MAB4) NPY1-NPY5 (forming a subfamily) and SETH6 (Inada et al., 2004; Lalanne et al., 2004; Furutani et al., 2007; Cheng et al., 2008). All these NRLs were shown to regulate important developmental processes such as tropisms (root and hypcotyl phototropism), embryogenesis and organogenesis (e.g. flowers and leaves). Several lines of evidence indicate that NRLs may control these processes by regulating auxin signalling. First, CPT1 (the rice ortholog of NPH3) is required for lateral auxin redistribution across coleoptiles (a pre-requisite for phototropic bending) (Haga et al., 2005). Second, based on epistasis and cell localisation results, NPY1 was proposed to co-regulate with the AGC kinase PINOID (PID) the cellular localisation of the auxin transporter PIN-FORMED (PIN) (Furutani et al., 2007). In fact, *NPY*s (*NPY1-NPY5*) appear to play key signalling steps with AGC kinases (*PID*, *PID2*, *WAG1*, *WAG2*) during auxin-mediated organogenesis (Cheng et al., 2008) (Robert and Offringa, 2008). Third, phot1 (which, like PID, is a member of the AGC VIII subfamily), controls BL-induced PIN1 cell relocalisation, and NPH3 may participate is this process since it is strongly associated with phot1 (Blakeslee et al., 2004; Pedmale and Liscum, 2007). These proposed similarities between NPY1 / PID and NPH3 / PHOT1 co-action have led to the hypothesis that phototropism and organogenesis are carried out via analogous molecular mechanisms (Furutani et al., 2007).

Unlike NPH3 which controls exclusively whole-organ developmental processes (phototropism and leaf flattening), RPT2 regulates phototropism and stomatal opening (a cell-autonomous process controlled by guard cell swelling) (Inada et al., 2004). Phots also control reversible cell swelling in braod bean pulivini, and by this means trigger BL-induced leaf movements (Inoue et al., 2005; Takemiya et al., 2006). How, at the molecular level, phots carry out these different types of processes is not yet well understood. One possibility is that phots regulate basal aspects of cell biology, such as the cytoskeleton and cell polarity. Phots may utilise specialized components such as NPH3 and RPT2 to translate such fundamental mechanisms into distinct physiological responses in the cell. This may form a basis for phot signalling branching. How the NRLs perform these proposed molecular roles at the biochemical level is not known (e.g. are they all involved in target degradation?). However, their well-known protein-protein binding motifs indicate that these proteins may function as scaffold proteins to recruit different sets of proteins, and this may confer phot signalling different specificities.

Phototropism and leaf flattening are developmental processes under hormonal regulation. One general concept is that phots regulate these processes by acting on hormone signalling. As briefly introduced, phots may utilise signalling elements such as NPH3 to modulate, for example, auxin transport via transporter's activities. In fact numerous connections (either direct molecular links or more distant genetic links) between phot signalling and auxin-regulated development are emerging. After a brief introduction on auxin-regulate development, I hereafter describe these connections.

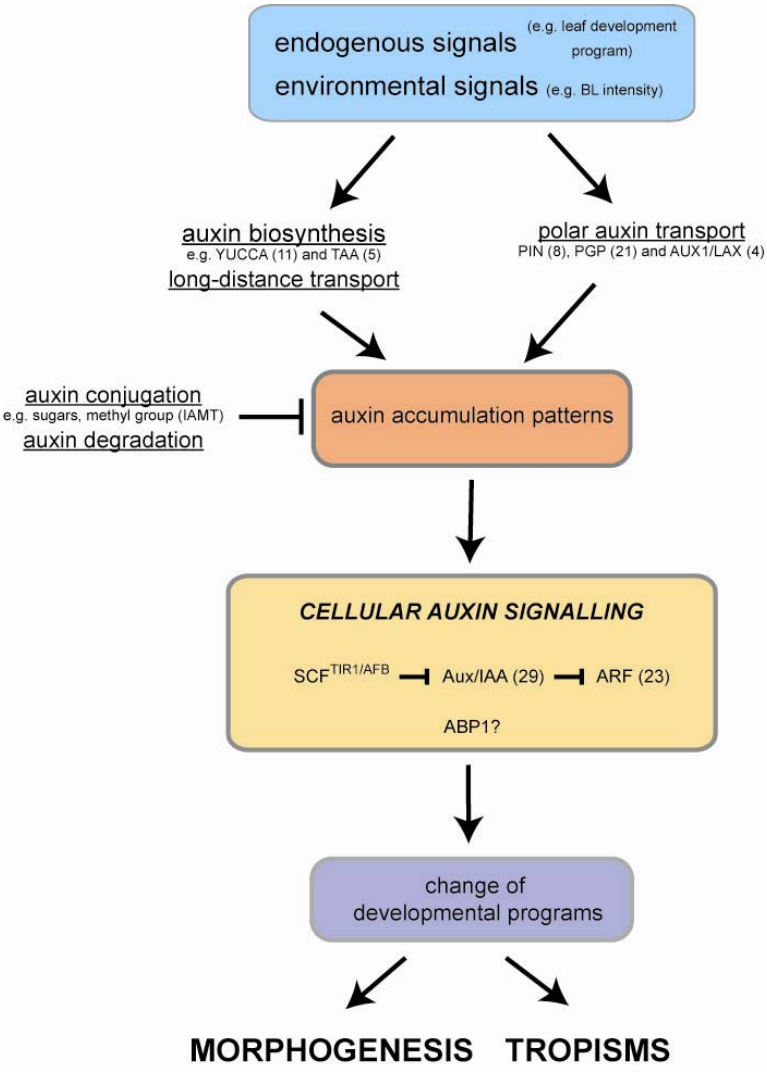


Figure 5: A framework of auxin-regulated plant development (based on Vanneste and Friml, 2009).

Environmental and developmental signals target auxin biosynthesis (via enzymes such as the flavin-monooxygenase-like YUCCA and the aminotransferases TAA) and transport (via the AUX1/LAX influx carriers and the P-glycoproteins (PGP) and PIN-FORMED (PIN) efflux carriers). This, in combination with auxin conjugation (with sugars, methyl groups or amino acids for instance) and degradation determines auxin accumulation patterns in tissues. In the nucleus, an interplay between homo and heterodimerization between the Aux1/IAA and ARF transcriptional regulators represents the core of auxin signalling. Aux1/IAAs bind to ARFs and repress their activities as transcriptional regulators. Auxin acts as molecular glue between Aux1/IAA and TIR1/AFB F-box proteins. This stimulates Aux1/IAA ubiquitination by the $SCF^{TIR1/AFB}$ E3 ligase and subsequent proteolysis by the proteasome. The cytoplasmic ABP1 (AUXIN BINDING PROTEIN1) protein is another auxin receptor that can also act on development (for instance during cell elongation in leaves) (Braun et al., 2008). Coordinated auxin signaling in whole tissues and organs triggers change in developmental programs leading to morphogenesis and tropisms. Multi-member gene families regulate all these steps. The number of genes in different families is indicated in brackets.

3. Phototropin signalling impinges on auxin homeostasis and signalling

3.1. Auxin is a central regulator of plant morphogenesis and tropism

Plants continuously monitor their environment and these external cues are translated into specific developmental changes. Two main developmental processes influenced by light are morphogenesis (e.g. inhibition of hypocotyls elongation and leaf expansion) and tropisms (e.g. hypocotyl phototropism and leaf solar tracking). Photomorphogenesis involves rather long-term processes while tropisms are rapid and reversible responses. These two aspects of development are intimately linked and confer to the plant great plasticity in shape and behavior.

Because plant cells are immobilized due to rigid cell walls, they rely on mobile signals to trigger tissue development. The simple indolic molecule auxin is one such signal and regulates cell division, expansion and differentiation. The action of auxin on cells is the combined result of (i) the preprogrammed status of the cell prior to auxin signaling and (ii) the auxin concentration. Thus, one central concept is that auxin regulation of whole tissues and organ development is based on distribution patterns (auxin gradients). A second fundamental concept is that auxin effect on a cell depends on the pre-programmed responsiveness of that cell (Vanneste and Friml, 2009).

Regulation of development by auxin is a multi-step process that involves many different molecular mechanisms. The combination of (i) local biosynthesis and catabolism, (ii) long-distance transport and (iii) short distance cell-to-cell transport, generates auxin gradients (Blakeslee et al., 2005; Stepanova et al., 2008; Tao et al., 2008). Auxin signaling mainly occurs in the nucleus and is based on regulated proteolysis of transcriptional regulators (Weijers et al., 2005). Additional auxin signal transduction mechanisms also occur in the cytoplasm and this second pathway leads to more rapid cellular responses (Braun et al., 2008). Globally, multigenic families control these processes and confer high versatility and robustness in auxin signaling. A summary of auxin signaling is presented in Figure 5.

Experiments with pharmacological inhibitors and auxin transport mutants revealed that cell-to-cell auxin transport is a crucial process in auxin-regulated development (Tanaka et al., 2006). The main feature of this type of auxin transport is its directionality (polar auxin transport – PAT). Specifically, the oriented transport of auxin in files of cells creates auxin gradient within tissues. The directional flow of auxin is based on two concepts: (i) the "trapping" of auxin in cells and (ii) the polar localization of auxin transporters in the cells. The first step is based on the chemiosmotic theory: in the acidic apopolasm, auxin (which is a weak acid) becomes protonated and can diffuse inside cells (Raven, 1975). This auxin import step is also aided and directed by the AUX1/LAX import carriers (Kramer and Bennett, 2006). Once inside the neutral cytoplasm, auxin becomes charged and is "trapped". As a consequence, its export strictly depends on the plasma membrane-localisation of auxin efflux carriers P-glycoproteins (PGPs) and PIN-FORMED (PINs). The direction of auxin efflux is subsequently governed by the polarity of these export carriers in the cell (Blakeslee et al., 2005). Coordinated polar localization of PINs and AUX1/LAXs in adjacent cells generates local auxin maxima that will trigger developmental processes in tissues (Scheres and Xu, 2006).

Since growth reorientation and organogenesis require auxin relocalisation, directional auxin efflux must be very modular. Such modulation is underpinned by the continuous cycling of auxin transporters between the plasma membrane and endosomal compartments (Geldner, 2009). The endososomal system is structured into connected intracellular networks comprising the trans-Golgi network, the early and late endosomal network and the vacuolar compartments (Geldner and Jurgens, 2006). Recent studies on auxin transporter intracellular localizations have shown that PINs, AUX1/LAXs and PGPs follow different cycling routes in the endomembrane system (Kleine-Vehn et al., 2006). The nature of these vesicular compartments and the molecular mechanisms controlling the trafficking of auxin carriers are just only starting to be elucidated (Robert et al., 2008). Auxin carrier localization and activities appear to be tightly regulated by several mechanisms including protein-protein interactions, post-translational modifications and membrane composition (Titapiwatanakun and Murphy, 2008). A simplified summary of trafficking mechanisms of PIN and AUX1/LAX proteins is shown in Figure 6.

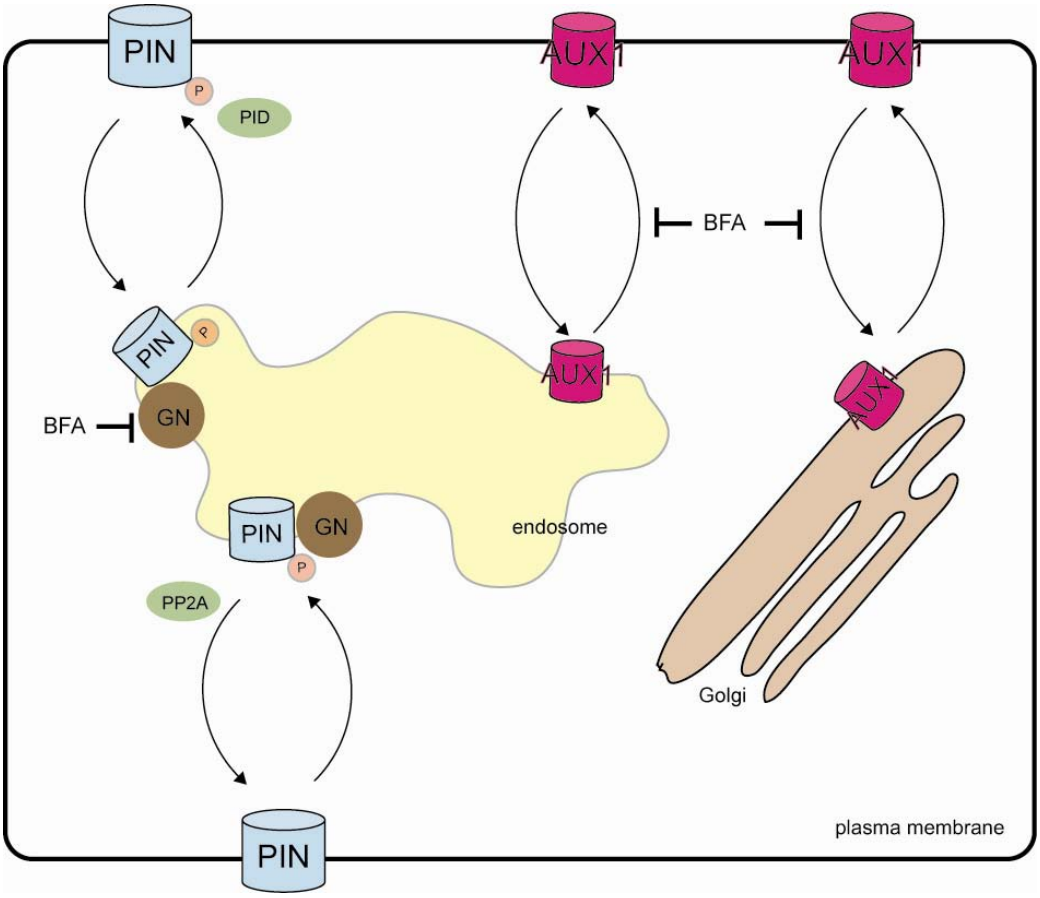


Figure 6: A simplified diagram of PIN and AUX1 cellular trafficking (based on Kleine-Vehn et al., 2006 and Weijers and Friml, 2009).

PIN proteins continuously cycle between the plasma membrane and endosomes. The ARF-GEF GNOM (GN) (which is inhibited by BFA) controls budding of the vesicles recycling back to the plasma membrane (Geldner et al., 2003). This constitutive PIN cycling allows rapid relocalisation of the auxin transporter (e.g. upon environmental or endogenous stimuli). PINs are phosphorylated and dephosphorylated by the AGC kinase PINOID (PID) and the PROTEIN PHOSPHATASE 2A (PP2A), respectively. This binary switch sorts the PIN proteins into the basal or apical trafficking pathways (Michniewicz et al., 2007).

AUX1 proteins exhibit either polar or non-polar distribution in the plasma membrane of cells depending on cell types. AUX1 can also accumulate in the Golgi apparatus and in endosomal compartments in a BFA-sensitive manner. This is mediated by mechanisms independent of GNOM (Kleine-Vehn et al., 2006).

From auxin biosynthesis to transcriptional regulation of auxin-responsive genes, each level of the hierarchy of auxin signaling is a potential target for modulation by environmental signals. In recent years, several links between phot signaling and auxin signaling have been uncovered. Hereafter I describe some of these links and put emphasis on emerging connections between phots and auxin transport.

3.2. Phototropism and leaf development are regulated by auxin

3.2.1. Phototropism

The Cholodny-Went theory states that asymmetric accumulation of auxin occurs in response to a tropic stimulus, and that this asymmetric gradient of auxin stimulates the differential growth response leading to curvature (Cholodny, 1924; Went and Thimann, 1937). This theory has been supported at the molecular level in several instances. First, phototropic bending is preceded by asymmetric transcription of auxin-responsive genes, including genes involved in the control of cell wall extensibility such as expansins (Esmon et al., 2006). Second, phototropic bending is tightly controlled by the co-action of the transcriptional activator NPH4 (NON-PHOTOTROPIC HYPOCOTYL 4) / ARF7 (AUXIN RESPONSE FACTOR 7) and its associated Aux/IAA repressors MSG2 (MASSUGU 2) / IAA19 and AXR5 (AUXIN-RESISTANT 5 / IAA1) (Harper et al., 2000; Tatematsu et al., 2004; Yang et al., 2004). Third, several auxin transporters are involved in phototropism, which is consistent with the need for asymmetric auxin transport in the phototropic stem.

Auxin is transported from the apex to the base of the hypocotyl through the phloem and the cortical tissues. One proposed function of auxin transporters during phototropism is to modulate the basipetal flux of auxin and activitely contribute to the lateral redirection of auxin on the flanks of the laterally illuminated stem. PIN3, PIN1, and PGP1, PGP19 are thought to control such auxin fluxes during phototropism (Friml et al., 2002; Noh et al., 2003; Blakeslee et al., 2004; Blakeslee et al., 2007). For example, lateral BL stimulates in a phot1-depedent fashion the delocalisation of

PIN1 away from the basal side of cells in vascular tissues (Blakeslee et al., 2004). This relocalisation is dependent on PGP19 which interacts with and stabilizes PIN1 on the plasma membrane (Noh et al., 2003; Titapiwatanakun et al., 2009). Such auxin carrier relocalisation is thought to modulate auxin basipetal transport and, in combination with laterally orientated efflux, to establish the tranversal auxin gradient. As previously described, the cycling of auxin transporters required for such relocalisation can be regulated by phospho / dephosphorylation of the carrier. Because of the concurrence in localization between the AGC kinase phot1 and PIN1 in BL-illuminated hypocotyls, one current hypothesis is that phot1 controls PIN1 delocalization via post-translational modification of PIN1 or its interactor PGP19 (Titapiwatanakun and Murphy, 2008; Wan et al., 2008).

AUX1 may have a modulation role on phototropism by affecting the symplasm – to – apoplasm partitioning of free auxin. This local modification in intracellular auxin concentration was proposed to vary the degree of auxin responsiveness of these cells, and ultimately phototropism (Falcone et al., 2007). It is interesting to point out that both AUX1 and phot2 can localize at the plasma membrane and in the Golgi apparatus, suggesting another possible molecular link between phot signaling and auxin transport (Kleine-Vehn et al., 2006; Kong et al., 2006). However, no reports on whether phot2 influences AUX1 activity have been reported so far.

In summary, recent major advances on mechanisms of auxin transporter cycling and phot receptors subcellular relocalisation have been made in parallel. These discoveries are starting to provide a molecular explanation of the Cholodny-Went theory. One apparent scenario is the modulation of auxin transporter cycling (and thus cell relocalisation) by phots. Clearly, these two classes of proteins undergo dynamic and constitutive cycling and relocalisation upon light stimulation. However, the pathways and concurrence of these trafficking events are still unclear, and how phot signaling directly regulates auxin carriers' activities is still poorly understood.

3.2.2. Leaf positioning and leaf flattening

Leaf positioning is mainly determined by the shape of the petiole connecting the plant's stem to the lamina. The interaction point between the lamina and the petiole is another site where leaf positioning is controlled. The petiole is highly responsive to light and gravity, and the effect of these external stimuli on petiole shape may be viewed as an analogous process than stem photo- or gravi-tropism. In fact, the Cholodny-Went theory was also shown to apply to petiole bending (Lyon, 1963). Pulvinar cells in the petiole-lamina connection point also mechanically influence lamina position via similar swelling processes than those found in stomata guard cells (Koller, 1990). This process is found in legume species for instance, but is absent in rosette-type plants like *Arabidopsis* which are devoid of pulvini. Phots are proposed to mediate BL-induced leaf positioning by acting on petiole shape in *Arabidopsis* and on pulvinar cells of broad bean (Inoue et al., 2005; Inoue et al., 2008a).

Leaf flattening may be seen as the result of coordinated expansion of different sets of tissues on the adaxial (upper) side, abaxial (lower) side and inner spaces of the leaf (Poethig, 1997; Hudson and Waites, 1998). Leaf expansion (size and shape) and thickness are highly responsive to light (Koller, 1990; Neff and Van Volkenburgh, 1994; Van Volkenburgh, 1999). Since RL and BL treatments of leaves modulate palisade mesophyl cell size and shape, and since BL restores flatness in RL-illuminated epinastic leaves, it is believed that phots may modulate leaf flattening by controlling the expansion of inner leaf cells (Takemiya et al., 2005; Lopez-Juez et al., 2007). However, the mechanisms of leaf flattening at a histological level are still unclear (Poethig, 1997; Tsukaya, 2008), and the involvement of phots in such cell regulation is still elusive (Lopez-Juez et al., 2007).

Since leaf positioning and leaf flattening are both the result of coordinated growth processes, it is not surprising that auxin tightly regulates these processes. For instance, exogenous auxin can induce epinasty (downwards curling/bending towards the abaxial side of leaf) of excises lamina strips (Keller and Van Volkenburgh, 1997). Mutations in several genes controlling auxin homeostasis also lead to epinasty or hyponasty (downwards curling/bending towards the adaxial side of leaf) (Li et al., 2007). Interestingly, some phototropism-impaired mutants also display leaf flattening and positioning defects (e.g. *nph3*, *nph4*, *iaa19* and *aux1*) supporting the notion that these developmental processes are regulated by similar mechanisms (Watahiki and

Yamamoto, 1997; Harper et al., 2000; Bainbridge et al., 2008; Inoue et al., 2008a). However unlike for phototropism, no detailed molecular studies were yet performed to analyse the mechanistic connections between phot signaling and auxin patterning in leaf tissues controlling the positioning and flattening processes.

In summary, phot-mediated developmental processes are tightly controlled by auxin signaling. In particular, regulation of auxin transporters localization and activities potentially represent a main target of phototropin signaling pathways. However, too many pieces of the puzzle are missing to obtain a clear picture of phot-regulated auxin transport, and the molecular mechanisms are still largely unknown.

4. The PHYTOCHROME KINASE SUBSTRATE family of proteins – important light signaling elements with a role in morphogenesis and tropism

4.1. PKS are important phy-signalling components during de-etiolation

The phy family of photoreceptors (phyA-E in *Arabidopsis*) is essential for sensing R and FR light. Phys have crucial functions in developmental processes such as seed germination, transition from dark growth to light growth (de-etiolation), and flowering. Phys also control shade avoidance and are important regulators of root and shoot phototropism and gravitropism (Chen et al., 2004). PhyA and phyB-E are classified into type I (light-labile) and type II (light stable) classes, respectively. Type II phys control the classic low fluence responses (LFRs) induced by R and partially reversed by FR. In contrast, phyA controls the very low fluence response (VLFR) which acts over a broad range of the visible spectrum (including blue light), and the high irradiance response (HIR) induced by FR. These three different signaling modes are functionally different and control different sets of light responses (Casal, 2000).

PKS1, the founding member of the *Arabidopsis* PKS family (PKS1-PKS4), was identified in a search for phy signaling elements that could directly interact with the COOH terminal part of phyA harboring a histidine-related kinase domain. PKS1 can interact with phyA and phyB. PKS1 is phosphorylated by phyA *in vitro* and its

phosphorylation is stimulated by red light *in vivo* (Fankhauser et al., 1999). PKS2 and PKS4 also interact with phyA and phyB *in vitro* (Lariguet et al., 2003; Schepens et al., 2008).

One important aspect of the *PKS* is the tight control of their expression by light. In dark-grown seedlings, *PKS1* expression is rapidly and highly induced under R and FR light (within 1h) and transcript levels steadily return to basal levels within 4-18 hours (Lariguet et al., 2003; Khanna et al., 2006; Molas et al., 2006; Molas and Kiss, 2008). *PKS4* expression rapidly decreases under R light and also returns to original levels within hours, whereas FR light leads to a slower and steady decline (Schepens et al., 2008). Light induction of *PKS2* appears different from *PKS1* and *PKS4*: it is much weaker and may be the result of circadian control (Lariguet et al., 2003). These patterns of *PKS* expression are predominantly controlled by the phyA VLFR pathway (Lariguet et al., 2003; Schepens et al., 2008). Consistent with their light-regulation at a molecular level, the PKS are important regulators of seedling de-etiolation (cotyledon opening and inhibition of hypocotyl elongation). During de-etiolation, PKS1 and PKS2 act in the phyA-mediated VLFR branch while PKS4 can act downstream phyA and phyB in VLFR, HIR and LFR branches (Lariguet et al., 2003; Khanna et al., 2006; Schepens et al., 2008).

4.2. PKS are important regulators of phot- and phy-mediated growth orientation

As described, PKS expression is highly modular under different light environments. In addition to a role in long-term photomorphogenesis such as de-etiolation, this also suggested that PKSs may play important roles during rapid adaptive responses (Lariguet et al., 2003; Khanna et al., 2006). Support for this hypothesis is the fact that *PKS* expression strongly localizes in tissues that involve strong differential growth. For instance, *PKS1* and *PKS4* are highly expressed in the hypocotyl elongation zone and *PKS1* is also expressed in root elongation region (Lariguet et al., 2003; Khanna et al., 2006; Boccalandro et al., 2008; Schepens et al., 2008). Asymmetric elongation in these regions leads to important adaptive responses such as phototropism and gravitropism.

Phys are well-known regulators of phototropism and gravitropism (Hangarter, 1997; Iino, 2006). Theys are proposed to modulate phototropic bending via at least two different mechanisms: (i) the attenuation of the gravitropic response which competes with phototropic bending (Lariguet and Fankhauser, 2004) and (ii) more direct mechanisms such as phot-signalling pathways and downstream auxin responsiveness (Stowe-Evans et al., 2001; Iino, 2006). Thus, because of their well-established roles during phyA signaling and their highly specific expression patterns in elongation zones, PKS function during organ growth orientation was extensively studied. Phototropism experiments were particular relevant since PKS1 and PKS4 expression is also strongly regulated by BL (Lariguet et al., 2003; Schepens et al., 2008).

R and FR light are well known to inhibit the gravitropic response (Hangarter, 1997). Recent work on gravi-reorientation of hypocotyls showed that this inhibition is in fact a consequence of the induced randomization of growth orientation by RL and FRL (Schepens et al., 2008). Under FR light, PKS4 acts negatively in this process in the phyA pathway. Thus, one proposed role for PKS4 is to act as an inhibitor of asymmetric growth (Schepens et al., 2008). PKS1 is also involved in RL-induced root phototropism (Molas and Kiss, 2008). Since asymmetric growth is a pre-requisite for tropic bending and organ-reorientation, the physiological roles of both PKS1 and PKS4 appear to be globally similar under RL.

The PKSs also positively regulate BL-induced phototropism. PKS1 plays an essential role in root phototropism (*pks1* resembles *phot1*) while in hypocotyl phototropism *PKS1* acts redundantly with *PKS2* and *PKS4* (*pks1pks2pks4* phenocopies *phot1*) (Lariguet et al., 2006; Boccalandro et al., 2008). This is consistent with the fact that PKS2 and PKS4 are exclusively expressed in aerial tissues (Lariguet et al., 2003; Schepens et al., 2008). PKS1 interacts with phot1 and the phot1 signalling element NPH3 *in vitro*, and these three proteins form a complex at the hypocotyl plasma membrane (Lariguet et al., 2006).

In both root and hypocotyl phototropism, curvature was preceded by an induction of *PKS1* expression that depended on phyA (Lariguet et al., 2003; Lariguet et al., 2006; Boccalandro et al., 2008; Schepens et al., 2008). Interestingly, genetic data indicate

that *PKS1* predominantly acts independently of phyA-modulation of gravitropism during BL-induced phototropism. Thus PKS1 appears to function predominantly in the phot1 pathway during BL-induced phototropism (Lariguet et al., 2006; Boccalandro et al., 2008).

In summary, the PKS family plays important functions during light-regulated growth processes. One particularly interesting property of these proteins is their capacity to bind both phy and phot photoreceptors and to act in different signaling pathways depending on the light conditions. In fact, *PKS1* loss-of-function and *PKS4* over-expression also affect growth orientation in darkness (Boccalandro et al., 2008; Schepens et al., 2008). This suggests that the PKS may have a role in asymmetric growth processes that is independent of light signalling.

5. Aim and methods

PKS-like genes are present in the genomes of all Angiosperms tested so far but appear to be absent in lower plants. The PKS are basic soluble proteins with no recognizable sequence motifs or signals for subcellular targeting. However, PKS1 was shown to be tightly associated with the plasma membrane but the anchorage mechanism is not yet known (Lariguet et al., 2006). On the other hand the subcellular localizations of PKS2, PKS3 and PKS4 have not been reported yet.

The fact that PKS1 can directly bind phot1 *in vitro* and is also associated with the phot1-interacting signaling element NPH3 *in vivo* suggests that PKS1 acts early in phot signaling. This raises an important question: are the PKS global regulators of phot signaling? If not, what branches of phot signaling do they act in?

Furthermore, since NPH3 function is closely linked with auxin redistribution, and since the PKSs are important regulators of orientated growth processes, another interesting question is: Are the PKS co-regulators of auxin transport?

The primary aim of my thesis was to globally analyse the role of the PKS in phot signaling. To do so, the involvement of each *PKS* gene in stomatal opening, chloroplast movements, leaf flattening and leaf positioning was investigated using a genetic approach.

Based on these results, I then used molecular and genetic approaches to investigate in more detail the function of PKSs in the phot signaling pathways they act in.

RESULTS

BRIEF INTRODUCTION TO RESULTS

The results are presented in three chapters.

In Chapter 1, published work on the role of the PKS in phototropism is presented (Lariguet et al., 2006). I begin by describing my contribution to this article and then present some related unpublished work that was done during the early stages of my thesis project. After summarizing the main findings, I propose a list of open questions raised by this work. I finally explain how these unresolved issues as well as early observations have contributed to shaping the aims of my thesis project.

In Chapter 2, I present our studies on the role of PKS in leaf flattening. This work has been submitted for review and is presented under the form of a manuscript.

In Chapter 3, I describe my results on the analysis of PKS function during leaf positioning. In this section, emphasis is given on the PKS that plays the most predominant roles in phot-mediated leaf responses: PKS2. The molecular mode of action of PKS2 during phot signalling is addressed in more details.

CHAPTER 1 – STUDY OF PKS ROLE DURING PHOTOTROPISM

1. Article – Lariguet et al., 2006

PHYTOCHROME KINASE SUBSTRATE 1 is a phototropin 1 binding protein required for phototropism

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Phototropism, or plant growth in response to unidirectional light, is an adaptive response of crucial importance. Lateral differences in low fluence rates of blue light are detected by phototropin 1 (phot1) in *Arabidopsis*. Only NONPHOTOTROPIC HYPOCOTYL 3 (NPH3) and root phototropism 2, both belonging to the same family of proteins, have been previously identified as phototropin-interacting signal transducers involved in phototropism. PHYTOCHROME KINASE SUBSTRATE (PKS) 1 and PKS2 are two phytochrome signaling components belonging to a small gene family in *Arabidopsis* (*PKS1–PKS4*). The strong enhancement of *PKS1* expression by blue light and its light induction in the elongation zone of the hypocotyl prompted us to study the function of this gene family during phototropism. Photobiological experiments show that the PKS proteins are critical for hypocotyl phototropism. Furthermore, PKS1 interacts with phot1 and NPH3 *in vivo* at the plasma membrane and *in vitro*, indicating that the PKS proteins may function directly with phot1 and NPH3 to mediate phototropism. The phytochromes are known to influence phototropism but the mechanism involved is still unclear. We show that PKS1 induction by a pulse of blue light is phytochrome A-dependent, suggesting that the PKS proteins may provide a molecular link between these two photoreceptor families.

Arabidopsis thaliana | NONPHOTOTROPIC HYPOCOTYL 3 | photomorphogenesis photoreceptors

Plants' survival depends on their ability to orient growth appropriately at the very beginning of their development. Plants need to reach the light and start photosynthesis before the seed reserves have been exhausted. They determine their direction of growth by sensing and responding to the gravity vector and the direction of light. These processes are called gravitropism and phototropism (1–3). *Arabidopsis thaliana* hypocotyls use gravity in darkness to orient their growth in the soil. But as soon as the seedlings perceive a weak source of light, even under the soil, gravitropism is repressed and phototropism predominates (3–5). Under low fluence rates of blue light, phytochrome A (phyA) is the predominant photoreceptor that triggers repression of gravitropism (6, 7). Light direction is perceived by the phototropin family [phototropin 1 (phot1) and phototropin 2 (phot2) in *Arabidopsis*] of UV-A/blue light sensors (2, 8). Phot1 is necessary and sufficient under a weak source of blue light, whereas phot1 and phot2 act redundantly to mediate phototropism under high blue light (9). Phot1 and phot2 are not only required for phototropism but also for chloroplast movement, stomatal opening, and leaf flattening. Together, these responses all are believed to maximize photosynthetic light capture while minimizing photodamage (8, 10). Phototropin-mediated responses are thus particularly important for normal plant growth under extreme (very low or very high) light conditions (11–13).

Despite the obvious importance of phototropism, the signaling mechanisms operating downstream of light perception are poorly understood. Light triggers a conformational change in the photoreceptor that activates its protein kinase activity, but very few specific phototropism signaling components have been identified (2, 14, 15). NONPHOTOTROPIC HYPOCOTYL 3 (NPH3) and ROOT PHOTOTROPISM 2 (RPT2) function as signal transducers in phototropism signaling (16–19). They belong to a plant-specific family of proteins possessing a BTB/POZ (broad complex, tramtrack, bric à brac/pox virus, and zinc finger) and a coiled-coil domain, both thought to be involved in protein–protein interaction (16). *nph3* null mutants show no phototropic curvature at any blue light fluence rates, whereas the *rpt2* mutant is impaired in phototropism only at high fluence rates (17, 18). Phot1, NPH3, and RPT2 all are associated with the plasma membrane, particularly in elongating cells (16, 20). NPH3 and RPT2 can physically interact with phot1 and each other (16, 18). Moreover, COLEOPTILE PHOTOTROPISM 1, a rice homologue of NPH3, acts upstream of the redistribution of auxin induced by unilateral illumination of the seedling, further indicating that these proteins function early in this signaling pathway (19). In addition to these components specifically acting in phototropism signaling, establishment of a gradient of auxin responsiveness is required to initiate asymmetric growth associated with not only phototropism, but also gravitropism (21).

The phytochromes modulate phototropism through mechanisms that remain to be molecularly elucidated (4, 5). Here we show that the phyA signaling components PHYTOCHROME KINASE SUBSTRATE (PKS) 1 and PKS2 (22) and PKS4, another member of this gene family in *Arabidopsis* (23), are required for phototropism. PKS1 is localized at the plasma membrane and can form a complex with phot1 and NPH3. Physiological analysis of *pks1*, *pks2*, and *pks4* loss-of-function mutants demonstrates that the PKS proteins are necessary for normal phototropism under weak intensities of blue light. Hence our findings define the PKS proteins as components of phot1 signaling and suggest that the PKS proteins may represent a link between phytochrome and phototropin signaling.

Results

The PKS Proteins Are Crucial for Hypocotyl Phototropism Under Low Fluence Rates of Blue Light. PKS1 and PKS2 are phytochrome-binding proteins acting as components of the very low fluence response (VLFR) branch of phyA signaling (22, 24). PKS1 expression is transiently induced by light precisely in the elongation zone of the root and hypocotyl (22). Elongation zones contain cells that elongate in response to tropic stimulations to

Conflict of interest statement: No conflicts declared.

Abbreviations: phot1, phototropin 1; NPH3, nonphototropic hypocotyl 3; PKS, phytochrome kinase substrate; phyA, phytochrome A; VLFR, very low fluence response.

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induce organ curvature (1, 2). Light induction of *PKS1* in the hypocotyl elongation zone (22) and strong up-regulation of *PKS1* by blue light (Fig. 4, which is published as supporting information on the PNAS web site) prompted us to test the involvement of PKS1 in phototropism. *PKS1* belongs to a small gene family that appeared at the emergence of angiosperms and consists of four members in *Arabidopsis* (*PKS1–PKS4*) (23). To test for possible functional redundancy among members of this gene family we isolated T-DNA insertion lines disrupting the coding sequence of *PKS4* (Fig. 5, which is published as supporting information on the PNAS web site) and constructed all possible mutant combinations among *pks1*, *pks2*, and *pks4*. There is currently no insertional mutant available in the *PKS3* gene.

The hypocotyls of *phot1* seedlings are randomly oriented when irradiated with a low fluence rate of blue light from above, because phyA represses gravitropism and phototropism is completely impaired in the absence of phot1 (6). We took advantage of this clear phenotype to test whether the PKS proteins play a role in phototropism. The growth orientation profile was determined for all of the available loss-of-function *pks1*, *pks2*, and *pks4* single, double, and triple mutants by using WT Col-O, *phyA*, *phot1*, and *nph3* as controls (Fig. 1*A*). Seedlings were classified into groups according to the angle of their hypocotyl relative to vertical (0–20°, 20–40°, 40–60°, and >60°). As reported (6), *phyA* hypocotyls were even more vertically oriented than the WT, and *phot1* hypocotyls were randomly oriented (Fig. 1*A*). The *pks1*, *pks2*, and *pks4* single mutants were subtly less vertically oriented than the WT. This tendency of random growth behavior was more pronounced in *pks1pks2*, *pks2pks4*, and particularly in the *pks1pks4* double mutants. Interestingly, the growth orientation profile of *pks1pks2pks4* hypocotyls was as random as in *phot1*, suggesting that, as with *phot1* mutants, the *pks1pks2pks4* mutants responded neither to light direction nor to gravity (Fig. 1*A*). This genetic analysis showed that PKS1, PKS2, and PKS4 had a function in determining the growth direction of hypocotyls. They seem to act in a redundant way, with PKS4 playing the major role. The similarity between the *phot1* and *pks* double- and triple-mutant phenotype suggested that the PKS proteins act positively in phot1 signaling.

To examine further whether the PKS proteins were implicated in phototropism, WT, *phot1*, *nph3*, and *pks1pks2pks4* seedlings were treated with unilateral light. Seedlings were illuminated for 3 days with a lateral source of low intensity blue light, and the final growth orientations were measured (Fig. 1*B*). As observed (6), WT hypocotyls were phototropic, whereas *phot1* hypocotyls no longer responded to the directional blue light and had an inhibited gravitropic response (Fig. 1*B*). As expected from previous studies the phenotype of *nph3* mutants was very similar to that of *phot1* mutants (16). As with the *phot1* and *nph3* mutants, hypocotyls of the *pks1pks2pks4* triple mutant did not direct their growth toward blue light and had an inhibited gravitropic response. This result indicates that PKS1, PKS2, and PKS4 were essential for phototropism but not for inhibition of gravitropism under long-term blue-light irradiation. When the different *pks* single, double, and triple mutants were grown in darkness, hypocotyls grew against the gravity vector as did the WT, *phot1*, and *nph3* mutants, whereas the agravitropic *arg1* mutant (25) was more randomly oriented (Fig. 6, which is published as supporting information on the PNAS web site). Taken together our results indicate that the *pks* mutants have a normal gravitropic response in darkness but are deficient for phototropism during long-term irradiation.

To test whether the PKS proteins are required for phototropism in etiolated seedlings stimulated by a short blue-light treatment, dark-grown seedlings were exposed to blue-light pulses (Fig. 1*C*). Under these conditions phot1 functions as the essential blue-light receptor mediating perception of directional light (26, 27). *phyA* mutants show a reduced phototropic response possibly because phyA is required to inhibit gravitropism and/or because the phytochromes are required to modulate the level or activity of phototropism signaling components (3, 6). Interestingly PKS1 induction by a pulse of blue light was phyA-dependent (Fig. 4*C*). In accordance with these expectations *phot1* mutants exhibited no detectable phototropic response to pulsed irradiation, whereas *phyA* mutants were ≈50%

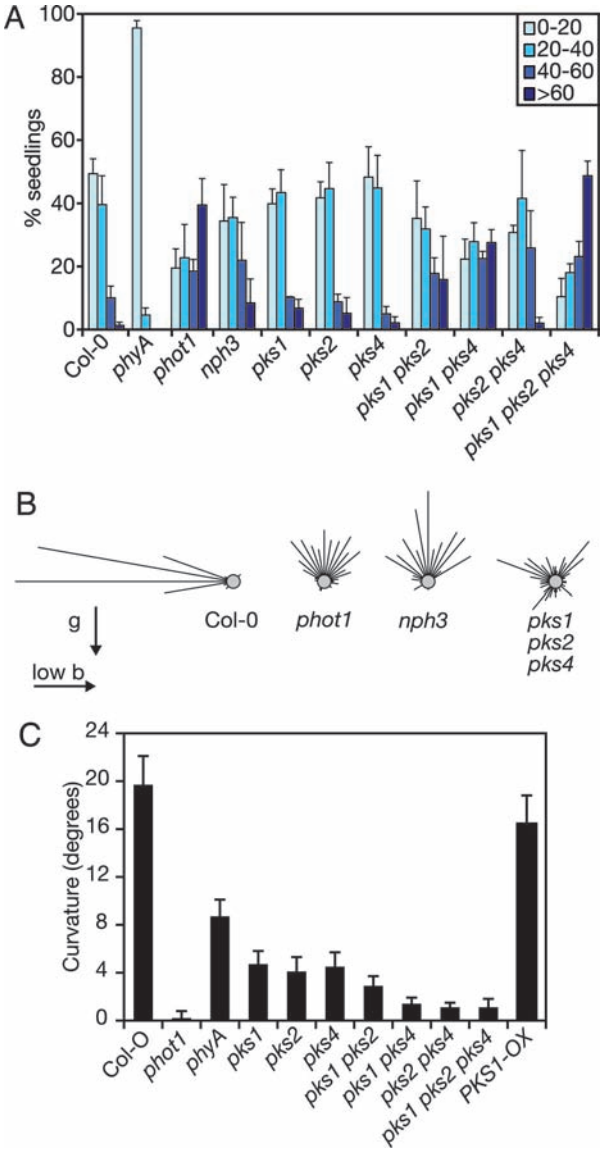

Fig. 1. PKS1, PKS2, and PKS4 are required for hypocotyl phototropism. (*A*) Hypocotyl growth orientation of Col-O, *phyA*, *phot1*, *nph3*, *pks1*, *pks2*, *pks4*, *pks1pks2*, *pks1pks4*, *pks2pks4*, and *pks1pks2pks4* grown on vertical plates for 3 days at 0.1 μmol·m^{-2}·s^{-1} blue light. The percentage of seedlings with hypocotyl angles relative to vertical (0°) is represented in four classes: 0–20°, 20–40°, 40–60°, and >60°. Data are average ± SD from three experiments with ≈50 seedlings for each experiment. (*B*) Hypocotyl growth orientation of Col-O, *phot1*, *nph3*, and *pks1pks2pks4* seedlings grown on vertical plates for 3 days subjected to continuous unilateral blue light (0.7 μmol·m^{-2}·s^{-1} coming from the left as indicated by the arrow). The repartition of the hypocotyl growth orientation is shown as circular histograms with 10° angle categories. The number of seedlings for each genotype is between 93 and 134. (*C*) Seedlings of the indicated genotypes were grown in darkness for 72 h and treated with six pulses of blue light (0.1 μmol·m^{-2} each) separated by 20 min of darkness. The hypocotyl phototropic curvature was determined 20 min after the last pulse. Data are average curvature angles ± SE with a minimum of 30 seedlings per genotype.

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as responsive as the WT (Fig. 1*C*). The *pks* single and multiple mutants exhibited phototropic responses that fell between those of the *phot1* and *phyA* mutants (Fig. 1*C*). The role of the different PKS proteins appeared to be partially redundant with the double mutants having a more pronounced phenotype than the single mutants (Fig. 1*C*). If the PKS proteins were influencing phototropism solely through a phyA-mediated mechanism we would have expected the *pks* mutants to exhibit phototropic responses at least as robust as those of the *phyA* mutant. Our results thus suggest that under pulsed conditions the PKS proteins function mainly in the phot1-dependent pathway. This interpretation is also consistent with the growth orientation of *pks1pks2pks4* triple mutants under long-term irradiation that is similar to that of *phot1* but distinct from the one of *phyA* (Fig. 1 *A* and *B*) (6). It is important to point out that etiolated *pks* mutants do not have a hypocotyl growth phenotype, indicating that their phototropism phenotype is not the result of a growth defect (22) (data not shown). Finally, overexpression of PKS1 did not lead to an increase in phototropic curvature, indicating that a higher level of PKS1 was not sufficient to enhance this physiological response (Fig. 1*C*).

PKS1 Is a Plasma Membrane-Associated Protein. Our physiological analysis demonstrated that PKS1, PKS2, and PKS4 are required for phot1-mediated phototropism under low fluence rates of blue light (Fig. 1). Primary sequence analysis of the PKS proteins indicates that they are basic soluble proteins devoid of a domain with a known function (24). In an attempt to address the molecular function of the PKS proteins, we examined the subcellular localization of PKS1 by using transgenic lines expressing PKS1-GFP. *PKS1* mRNA is transiently light-induced in the elongation zone of hypocotyls and roots of etiolated seedlings (22). Microscopic examination of transgenic seedlings expressing *PKS1*:*PKS1-GFP* confirmed this observation at the protein level (Fig. 7, which is published as supporting information on the PNAS web site, and data not shown). By using confocal microscopy we observed that the PKS1-GFP signal was mainly at the periphery of the cells, distinct from the subcellular localization of soluble GFP but very similar to the subcellular localization of plasma membrane-localized GFP (Fig. 2 *A*–*C*). Interestingly, this tissue-level and subcellular localization of PKS1-GFP is very similar to that of phot1 and NPH3, which is associated with the plasma membrane (16), and strongly expressed in elongating cells of etiolated hypocotyls (20) (Fig. 8, which is published as supporting information on the PNAS web site).

To examine whether, like phot1 and NPH3, PKS1 was membrane-associated we prepared microsomal and cytoplasmic fractions from 3-day-old WT seedlings. Cell fractionations were performed either from etiolated seedlings or after an additional 4 h of white-light treatment to allow induction of PKS1 protein expression (22). Phot1 was used as a positive control for microsomal proteins (20). As described (20), phot1 was membrane-associated in dark-grown seedlings, and a small fraction of phot1 was released into the cytosol upon light exposure. PKS1 protein accumulation was induced in seedlings exposed to white light compared with seedlings grown in darkness (22) (Fig. 2*D*). In both conditions PKS1 was detected mainly in the microsomal fraction, suggesting that PKS1 was associated with membranes (Fig. 2*D*).

PKS1, like all of the other PKS proteins, lacks any obvious membrane attachment sequence (24). To determine whether PKS1 is a peripheral membrane protein, pellets of microsomal fractions were treated with high salt, alkali buffer, or the detergent Triton X-100. PKS1 could only be released from microsomes by solubilization with 1% Triton X-100 (Fig. 2*E*), suggesting that PKS1 is actually membrane-anchored rather than a peripheral membrane protein. This possibility was confirmed by using a Triton X-114 partitioning experiment that allows the separation of hydrophilic proteins in the aqueous phase from lipophilic proteins in the detergent phase (28) (data not shown).

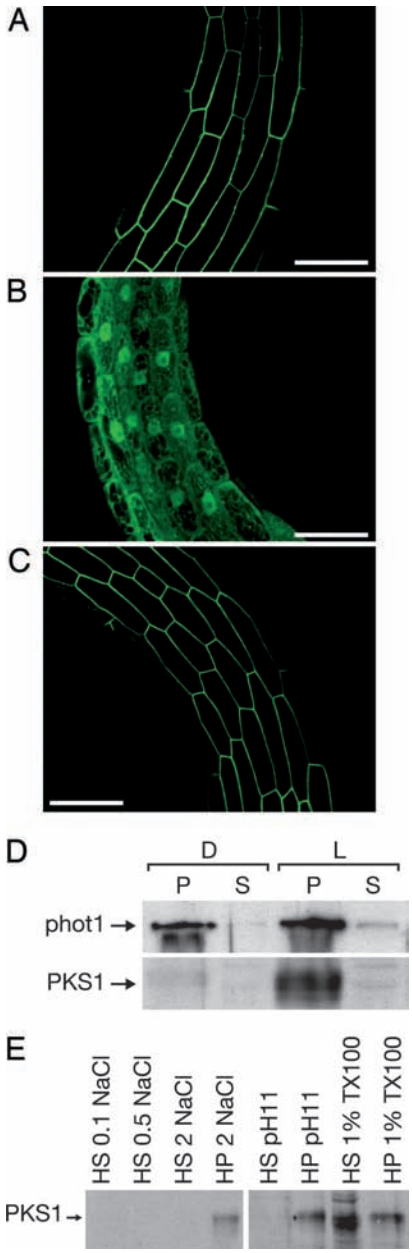

Fig. 2. PKS1 protein is associated with the plasma membrane. (*A*) Localization of PKS1-GFP in 2-day-old etiolated *35S:PKS1-GFP* seedlings. The seedling was imaged with a confocal microscope with a ×40 objective with a 488-nm excitation line. (*B*) Localization of GFP in 2-day-old etiolated *35S:GFP* seedlings. The seedling was imaged as above. (*C*) Localization of plasma membrane-targeted GFP in 2-day-old etiolated *35S:GFP-LTI6b* seedlings. The seedling was imaged as above. (Scale bars: 50 μm.) (*D*) Immunoblot analysis of PKS1 localization after cell fractionation of etiolated seedlings (D) or etiolated seedlings treated with 4 h of white light (L). Microsomal (P) or cytosolic (S) fractions were separated by SDS/PAGE, transferred onto nitrocellulose, and subjected to Western blot analysis by using phot1- or PKS1-specific antibodies. (*E*) Immunoblot analysis of PKS1 localization after solubilization of microsomal fractions from etiolated seedlings treated for 4 h with white light. Microsomal pellets were treated with 0.1 M NaCl, 0.5 M NaCl, 2 M NaCl, carbonate buffer (pH 11), or 1% Triton X-100. HS (high speed supernatant) corresponds to the solubilized proteins and HP (high speed pellet) corresponds to the proteins still associated with the microsomes. Those fractions were separated by SDS/PAGE, transferred onto nitrocellulose, and subjected to Western blot analysis using a PKS1-specific antibody.

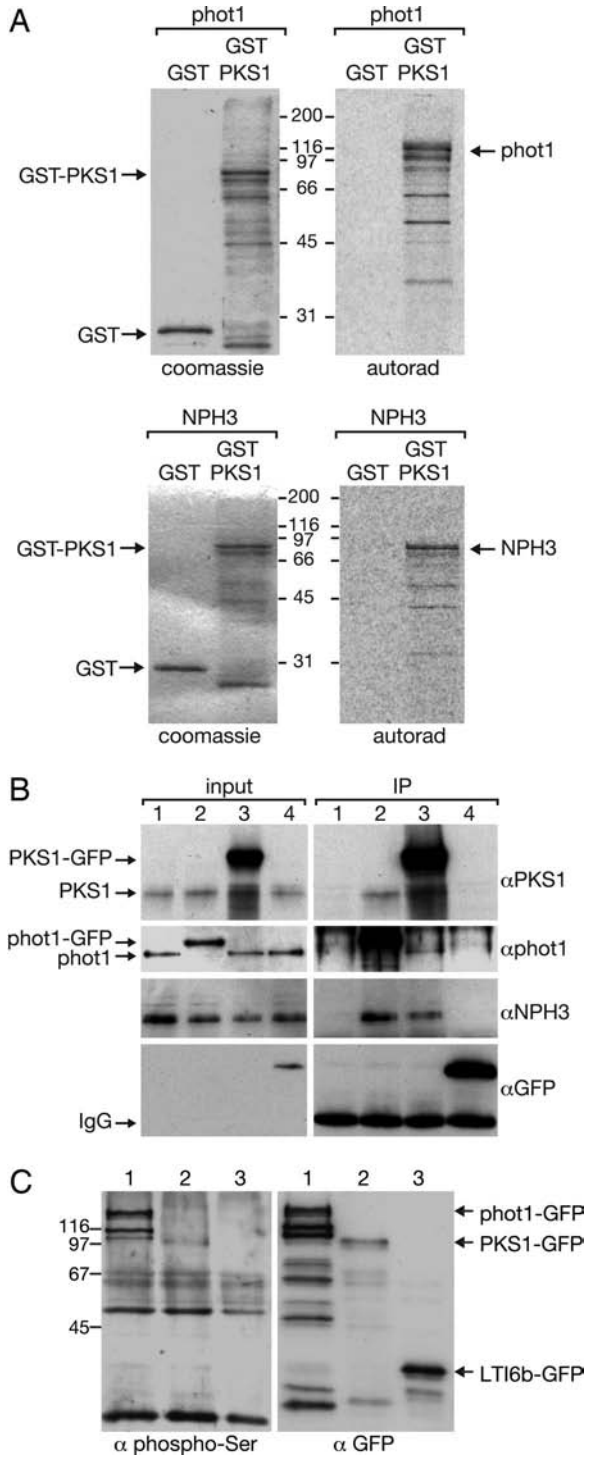

Fig. 3. PKS1 interacts with phot1 and NPH3 *in vitro* and *in vivo*. (*A*) PKS1 interacts with phot1 and NPH3 *in vitro*. Bacterially produced GST or GST-PKS1 were bound onto glutathione-agarose beads and incubated with *in vitro*-transcribed and -translated PHOT1 or NPH3. Beads were extensively washed, and proteins binding to the beads were eluted with reduced glutathione and separated by SDS/PAGE. (*Left*) The Coomassie blue-stained gels. (*Right*) Autoradiograms of the same gel. Note: GST-PKS1 is unstable in *Escherichia coli*, leading to a number of breakdown products in addition to the ≈80-kDa protein full-length fusion protein. *In vitro*-transcribed and -translated NPH3 and PHOT1 gave rise to a number of smaller proteins that are the result of either degradation or internal translation initiation. (*B*) PKS1 interacts with phot1 and NPH3 *in vivo*. Solubilized microsomal fractions were prepared from etiolated seedlings treated for 150 min with white light. The following genotypes were used in this assay: Col (lanes 1), *PHOT1*:*PHOT1-GFP phot1* (lanes 2), *35S*:*PKS1-GFP* (lanes 3), and *35S*:*LTI6b-GFP* (lanes 4). An aliquot was mixed 1:1 with 2XFSB (input). These extracts were immunoprecipitated with a covalently attached anti-GFP antibody coupled to agarose beads and specifically bound proteins eluted with 2XFSB (IP). Proteins were separated on SDS/PAGE, Western-blotted, and probed with various antibodies as described in *Materials and Methods*. (*C*) PKS1-GFP and phot1-GFP were phosphorylated *in vivo*. Immunoprecipitates of *PHOT1*:*PHOT1-GFP phot1* (lanes 1), *35S*:*PKS1-GFP* (lanes 2), and *35S*:*LTI6b-GFP* (lanes 3) were separated by SDS/PAGE and Western-blotted as above but probed with anti-GFP or anti-phospho-Ser/Thr antibodies.

Molecular Interactions Among PKS1, Phot1, and NPH3. Phot1, NPH3, and PKS1 all localize to the plasma membrane and are involved in phototropic responses (16, 20) (Figs. 1 and 2). We therefore decided to test whether those proteins interact with each other. We first examined whether PKS1 was able to bind phot1 and NPH3 *in vitro* by using a GST pull-down assay (Fig. 3*A*). Bacterially produced GST or GST-PKS1 fusions were bound to glutathione-agarose beads. The beads were incubated with ^{35}S-Met-labeled *in vitro*-transcribed and -translated PHOT1 or NPH3. The bound proteins were separated by SDS/PAGE, and the stained gel was subjected to autoradiography. This experiment showed that GST-PKS1 could interact with phot1 and NPH3, whereas GST did not interact with either of them (Fig. 3*A*).

To determine whether these interactions also occur *in vivo* we performed coimmunoprecipitation experiments with protein extracts from etiolated *Arabidopsis* seedlings treated for 150 min with white light to induce PKS1 expression and activate the phototropins (Fig. 3*B*). Microsomes were isolated from four different genotypes: Col-O, a transgenic line expressing the phot1-GFP fusion driven by the *PHOT1* promoter (*phot1*, *PHOT1*:*PHOT1-GFP*) (20), a line overexpressing PKS1-GFP (*35S*:*PKS1-GFP*) (24), and finally a line expressing a plasma membrane-localized GFP fusion protein (GFP fused to LTI6b) (29). Solubilized microsome extracts were incubated with an anti-GFP antibody linked to agarose beads. The beads were extensively washed, and the proteins specifically bound to the anti-GFP beads were analyzed by immunoblotting. Phot1-GFP, PKS1-GFP, and LTI6b-GFP all were present in the microsomal fraction and efficiently immunoprecipitated by the anti-GFP antibody (Fig. 3*B*). Given that the seedlings were exposed to white light before and during the immunoprecipitation experiment, we expected PKS1-GFP and phot1-GFP to be phosphorylated (2, 8). To test the phosphorylation status of phot1 and PKS1 we probed the immunoprecipitated proteins with an anti-phospho Ser/Thr antibody and an anti-GFP antibody as a loading control (Fig. 3*C*). PKS1-GFP and phot1-GFP were recognized by the anti-phospho Ser/Thr antibody, whereas LTI6b-GFP was not (Fig. 3*C*). This result indicates that phot1-GFP and PKS1-GFP were indeed phosphorylated during the *in vivo* immunoprecipitation and ruled out the possibility that the phosphorylation occurred on the GFP moiety.

Interestingly, PKS1 coimmunoprecipitated with phot1-GFP and phot1 coimmunoprecipitated with PKS1-GFP, whereas neither of those proteins were present in the Col-O and LTI6b-GFP controls, showing that phot1 and PKS1 interact *in vivo* (Fig. 3*B*). Moreover, NPH3 coimmunoprecipitated with both phot1-GFP and PKS1-GFP, confirming the previously described interactions between NPH3 and phot1 (16) and the *in vitro* interaction we observed between NPH3 and PKS1 (Fig. 3). Both NPH3 and PKS1 coimmunoprecipitated with phot1-GFP (Fig. 3*B*). Conversely, NPH3 and phot1 both coimmunoprecipitated with PKS1-GFP, whereas NPH3 protein was not pulled down in control WT and LTI6b-GFP-expressing seedlings (Fig. 3*B*). The three proteins were thus present as a complex in solubilized microsomes. Finally, PKS1-GFP and

phot1-GFP did not coimmunoprecipitate the membrane-associated protein DET3 (30), highlighting the specificity of the interactions observed here (data not shown). It is noteworthy that the phot1–PKS1 interaction was observed in phot1–GFP-expressing plants where both proteins were present at WT levels (20) (Fig. 3*B*). The physical interaction thus occurred *in planta* at physiological concentrations of the two proteins. Our molecular data thus indicate that PKS1, NPH3, and phot1 are likely to form a complex at the plasma membrane. This observation is entirely consistent with and supports our physiological data showing that the PKS proteins are important for phototropism.

Discussion

Our photobiological experiments establish an important role for the PKS proteins during hypocotyl phototropism. The facts that PKS1 and PKS2 act in phyA signaling (22) and that *phyA* mutants are impaired in phototropism (3–5) suggest, at first glance, that the phototropism phenotype of *pks* mutants may be exclusively caused by alterations in phyA signaling. Two distinct sets of observation make this hypothesis unlikely: first, *pks1* and *pks2* mutants have an increased phyA VLFR when treated with pulses of far red light, whereas the *pks1pks2* double mutant shows a normal VLFR (22). In contrast, in response to a pulse of blue light the *pks1* and *pks2* mutants have a weaker phototropic response than *phyA*, a phenotype that is further enhanced in the *pks1pks2* double mutant (Fig. 1*C*). The phenotypes of *pks1* and *pks2* mutants are thus distinct when comparing far red and blue light. The PKS proteins appear to function as negative regulators of the phyA-VLFR (22), but positive regulators of phototropism in blue light (Fig. 1). Second, under long-term irradiation experiments the *pks* mutants behaved similarly to *phot1* and *nph3* mutants and very differently from the *phyA* mutant (Fig. 1 *A* and *B*). We have previously proposed that the reduced phototropic response of *phyA* mutants results from a reduced inhibition of gravitropism (6, 7). However, in contrast to *phyA* mutants, *phot1*, *nph3*, and *pks1pks2pks4* mutants clearly show an inhibition of the gravitropic response but exhibit no phototropic response under long-term low fluence rate blue-light illumination (6, 7) (Fig. 1*B*). The most parsimonious interpretation of these results is that the PKS proteins are positive regulators of phot1 signal transduction in blue light.

Given that phototropin signaling components are differentially required for the different phototropin responses (2, 8), it will be interesting to test whether the PKS proteins are also important for additional phototropin responses. PKS1, PKS2, and PKS4 do not appear to control leaf flattening by themselves, because *pks1pks2pks4* triple mutants have WT leaves that are very easy to distinguish from the curled leaves of *phot1phot2* double mutants (Fig. 9, which is published as supporting information on the PNAS web site). Future experiments should determine whether the PKS proteins regulate chloroplast movements and stomatal aperture.

The interpretation of our genetic results functionally coincides with the tissue distribution, subcellular localization, and protein–protein interaction data obtained for PKS1 (Figs. 2, 3, and 7). PKS1, phot1, and NPH3 are highly expressed in the hypocotyl elongation zone (20, 22) (Figs. 7 and 8). All three proteins are rather tightly associated with the plasma membrane through a mechanism that remains to be identified (16, 20) (Fig. 2). Finally, PKS1 strongly interacts with phot1 and NPH3 both *in vivo* and *in vitro* (Fig. 3). The fact that phot1-GFP can interact with PKS1 *in vivo* when both proteins are expressed at WT concentrations is a strong indication that this interaction is physiologically meaningful.

The existence of phototropin signaling elements that would be induced by the phytochromes has been postulated (3). Phytochrome-mediated induction of *PKS1* and *PKS2* expression (22) may thus partly explain the reduced phototropism in *phyA* mutants in response to blue-light pulses (Fig. 1*C*) (27). Indeed, PKS1 protein and mRNA are light-induced by a phyA-dependent VLFR (22). Moreover, we have shown that a pulse of blue light induces PKS1 protein levels and that this induction is lost in *phyA* mutants (Fig. 4*C*). Thus a pulse of blue light suffices to trigger both phototropism and phyA-dependent induction of PKS1. There may be additional levels of regulation whereby the PKS proteins could enable a coordination of phytochrome and phototropin action. Our coimmunoprecipitation results indicate that, under our assay conditions, both phot1 and PKS1 were phosphorylated (Fig. 3*C*). Phosphorylation may thus represent another level of regulation of this interaction *in planta*. We would, however, like to point out that *in vitro*-transcribed and -translated phot1 can interact with bacterially produced PKS1 (Fig. 3*A*), suggesting that plant-specific phosphorylation is not a prerequisite for this interaction. Finally, given that PKS1 and PKS2 are capable of interaction with the phytochromes *in vitro* (22) and that our data show that PKS1 interacts with phot1 *in vivo* (Fig. 3*B*), the PKS proteins may represent a link between these two photoreceptor families that have long been known to cooperate during the early steps of phototropism (3, 4). Such a cooperation between the phytochromes and phototropins is not incompatible with the independent effects we have observed for phyA and phot1 in the control of hypocotyl growth orientation in long-term experiments (6).

Materials and Methods

Plant Material and Growth Conditions. The Columbia (Col-O) ecotype of *A. thaliana* was used as the WT. All of the mutant alleles were in the Col-O background. The mutants were the following: *phot1-5* (31), *phyA-211* (32), *nph3-6* (16), *arg1-42* (Salk T-DNA insertion allele in *ARG1* from the laboratory of P. Masson, University of Wisconsin, Madison), *pks1-1*, *pks2-1* (22), and *pks4-1* (this study). Seeds were surface-sterilized and plated as described (6). With the exception of pulse-light experiments (see below), experiments were performed with continuous blue light at 22°C as described (6).

Generation of Mutants. The *pks4-1* mutant was identified by PCR-screening 40,000 T-DNA insertion lines using the *PKS4* (At5g04190)-specific primer CF259 (5′-GGAATCATCTC-CCAAGTTCCCAACTCGTGA-3′) and the T-DNA-specific primer JMLB1 (5′-GGCAATCAGCTGTTGCCCGTCTCACT-GGTG-3′). The PCR conditions were as described (33). The exact insertion site, determined by sequencing the PCR product, was after the 114th codon. The kan^R/kan^S ratio indicated the presence of a single T-DNA in the line, and the line was backcrossed to Col-O before future analysis. *pks4-1* was genotyped by PCR using a primer pair that detects the presence of the T-DNA (JMLB1, 5′-GGCAATCAGCTGTTGCCCGTCTCACTGGTG-3′ and CF329, 5′-CTTGGGACTCGTAGGATTCA-3′) and a primer pair to test for homozygocity (CF329 and CF262, 5′-CAATGGCG-CAAACTACTGTC-3′). The phenotypes observed for *pks4-1* were confirmed with *pks4*-2, a second allele obtained from the GABI collection (line 312E01) (I.S. and C.F., unpublished work) (34). *pks* double and triple mutants were obtained by crossing. Genotyping of *pks1* and *pks2* was performed as described (22), and *pks4-1* genotyping was performed as described above.

Hypocotyl Growth Orientation. For long-term irradiation experiments seedlings were grown on vertically oriented half-strength Murashige and Skoog plates treated and measured as described (6). Phototropism in response to pulses of blue light was performed as described (27).

Transgenic Plants. Transgenic lines expressing PKS1-GFP under the control of the *PKS1* promoter were obtained by cloning a

4.3-kb sequence 5′ of the PKS1 initiator ATG upstream of the PKS1-cDNA fused to GFP5-S65T and the rbcs terminator into pPZP212 (35) in the EcoRI and HindIII cloning sites, to give rise to pCF334. pCF202 is the same construct but with a 35S promoter. These constructs were transformed into Col-O by *Agrobacterium*-mediated transformation by using the spray method (36). Single insertion lines were selected based on the ratio of kan^R/kan^S and homozygous lines were used for the study. The *phot1-5*, *PHOT1:PHOT1-GFP* line was provided by Winslow Briggs (Carnegie Institution of Washington, Stanford, CA) (20). Seedlings overexpressing the GFP-LTI6b fusion and PKS1 were as described (29).

Microscopy and Biochemical Techniques. Detailed procedures are provided in *Supporting Text*, which is published as supporting information on the PNAS web site.

We thank Winslow Briggs for providing *PHOT1:PHOT1-GFP phot1* seeds and the phot1 antibody; Patrick Masson for providing *arg1-42* seeds; Karen Schumaker (Universität Tübingen, Tübingen, Germany) for DET3 antibodies; Bernd Weisshaar (Max-Planck-Institut für Züchtungsforschung, Cologne, Germany) for providing the GABI line 312E01 generated in the context of the GABI-Kat program; Ariane Honsberger, Angélique Vaucher, and Dany Rifat for technical support; the University of Lausanne microscopy platform for help with confocal microscopy; Nicolas Roggli for artwork; and Christian Hardtke and two anonymous reviewers for helpful suggestions on the manuscript. This work was supported by the University of Lausanne (C.F.), Swiss National Science Foundation Grant PP00A-103005, a National Center of Competence in Research Plant Survival grant (to C.F.), a Marie Heim Vögtlin Program fellowship (to P.L.), National Science Foundation Grants IBN-0415970 and IBN-0114992 (to E.L.), long-term fellowships from the EMBO and the Roche Foundation (to I.S.), a Toyobo Biotechnology Foundation postdoctoral fellowship (to C.K.), and a University of Missouri Life Sciences Predoctoral Fellowship (to D.H.).

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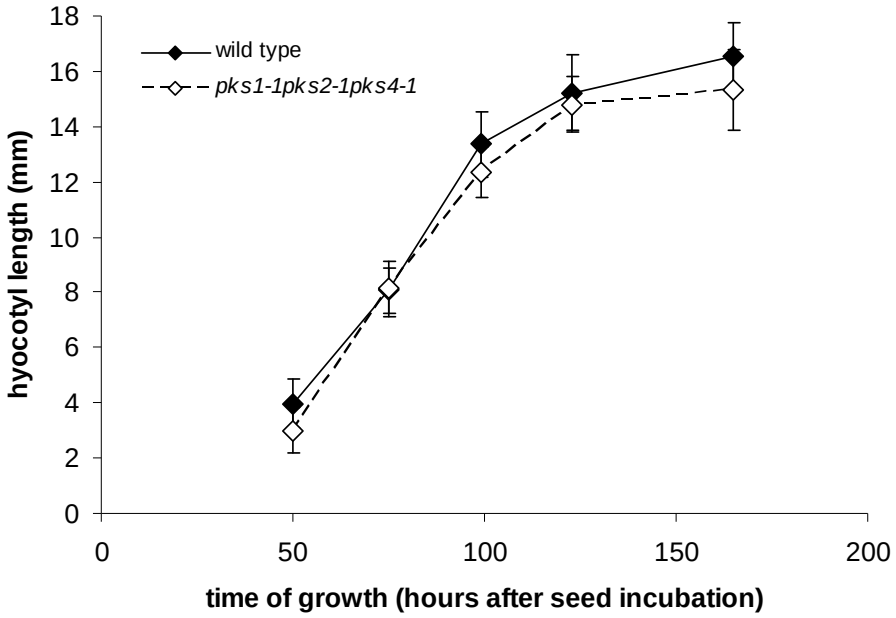

Figure 1: Kinetics of hypocotyl growth in etiolated *pks1pks2pks4* mutant and wild type.

Seeds were stratified for 3 days in dark at 4°C and then exposed to 80 µmol.m^{-2}.s^{-1} white light for 8 hours to synchronise germination. At each time point, dark-growing seedlings were collected and the length of their hypocotyls measured using the ImageJ image analysis software. Data shows mean ± standard deviation for 38<n<49 seedlings, one experiment.

2. Contributions to the article

2.1. Defective phototropism in pks1pks2pks4 *is not due to impaired hypocotyl elongation*

A pre-requisite for organ bending is the potential for asymmetric growth (or elongation) along the axes of that organ (Esmon et al., 2005; Whippo and Hangarter, 2006). To control that defective hypocotyl phototropism in *pks* mutants was not a consequence of defective hypocotyl elongation, kinetics of growth in *pks1pks2pks4* and wild type etiolated seedlings of similar growth were compared. Results clearly show that seedlings had similar hypocotyl elongation rates when grown in the dark (Figure 1). The growth curve covers the development stages of seedlings in which phototropism were assayed (in both long-term and short-term phototropism experiments) further indicating that impaired phototropism was an unlikely consequence of elongation defects (Figure 1 of article).

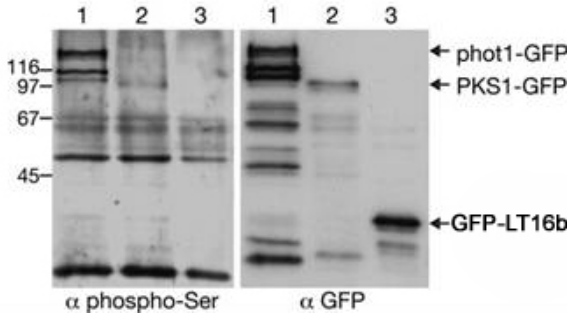

Figure 2: Figure 3-C of article -"PKS1-GFP and phot1-GFP were phosphorylated *in vivo*".

Immunoprecipitates of PHOT1;;PHOT1-GFP phot1 (lanes 1), 35S::PKS1-GFP (Lanes2) and 35S::GFP-LT16b (lanes 3) were separated by SDS-PAGE, Western-blotted and probed with anti-GFP or anti-phospho-Ser/Thr antibodies.

2.2. PKS1 and phot1 in immunoprecipitation complexes are phosphorylated

This experiment was done with Isabelle Schepens.

Genetic studies showed that *PKSs* control phototropism and molecular studies showed that PKS1 can associate with phot1 and the essential signaling component NPH3 at the plasma membrane (Figures 1-3 of article). We then decided to test the phosphorylation status of PKS1 and phot1 because it provided information about the biochemical state of these proteins in the complex. To do so we analysed PKS1-GFP and phot1-GFP and GFP-LT16b proteins obtained in high amounts from anti-GFP immunoprecipitation elutions (IP). These proteins were obtained from 3-day-old dark-grown seedlings illuminated with 80 µmol.m^{-2}.s^{-1} WL for 150 mins. Light signalling pathways were thus activated in these seedlings. Using anti-phospho-Ser/Thr antibodies we could show that PKS1-GFP and phot1-GFP were phosphorylated but the GFP moiety of the tagged proteins was not (Figure 2). This result indicates that in illuminated seedlings both PKS1 and phot1 were phosphorylated in the same complex *in vivo*, suggesting that these proteins were present in some activated form in the complex (Fankhauser et al., 1999; Cho et al., 2007; Christie, 2007; Inoue et al., 2008b). It is not known yet whether PKS1 phosphorylation was regulated by phot1, or phyA, or both *in vivo*. As discussed in the article, the regulation of PKS1 phosphorylation by phyA may represent another means by which phyA modulates phototropism (Iino, 2006).

Figure 3: Adapted from Supporting Figure 9 of article – "*pks1pks2pks4* triple mutants have WT-shaped leaves".

(A) WT (Col-0), *pks1pks2pks4* triple mutants, and *phot1phot2* double mutants (Lariguet and Fankhauser, 2004) were grown for 31 days at 22°C and 60% relative humidity in a 16-h-light (120 $\mu mol.m^{-2}.s^{-1}$ WL), 8-h-dark photoperiod.

(B) Adaxial view of three mature leaves of one representative plant.

2.3. PKS *loss-of-function mutants do not show obvious leaf epinasty phenotype*

Because phototropism is abolished in the *pks1pks2pks4* mutant (*pks1pks2pks4* phenocopies *phot1*; Figure 1 of article) we asked whether *PKS* genes could also play crucial roles in other phototropin-regulated processes. As shown in Figure 3, *pks1pks2pks4* does not resemble *phot1phot2* and has wild-type leaves, suggesting that PKS1/PKS1/PKS4 are not strictly required for leaf flattening.

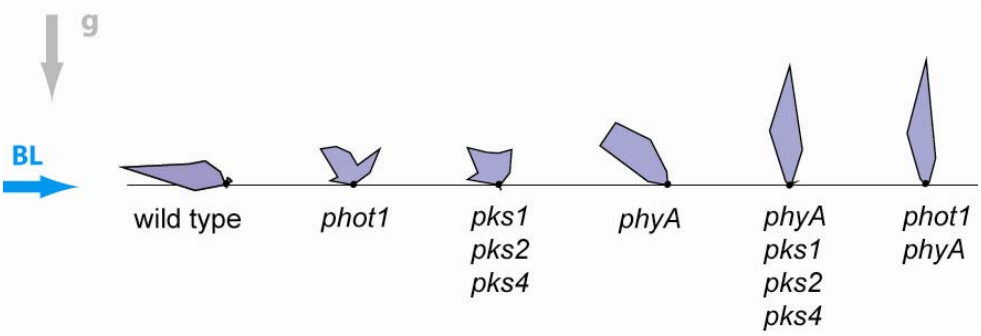

Figure 4: *phyApks1pks2pks4* growth orientation resembles *phyAphot1*.

Hypocotyl growth orientation of seedlings grown on vertical plates for 67 hours subjected to continuous unilateral blue light (0.7 µmol.m^{-2}.s^{-1}, blue arrow). Grey arrow indicates the direction of gravity vector. The repartition of the hypocotyl growth orientation is shown as radar histograms where distance from origin (black dot) is proportional to frequency of hypocotyl growth orientation angles. Data shows angle measurement for 36<n<46 seedlings per plate, 2 plates, one experiment.

3. Other experiments related to the study of PKS role in phototropism

3.1. The phyApks1pks2pks4 *phenotype supports a role for PKS in the phot1 pathway*

An early aim of my PhD thesis was to analyse the role of *PKS* in hypocotyl growth orientation (phototropism and gravitropism). Since the PKS were previously shown to regulate phyA signalling (Lariguet et al., 2003) and since phyA can strongly modulate phototropism (Hangarter, 1997; Lariguet and Fankhauser, 2004), one initial hypothesis was that the PKS might control phototropism by acting downstream of phyA. However, as discussed in the article, this hypothesis is highly unlikely for two main reasons: (i) *pks* phenotypes are stronger than the *phyA* phenotypes (they resembled more *nph3* and *phot* than *phyA*) and (ii) PKS1 / PKS2 play antagonistic roles during phyA-regulated de-etiolation (Lariguet et al., 2003) while these two proteins act redundantly during phototropism (Figures 1-A, C in article).

To further test the contribution of PKS1/PKS2/PKS4 in phyA- and phot1-regulation of hypocotyl growth orientation under lateral blue light, the phenotype of *phyApks1pks2pks4* was analyzed in long-term phototropism experiments. As shown in Figure 4 *phyApks1pks2pks4* phenocopied *phyAphot1*, supporting the hypothesis that the PKS predominantly act in the phot1 pathway under these conditions.

To further verify whether the PKS could play a role downstream of phyA in addition to phot1, epistasis study between *phot1* and *pks1pks2pks4* were still required (phenotype of the *phot1pks1pks2pks4* mutant). In fact, *pks1pks2pks4* hypocotyls appeared less gravitropic than *phot1* hypocotyls, which remained weakly gravitropic (Figure1B of the article). This suggested that the PKS might have (for instance) a negative role in phyA-mediate inhibition of gravitropism (Lariguet and Fankhauser, 2004). These interesting genetic experiments were continued by other members of the lab with the aim of understanding in detail the role of the PKS in light signalling pathways controlling hypocotyls growth orientation.

4. Conclusions and open questions that shaped my thesis project

The main conclusions of this article are:

- PKS1 represents a new phot1 signaling element that acts in phototropism
- *PKS* family members have redundant roles in this process
- The *PKS* family probably represent a key step in phot1 signaling pathway controlling phototropism
- PKS1 forms a complex with the phot1 signalling component NPH3 at the plasma membrane
- PKS1 represents a link for phyA modulation of phototropism. One mechanism of this link is the control of *PKS1* expression by phyA under BL
- Phototropism is another developmental process controlled by the PKS

Open questions that arise from this study are:

1/ Since PKS1 directly interacts with phot1 *in vivo*, are the PKS global regulators of phototropin signalling?

2/ What is the molecular mode of action of PKS1 with NPH3 in the phot1 pathway controlling phototropism?

3/ How are the PKS involved in the coordinated regulation of phototropism (and other photomorphogenesis processes) by both phy and phot signalling pathways?

During the early stages of my thesis project I performed experiments related to Questions 2 and 3. For instance, Isabelle Schepens and I analyzed the influence of different photoreceptors on transcript and protein levels of PKS1 in response to a blue-light-induced VLFR. I also continued to study the role of PKS in the regulation of hypocotyl growth orientation (data not shown).

In parallel, I received from Patricia Lariguet and Martine Trevisan some *nph3pks* mutants that displayed highly epinastic leaves. This interesting phenotype attracted our attention towards Question 1: the PKS may act more globally in phototropin signalling since leaf flattening is another important process controlled by phots (Sakamoto and Briggs, 2002; Takemiya et al., 2005; Inoue et al., 2008a). We thus decided to systematically analyze the role of each *PKS* in phot-controlled processes, namely chloroplast movements, stomatal opening, leaf flattening and leaf positioning.

Chapters 2 and 3 present the results of these studies

CHAPTER 2 – STUDY OF PKS ROLE IN LEAF FLATTENING

1. Submitted article

The *Arabidopsis* PHYTOCHROME KINASE SUBSTRATE genes are phototropin signalling components involved in auxin-regulated leaf flattening

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This work was submitted to the journal Plant Cell for review. The structure of this Chapter is that of a submitted manuscript.

Abstract

In *Arabidopsis thaliana*, phototropins (phot1 and phot2) fine-tune the photosynthetic status of the plant in response to environmental light variations by controlling several important adaptive processes. These processes include phototropism, leaf flattening, stomatal opening and chloroplast movements. The PHYTOCHROME KINASE SUBSTRATE (PKS) family comprises four members in Arabidopsis (PKS1 to PKS4). PKS1 acts as a phot1 signalling element during phototropism. Here we show that PKS1 and PKS2 are also required for leaf flattening, but PKS1/2/4 are dispensable for chloroplast movements and stomatal opening. PKS1 and PKS2 act selectively in the same set of phot-controlled processes than the signaling element NON PHOTOTROPIC HYPOCOTYL 3 (NPH3). Taking advantage of the different phenotypes of the epinastic *phot1phot2* and *nph3pks1pks2pks4* mutants we found that leaf flattening contribute greatly to plant growth, even under favourable light conditions. Genetic and molecular studies indicated that PKS1, PKS2 and NPH3 can act in both phot1 and phot2 pathways controlling leaf flattening. In addition, several lines of evidence indicate that PKS protein function in auxin-regulated leaf flattening. Together with previous findings, these results indicate that the PKS represent another important family of phototropins signaling proteins that specifically regulate asymmetric growth processes. We propose that PKS2, PKS1 and NPH3 provide a link between phot-mediated light signalling and auxin-regulation of leaf flattening.

Introduction

Plants constantly monitor the light in their natural environment to optimize light capture for photosynthesis and growth (e.g. shade avoidance and phototropism) and to time important developmental transitions (e.g. germination and flowering) (Neff et al., 2000; Briggs and Christie, 2002; Franklin and Whitelam, 2005). To do so, plants utilize photoreceptors that allow them to sense changes in light period, direction, wavelength composition and intensity. In higher plants, the main types of photoreceptors are the red/far-red light-absorbing phytochromes and the UV-A/blue light-sensing phototropins, cryptochromes and Zeitlupe protein families (Chen et al., 2004; Jiao et al., 2007; Demarsy and Fankhauser, 2009). The signaling pathways triggered by these photoreceptors are integrated to fine-tune the plant's responses to the ever-changing light environment (Casal, 2000; Franklin and Whitelam, 2004; Iino, 2006).

In Arabidopsis, phototropin1 (phot1) and its paralog phot2 were respectively discovered as primary photoreceptors for blue light-induced hypocotyl phototropism and for high light-induced chloroplast avoidance movements (Liscum and Briggs, 1995; Huala et al., 1997; Jarillo et al., 2001; Kagawa et al., 2001). Subsequent studies have shown that phototropins regulate a wide set of adaptive processes including chloroplast accumulation under low light, stomatal opening, leaf flattening, leaf positioning and root phototropism (Sakai et al., 2000; Kinoshita et al., 2001; Sakai et al., 2001; Sakamoto and Briggs, 2002; Takemiya et al., 2005; Inoue et al., 2008a). Thus, phototropins are believed to optimize the photosynthetic status of plants particularly under unfavorable environments such as extremely high light, weak illumination, and drought (Kasahara et al., 2002; Takemiya et al., 2005; Galen et al., 2007).

Phot1 and phot2 regulate these processes selectively and in a fluence-dependent manner. Phot1 mediates the chloroplast accumulation, leaf positioning, leaf flattening and phototropic responses under very low light. Under higher light intensities, phot2 acts redundantly in these processes (Sakai et al., 2001). Phot2 also specifically controls the chloroplast avoidance response induced by high light. For stomatal

opening, phot1 and phot2 act redundantly over a broad range of light intensity (Kinoshita et al., 2001; Doi et al., 2004). How phot1 and phot2 selectively and co-ordinately control such a broad variety of processes is a fascinating and important question.

Phototropins are Ser/Thr kinases belonging to the AGC family (cAMP-dependent protein kinase, cGMP-dependent protein kinase, and phospholipids-dependent protein kinase C) (Bogre et al., 2003). Two LOV (light, oxygen, or voltage) photosensory domains that bind to the blue light-absorbing chromophore FMN (flavin mononucleotide) induce and regulate the kinase activity (Harper et al., 2003; Christie, 2007). Phototropin activation and signaling has been well studied at the level of the photoreceptor itself (Tokutomi et al., 2008; Demarsy and Fankhauser, 2009). However, downstream signaling is less well described. For instance, no direct substrate for the kinase activity has been identified so far *in planta* (apart from the phots themselves) (Sullivan et al., 2008; Inoue et al., 2008b) . Nonetheless, several phototropin signaling components have been identified (Christie, 2007). These include NON-PHOTOTROPIC HYPOCOTYL3 (NPH3) and ROOT PHOTOTROPISM2 (RPT2) which are part of the 33-member NPH3/RPT2-like (NRL) Arabidopsis protein family (Motchoulski and Liscum, 1999; Sakai et al., 2000; Celaya and Liscum, 2005). All NRLs harbour NPH3-like motifs and one or both of the protein-protein interaction domains BTB/POZ (broad complex, tramtrack, bric à brac/pox virus and zing finger) domain and coiled-coil (Motchoulski and Liscum, 1999). Although the biochemical function of these proteins is not yet clearly established, NRL genes are known to accomplish important and diverse roles in plant development (e.g. tropism and organogenesis) (Motchoulski and Liscum, 1999; Sakai et al., 2000; Lalanne et al., 2004; Stone et al., 2005; Cheng et al., 2007; Furutani et al., 2007; Cheng et al., 2008). NPH3 interacts directly with phot1 but is not required for all the phototropin responses (Motchoulski and Liscum, 1999; Inada et al., 2004; Pedmale and Liscum, 2007). Indeed, NPH3 acts in phot1-controlled phototropism and leaf positioning and phot2-mediated phototropism but is dispensable for chloroplast positioning and stomatal opening (Inada et al., 2004; Inoue et al., 2008a). RPT2 also interacts with phot1 but regulates a different subset of phot1-controlled processes than NPH3 (i.e. phototropism and stomatal opening) (Inada et al., 2004). Thus, signaling downstream of the phototropins branches quickly and phot1 and

phot2 appear to recruit different signaling components to regulate different blue light responses.

On the one hand, chloroplast movements and stomatal opening are rapid, cell autonomous and reversible processes. On the other hand, phototropism and leaf flattening are slower asymmetric growth processes that require coordinated cell expansion and division. The functional selectivity of some signaling elements indicates that phots may utilizes pecific components to carry out blue light responses of specific nature (Inada et al., 2004). The phototropic response is triggered by blue light-induced auxin redistribution and signaling across the stem (Friml et al., 2002; Blakeslee et al., 2004; Esmon et al., 2006). CPT1 (the rice homolog of NPH3) controls auxin redistribution in phototropic coleoptiles (Haga et al., 2005). Interestingly, recent reports have shown that another NRL (NPY1/MAB4/ENP) is implicated in auxin-regulated organogenesis (Cheng et al., 2007; Furutani et al., 2007; Cheng et al., 2008). In addition, leaf flattening also involves auxin signaling and is regulated by NPH3 (Keller and Van Volkenburgh, 1997; Harper et al., 2000; Zhao et al., 2001; Kinoshita et al., 2003; Qin et al., 2005; Braun et al., 2008; Inoue et al., 2008a). Thus, NRL proteins may represent a link between phot signaling and auxin-regulated development. However, our knowledge on how phot signalling modulate auxin-regulated development is still limited (Whippo and Hangarter, 2006) . Identifying new signaling components will help us to characterize mechanistically how phot signaling controls plant development.

PHYTOCHROME KINASE SUBSTRATE (PKS) proteins are phytochrome signaling components that regulate developmental processes such as de-etiolation, root gravitropism and hypocotyl growth orientation (Fankhauser et al., 1999; Lariguet et al., 2003; Khanna et al., 2006; Boccalandro et al., 2008; Molas and Kiss, 2008; Schepens et al., 2008). PKS1, PKS2 and PKS4 interact with phyA and PKS1 is phosphorylated by phyA *in vitro* (Fankhauser et al., 1999; Lariguet et al., 2003; Schepens et al., 2008). Recently, it has been shown that PKS1 also interacts with phot1 and NPH3 *in vivo*, and is required for phot1-mediated root and hypocotyl phototropism (Lariguet et al., 2006; Boccalandro et al., 2008). The importance of PKS proteins for root and hypocotyl phototropism prompted us to test their involvement in phototropin-mediated responses more globally. Our results show that

PKS proteins, similarly to NPH3, are not required for chloroplast movements or stomatal opening, but are involved in leaf flattening. Epistasis and molecular results expand the known functions of PKS1, PKS2 and NPH3 to phot2-mediated leaf morphology. Finally, several elements connected PKS proteins with auxin-mediated leaf flattening.

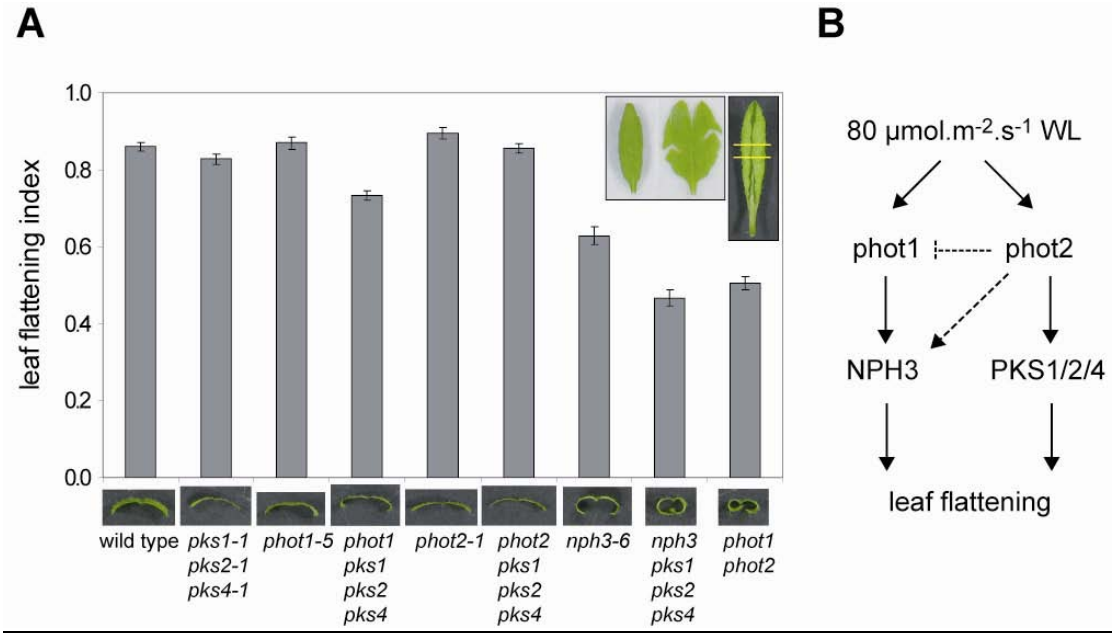


Figure 1: ***PKS1/2/4*** **are required for normal leaf flattening and act in the phot2 pathway.**

(A) Leaf flattening in the *pks1pks2pks4* mutant. Plants were grown for 25 days (until wild type reached growth stage 1.11, Boyes et al., 2001) at 21°C under 80±8 µmol.m^{-2}.s^{-1} white light. The degree of leaf flattening (leaf flattening index) is expressed as the ratio of the projection areas of leaf number 5 before (left inset – left) and after (left inset – right) manual uncurling. Graph shows the average value ± 95% confidence intervals for 17 or 18 plants. Lower pictures illustrate leaf curling. The sections were obtained from leaf number 8 (right inset) of 30-day-old plants.

(B) Epistasis data position *PKS1/2/4* within the phot2 pathway. Phot1 and phot2 pathways both trigger leaf flattening under 80 µmol.m^{-2}.s^{-1} WL conditions. *PKS1/2/4* loss-of-function leads to enhanced leaf epinasty when the phot1 signalling pathway is disrupted (*phot1* or *nph3* sensitized backgrounds). *PHOT2* loss-of-function in wild type or *pks1pks2pks4* backgrounds generates flatter leaves, suggesting that phot2 may slightly inhibit the phot1 pathway (dashed line). The intermediate phenotype of *nph3* indicates that NPH3 has a significant role in the phot2 pathway (dashed arrow).

Results

PKS1/2/4 act in a subset of phototropin-mediated processes

Since PKS1/2/4 are required for phototropism and PKS1 is associated with phot1 in vivo, we asked whether PKS1/2/4 are global regulators of phototropin signaling by looking at other phototropin-mediated responses. For this, we took a genetic approach and analyzed leaf flattening, chloroplast movements and stomatal opening in the *pks1pks2pks4* triple mutant. Our analysis excluded *PKS3* for which no null mutants were available. Since phot1 and phot2 can act redundantly in these processes we also included the *phot1pks1pks2pks4* and *phot2pks1pks2pks4* quadruple mutants in our analyses (Sakai et al., 2001; Takemiya et al., 2005). These mutants also enabled us to position the PKS within the phot pathways.

PKS1/2/4 are involved in leaf flattening

Under our experimental conditions (80 µmol.m^{-2}.s^{-1} WL; 16 hours light / 8 hours darkness photoperiods), phot1 and phot2 mediated leaf flattening redundantly because leaves curled only in the *phot1phot2* double mutant and not in the single mutants (Figure1; Sakamoto and Briggs, 2002). Leaves of *pks1pks2pks4* and *phot2pks1pks2pks4* mutants did not show obvious defects in leaf flattening (Figure 1A; Lariguet et al., 2006). However, *phot1pks1pks2pks4* laminas curled downwards (epinasty) near their margins (Figure 1A). Thus, an effect of *pks1pks2pks4* mutations became visible in plants that had an impaired phot1 pathway. To study further the role of *PKS* in phot-regulated leaf flattening we crossed *pks* mutants with the phot1-signalling mutant *nph3* (Inoue et al., 2008a). Under our experimental conditions, the *nph3* mutant already had epinastic laminas and the *nph3pks1pks2pks4* mutant had very curled laminas that resembled phototropin-deficient plants (Figure 1A). Together, these data indicate that *PKS1/2/4* act in leaf flattening within the phot2 pathway (Figure 1B).

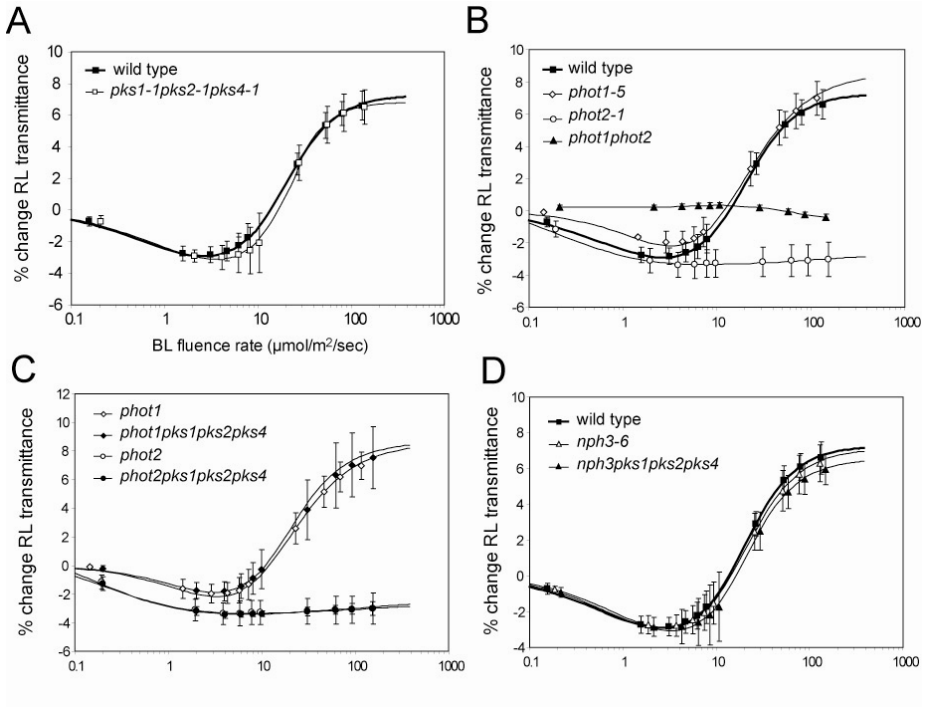


Figure 2: PKS1, PKS2 and PKS4 are dispensable for blue light induced chloroplast movements

Plants were grown for six weeks at 24°C under 100-120 µmol.m^{-2}.s^{-1} white light (WL) with a 12h/12h light/dark photoperiod. Leaves were dark-adapted for 18 hours and then exposed to a progressive increase of BL (450nm) fluence rate from 0.1 to 120 µmol.m^{-2}.s^{-1}. Plots show dose response curves corresponding to the change (in percent) of RL (660nm) transmittance of the leaves relative to the average transmittance measured in dark-treated leaves. Data points show average ± SD of 9<n<13 plants. Curves were fitted on data points using a mathematical equation (see *Material and Methods).*

PKS1/2/4 are dispensable for normal stomatal opening and chloroplast movements

To test whether PKS1/2/4 were involved in phototropin-controlled processes other than phototropism (Lariguet et al., 2006) and leaf flattening (Figure 1), we analyzed blue light-induced stomatal opening and chloroplast movements in *pks1pks2pks4*, *phot1pks1pks2pks4* and *phot2pks1pks2pks4* mutants .

To study chloroplast movements, we measured the red light transmittance of leaves in response to a gradual increase of blue light fluence rate. This method provided an indirect but quantitative means to monitor chloroplast movements into the accumulation (low light response) and avoidance (high light response) positions (Inoue and Shibata, 1973; Trojan and Gabrys, 1996; DeBlasio et al., 2003). As previously reported, phot1 and phot2 controlled redundantly the accumulation response while only phot2 mediated the avoidance response (Sakai et al., 2001; Figure 2B). *pks1pks2pks4* plants showed no signs of impaired chloroplast movements (Figure 2A), and *phot1pks1pks2pks4* and *phot2pks1pks2pks4* looked essentially like *phot1* and *phot2*, respectively (Figure 2C). These results clearly show that PKS1/2/4 did not play important roles in phot1 or phot2 pathways mediating the low light (accumulation) response and were not required for the phot2-mediated high light response (Figure 2C). NPH3 was previously shown to be dispensable for chloroplast movements (Inada et al., 2004). Under our experimental conditions, the epinastic *nph3* and *nph3pks1pks2pks4* mutants also showed normal chloroplast movements indicating that NPH3 and PKS1/2/4 did not act redundantly in this process (Figure 2D; Inada et al., 2004).

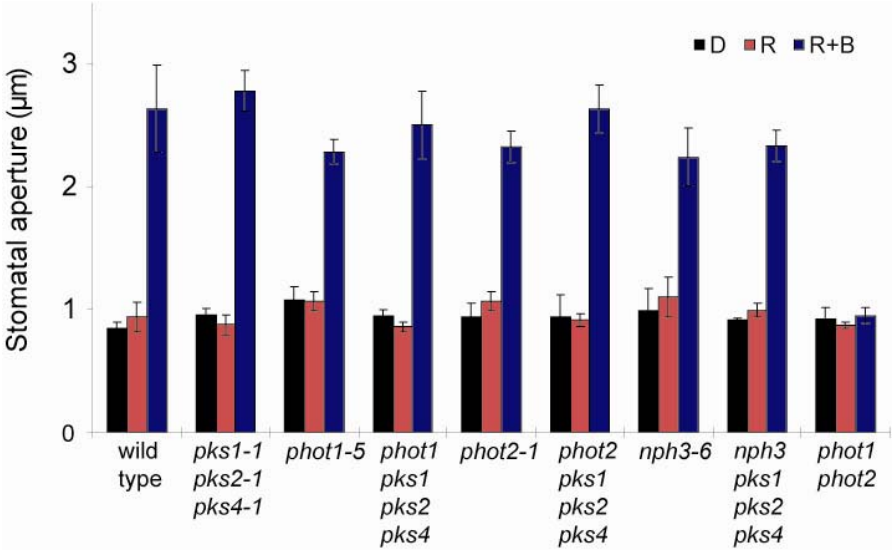
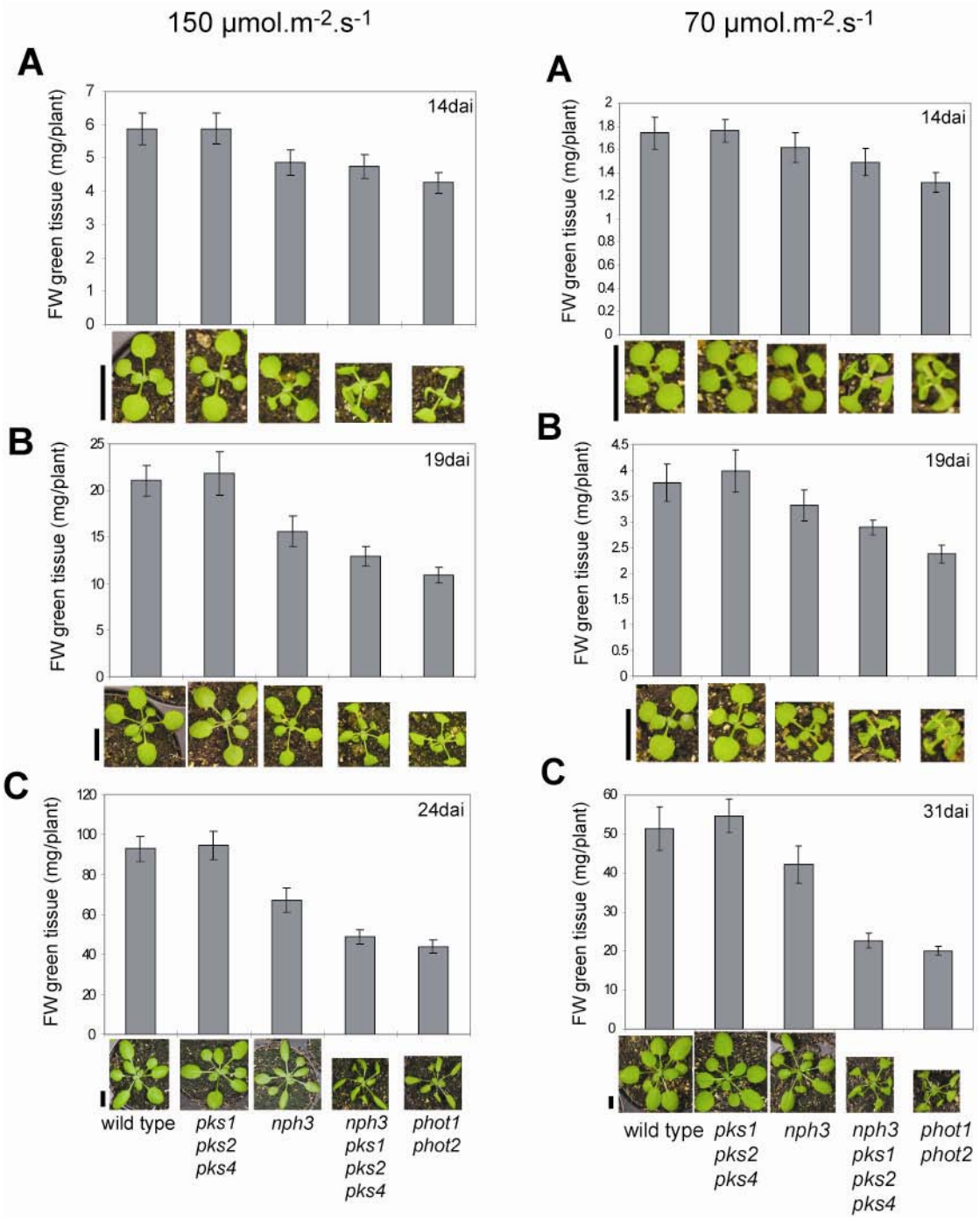


Figure 3: PKS1, PKS2 and PKS4 are dispensable for blue light induced stomatal aperture

Isolated epidermal peels were obtained from rosette leaves of 4-week-old plants and irradiated for 3hrs at 24°C under red light (60 µmol.m^{-2}.s^{-1}. RL) or red light (50 µmol.m^{-2}.s^{-1}) and blue light (10 µmol.m^{-2}.s^{-1}, RL + BL). The average aperture of 45 stomata was calculated per experiment. The graph shows average ± SD of three separate experiments.

To test phototropin-mediated stomatal opening, we applied blue light onto isolated stomata obtained from rosette leaf epidermal peels. We superimposed red light in the assay because red light increases the blue light response of guard cells (Shimazaki et al., 2007). Red light alone did not induce stomatal opening in wild type or mutants (Figure 3). However, the addition of blue light caused a two- to three-fold increase in the size of stomata pores in wild type (Figure 3). Under these conditions phot1 and phot2 redundantly controlled the response (Kinoshita et al., 2001). We could not detect significant reductions in stomatal aperture in *pks1pks2pks4*, *phot1pks1pks2pks4* or *phot2pks1pks2pks4* mutants indicating that PKS1/2/4 were not required for phot1 or phot2 signaling during stomatal opening (Figure 3). As for chloroplast movements, the epinastic *nph3* and *nph3pks1pks2pks4* mutants had functional guard cells meaning that PKS1/2/4 did not act redundantly with NPH3 during blue light-induced stomatal opening (Figure 3; Inada et al., 2004).

Taken together, our genetic experiments show that PKS1/2/4 are dispensable for phototropin-mediated chloroplast movements and stomatal opening, but are required for normal leaf flattening and phototropism (Figure 1; Lariguet et al., 2006). Interestingly, PKS1/2/4 acted in the same subset of responses than NPH3 (Inada et al., 2004). Moreover, the striking curled leaf phenotype of *nph3pks1pks2pks4* suggests that these two types of proteins act coordinately in this process (Figure 1).



Figures 4: Growth of wild type and epinastic mutant plants under two different intensities of WL.

Plants were grown on soil at 21°C under 150±15 µmol.m^{-2}.s^{-1} (left) or 70±8 µmol.m^{-2}.s^{-1} (right) WL with a 16hrs/8hrs light/dark photoperiod and were shuffled around to even out the effects of varying microenvironments. Fresh weight of green tissue was measured at 14 (A), 19 (B) and 24 or 31 (C) days after incubation (dai). Graphs show average values ± 95% confidence intervals for 20<n<36 plants. Lower pictures show one representative plant. Bar=1cm.

Contribution of leaf flattening to plant growth under intermediate WL fluence rates

Takemiya and co-workers have shown that under low photosynthetically active radiation (PAR - 25 μmol.m^{-2}.s^{-1}white light (WL)), phot1 and phot2 promoted photosynthesis and plant growth by driving chloroplast positioning into the accumulation position, opening stomata and flattening leaves (Takemiya et al., 2005). In the same study under higher PAR (70 μmol.m^{-2}.s^{-1} WL), *phot1phot2* mutants displayed flat leaves and normal plant growth. These results suggested that phototropins mediate plant growth enhancement specifically in low light environments. Interestingly, under our experimental conditions (80 μmol.m^{-2}.s^{-1} WL), *phot1phot2* displayed highly curled leaves (Figure 1). The fact that chloroplast movement and stomatal opening were also abolished in *phot1phot2* even under high fluence rates of blue light encouraged us to test whether phototropin-deficient plants also suffered reduced plant growth under intermediate PAR (70 and 150 μmol.m^{-2}.s^{-1} WL) (Figures 2B and 3). In addition, we used the properties of the *nph3pks1pks2pks4* mutant to investigate the contribution of leaf flattening in plant growth under these favourable light conditions.

Under 150 μmol.m^{-2}.s^{-1} WL, cotyledons and true leaves of *phot1phot2* mutant plants curled downwards throughout plant development (Figure 4 A-C left). In parallel, we observed a gradual decrease in green tissue fresh weight of *phot1phot2* relative to wild type plants over a ten-day period (Figure 4 A-C left). We found a similar trend under 70 μmol.m^{-2}.s^{-1} WL (Figure 4 A-C, right). Although the cotyledons and true leaves of *nph3pks1pks2pks4* plants appeared as epinastic as *phot1phot2* plants (Figure 4, see also Figure 1), *nph3pk1pks2pks4* plants were slightly heavier than *phot1phot2* (Figure 4). These results show a correlation between reduced leaf flattening and reduced plant growth, and also suggest that functional chloroplast movements and stomatal opening in *nph3pks1pks2pks4* slightly contributed to plant growth.

Cotyledons and leaves of *nph3* mutant plants showed an intermediate degree of epinasty between wild type and *nph3pks1pks2pks4* (Figures 1 and 4). Interestingly, while *nph3pks1pks2pks4* plants appeared as heavy as *nph3* at early stages of growth, the quadruple mutant accumulated gradually less weight compared to *nph3* over time

(Figure 4 A-C. left and right). These observations show again a correlation between leaf flattening and accumulation of fresh weight.

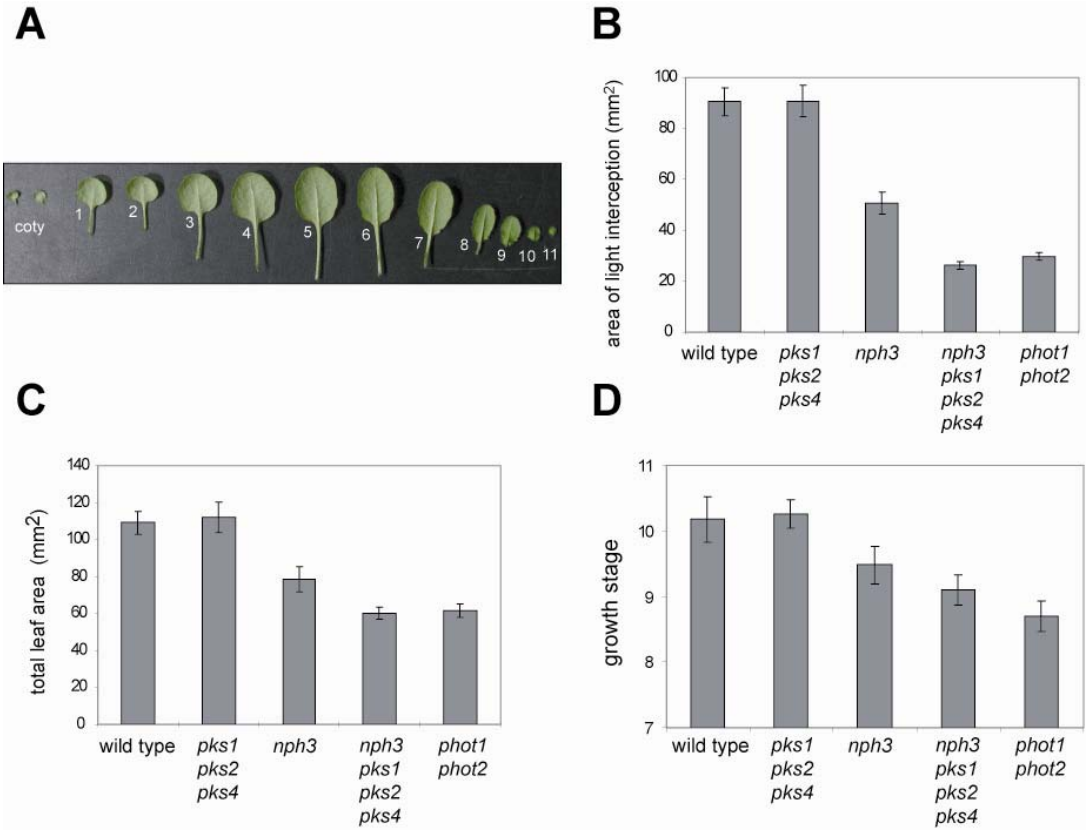


Figure 5: Morphology of leaves of wild type and epinastic mutant plants.

Leaves of plants from Figure 4C (left) were analyzed.

A/ Heteroblasty of a wild type plant. Cotyledons (coty) and true leaves number one to eleven (1-11) are shown. Note the difference in size and shape between juvenile (one to three), transition (four to five) and adult (six and onwards) leaves. Leaf number five appeared large and well expanded.

B/ Area of light interception of leaf number 5. Projection area of the leaf when viewed from above was calculated (as shown in Figure 1 - left inset). This area corresponds to the leaf surface that would capture light if light was applied from above.

C/ Total area of manually uncurled leaf number five (as shown in Figure 1 left inset).

D/ Growth stage reached by plants at the time when leaf number 5 was analysed. Number of the last leaf longer than 1mm was used as an indicator of development (Boyes et al., 2001).

Plots show average ± 95% confidence intervals for $20<n<36$ plants.

Similar trends were found for plants grown under 70 $\mu mol.m^{-2}.s^{-1}$ (Figure 4 C – left) (data not shown).

Physiological analysis of wild type and curled leaves

We reasoned that diminished plant growth observed in epinastic mutants could be the consequences of (i) reduced light capture leading to reduced photosynthesis and also (ii) a basal defect in leaf expansion. To address these hypotheses we analyzed the morphology and physiology of whole leaves.

Morphology studies were done on leaf number 5 of plants that had reached growth stage 1.11 (Figure4 C left and right) because this leaf was well expanded and certainly contributed highly to plant vegetative vigour (Figure 4C and 5A; (Kerstetter and Poethig, 1998)). The area of light interception by *nph3pks1pks2pks4* and *phot1phot2* leaves was three-fold lower than wild type or *pks1pks2pks4* leaves. *nph3* showed a two-fold reduction (Figure 5B). Interestingly, the total area of *nph3pks1pks2pks4* and *phot1phot2* leaves was also smaller than wild type (50% of wild type size) and *nph3* also showed a 30% decrease in size (Figure 5C). Thus, slower plant growth in the mutants correlated with both reduced light capture and reduced leaf expansion. One simple interpretation of this data is that plants produced smaller leaves because they suffered reduced photosynthesis and overall growth as a consequence of limited light capture. This hypothesis is consistent with the fact that epinastic mutants also developed more slowly than wild type-like plants (Figure 5D). Nonetheless, one cannot exclude the possibility that basal developmental defects also hindered leaf expansion and overall plant growth in a photosynthesis-independent fashion. To test the latter hypotheses we analysed gas exchanges in intact leaves.

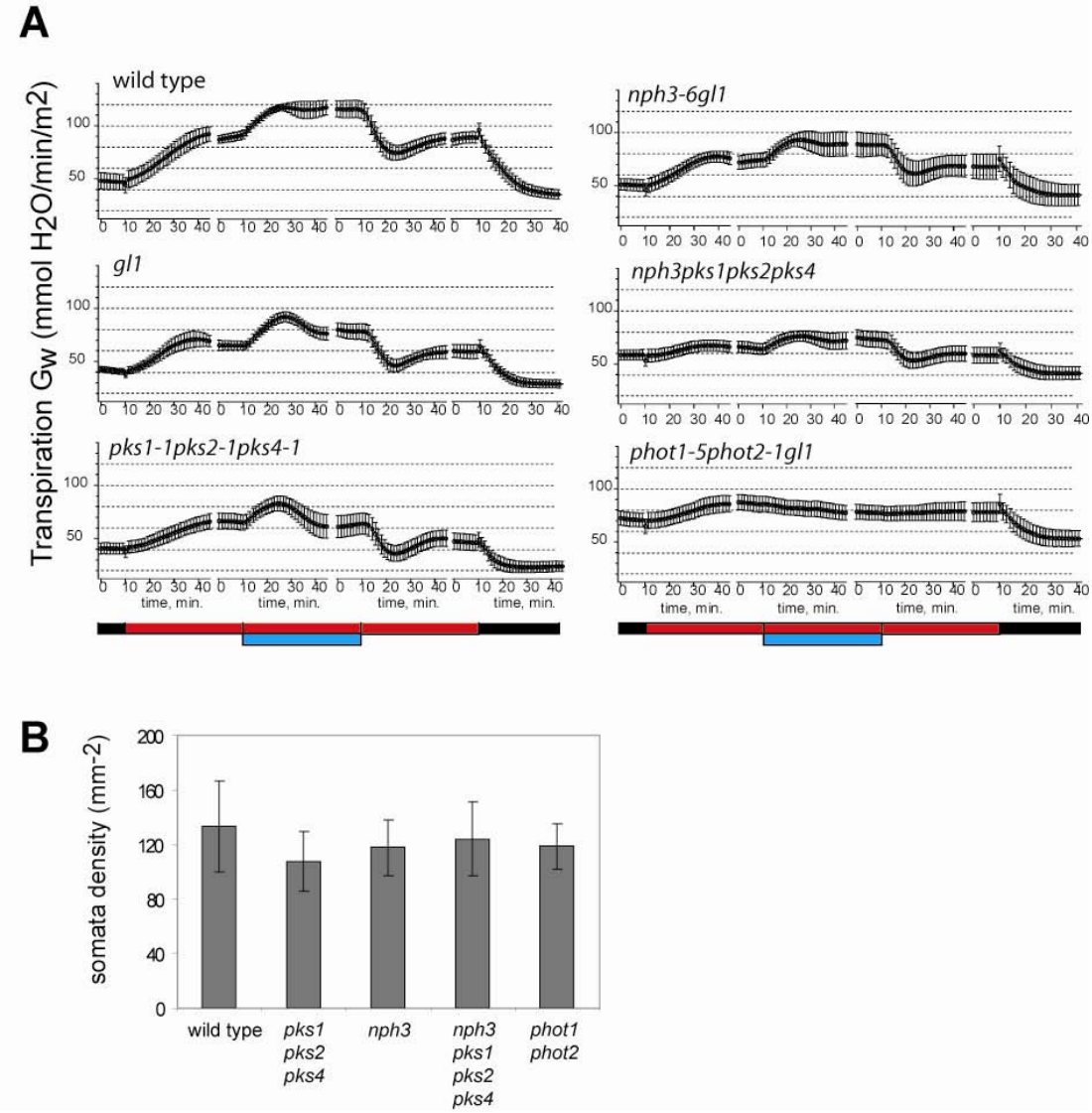


Figure 6: Epinastic leaves are impaired in leaf transpiration.

(A) Light-induced transpiration. Plants were grown for 8-10 weeks under 200 µmol.m^{-2}.s^{-1} WL with an 8/16hrs and 22/16°C light/dark photoperiod. Mature leaves were analyzed by infrared gas analysis technique. Leaves were dark-adapted (dark bar) then illuminated on their adaxial side with 500 µmol.m^{-2}.s^{-1} RL (red bar) and 25 µmol.m^{-2}.s^{-1} BL (blue bar) on a 21mm-wide stretch approximately 5mm from the apex of the leaf. Gas exchange was measured on the abaxial side over time.
Graphs show average ± SE of 5<n<9 plants.
(B) Stomatal density on abaxial epidermis. Prints were obtained from the fifth leaves of plants grown as in Figure 1 and observed under 100× magnification. Average stomatal density was calculated by counting the number of stomata within a measured area comprising 60-120 epidermal pavement cells. Plots show average ± SD of different regions per leaf (margin to midvein, apex to base) of 5 leaves.

To further analyze the consequences of leaf epinasty whole-leaf physiology, we analyzed RL and BL-induced transpiration in whole leaves using gas exchange assays. As shown in Figure 6, all mutants except *phot1phot2* responded to the addition of blue light. This result indicates that blue-light induced stomatal opening data previously obtained for isolated guard cells were meaningful in a whole-leaf context (Figure 3). Interestingly, the highly epinastic *nph3pks1pks2pks4* mutant displayed reduced transpiration compared to *nph3, pk1pks2pks4* and wild type leaves, and this was not due to lower stomata density (Figure 6A and B). This suggests that leaf curling had an effect on leaf gas exchange. Since stomatal opening is a limiting step for CO_2 assimilation by photosynthesis, we asked whether the epinastic *nph3pks1pks2pks4* also showed reduced photosynthesis activity (Roelfsema et al., 2002; Roelfsema and Hedrich, 2005). Using the gas exchange assay we observed that this was indeed the case (data not shown). Although these results do not enable us to determine whether leaf epinasty had a primary consequence on stomatal opening potential or on photosynthesis itself, they nonetheless correlate with the fact that the epinastic *nph3pks1pks2pks4* mutant suffered slower growth. These observations support the notion that leaf morphological changed in epinastic mutants affect overall photosynthesis and growth. However, it is difficult to determine the means by which leaf curling impairs photosynthesis. Indeed, one possible scenario is that guard cell swelling potential and gas diffusion are affected by the curvature in whole epinastic leaves. Another scenario is that smaller light capture area also limited photosynthesis and transpiration.

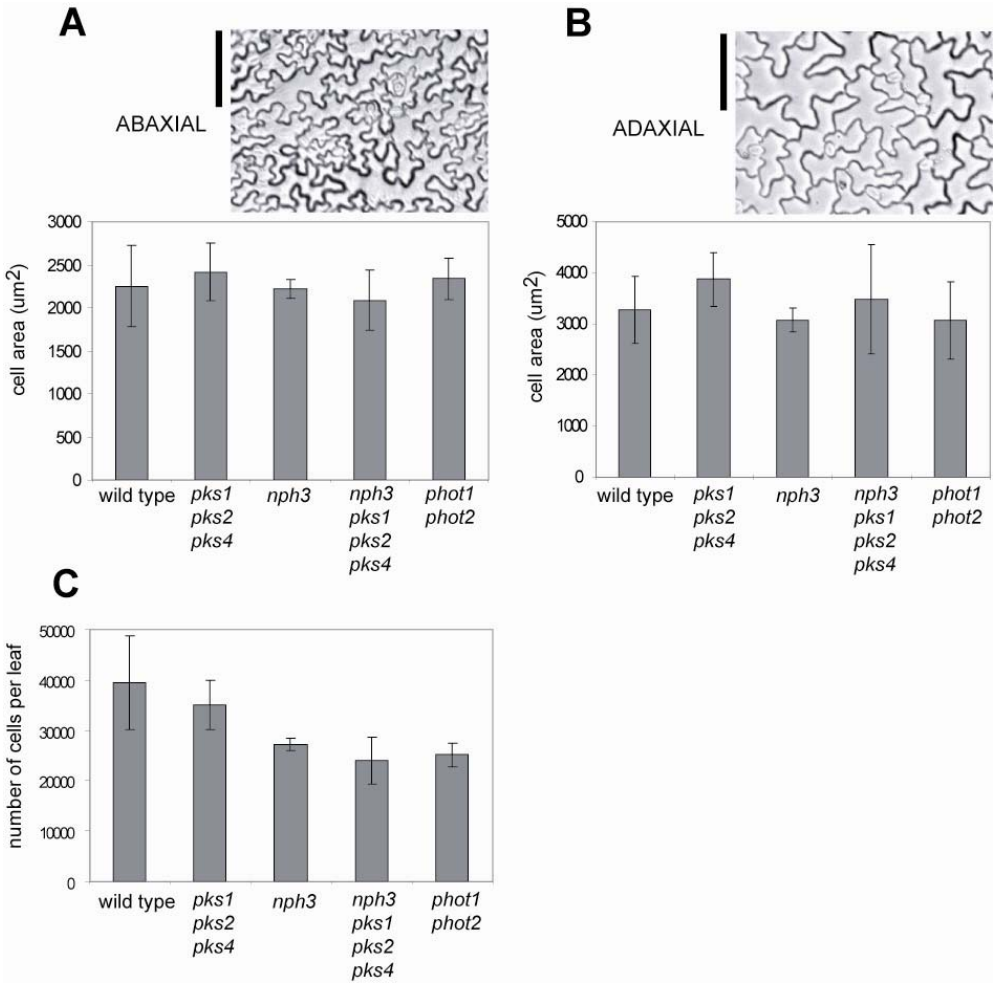


Figure 7: Analysis of epidermal pavement cell size in wild type and curled leaves

Similar plants as in Figure 1 were analyzed. Epidermal prints were obtained using nail polish and observed under 100× magnification. Cell size was determined by measuring the area of a region comprising 60-120 cells and dividing this area by the number of cells. Plots show average ± SD of different regions per leaf (margin to mdivein, apex to base) of 5 leaves.

A/ abaxial cell size of leaf number 5.

B/ adaxial cell size of leaf number 6.

C/ Number of cells per leaf number 5. Values are the product of total leaf area (determined as in Figure 1) and epidermal cell density on the abaxial side (mm^{-2}).

Bar=100µm.

Epidermal cell size and number in wild type and epinastic leaves

To test further the hypothesis that *nph3pks1pks2pks4* and *phot1phot2* epinastic plants suffered severe consequences on growth because of basal defects in development, we analysed cell division and cell expansion patterns of leaf epidermises. The epidermis is a particularly relevant tissue to analyse because it restricts growth (Savaldi-Goldstein and Chory, 2008)

No significant differences in cell size on either leaf 5 abaxial or leaf 6 adaxial epidermises could be identified in *nph3pks1pks2pks4* compared to wild type, *pks1pks2pks4* or *nph3* (Figure 7). Furthermore, the average size of pavement cells was similar from apex to base and from margin to midvein in both epinastic and wild type plants indicating that these leaves were not seriously delayed in their development (Donnelly et al., 1999; Autran et al., 2002). However, the abaxial epidermis of curled leaves number 5 had fewer cells than wild type leaves (Figure 7C). Thus, the reduced leaf size in both *nph3pks1pks2pks4* and *phot1phot2* epinastic mutants may be due to reduced cell division rather than lower cell expansion. However, it is difficult to determine whether such cellular defects in the epinastic mutants are the cause for downwards leaf curling.

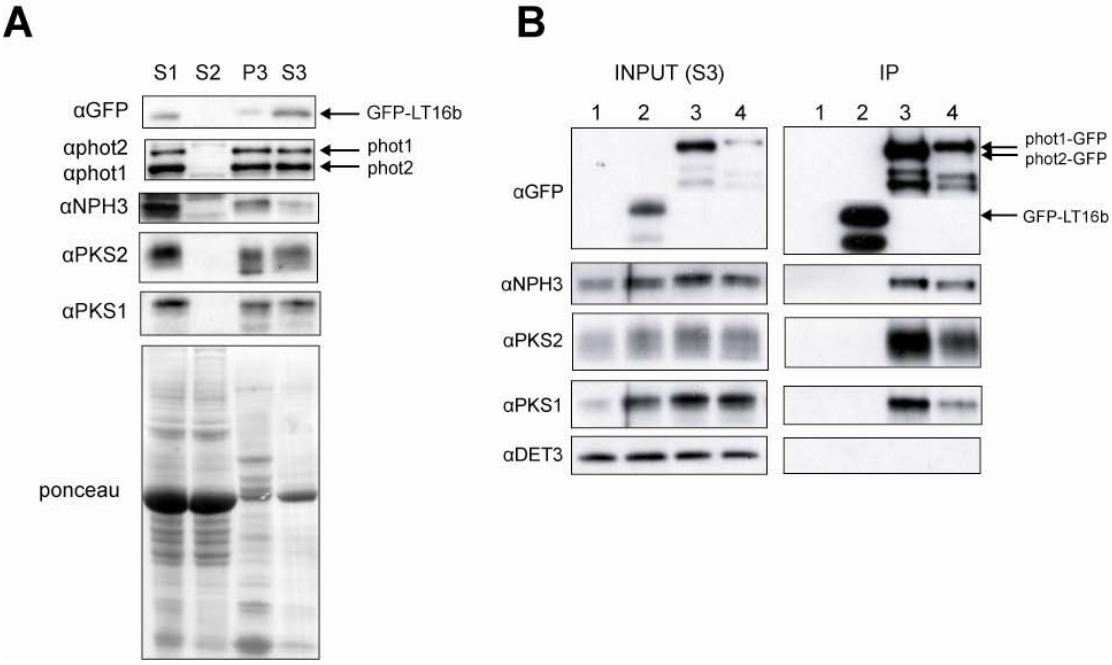


Figure 8: PKS2, PKS1 and NPH3 are associated with phot1 and phot2 *in vivo*.

A/ Co-localisation of PKS1, PKS2, NPH3 and phot1/phot2 in insoluble protein fractions. Microsomal fractions were prepared from green tissues of 14-day-old plants (S1, total protein extract; S2, soluble fraction after ultracentrifugation). The microsomal pellet was resuspended with 0.5% (v/v) Triton X-100 (P3, pellet after detergent treatment; S3, soluble fraction after detergent treatment).

B/ PKS1, PKS2 and NPH3 co-immunoprecipitate with phot1-GFP and phot2-GFP. The following genotypes were analyses: wild type (lanes 1), *35S:GFP-LT16b* (plasma-membrane associated protein, lane 2), *PHOT2:PHOT2-GFP phot1-5phot2-2 (lane 3*), *PHOT1:PHOT1-GFP phot1-5* (lanes 4). An aliquot of solubilized microsomal proteins (S3) was mixed 1:1 with Laemmli sample buffer and used as input (INPUT). Proteins from S3 fraction were subjected to anti-GFP immunoprecipitation using anti-GFP antibodies covalently coupled to magnetic beads. Beads were extensively washed and specifically bound proteins were eluted with boiling Laemmli sample buffer (IP). Proteins were separated on SDS/PAGE, Western-blotted, and probed with various antibodies as described in Materials and Methods. DET3 was used as a loading control.

PKS2, PKS1 and NPH3 are associated with both phot1 and phot2 in leaves

Previous genetic results indicate that PKS1/PKS2/PKS4 act within the phot1 pathway during phototropism and within the phot2 pathway during leaf flattening. To better understand how the PKS may act in phot signalling during leaf flattening we then decided to analyse protein complexes extracted from leaves. In young etiolated seedlings PKS1 was found in the same complex as with phot1 and NPH3 and consistently acted in the phot1 pathway (Lariguet et al., 2006).

PKS2 co-fractioned with phot1, phot2, NPH3 and PKS1 and a plasma membrane-associated protein fused to GFP (GFP-LTi6b; (Cutler et al., 2000)) in insoluble microsomal pellets and were similarly released into solution by detergent treatment (Figure 8A; Sakamoto and Briggs, 2002; Lariguet al., 2006; see Chapter 3 Figure 15C for method). Interestingly PKS2, PKS1 and NPH3 all co-immunoprecipitated with phot1-GFP and phot2-GFP, but not with GFP-LTi6b (Figure 8B). These results show that PKS1 and NPH3 can associate with phot2 in addition to phot1 (Lariguet et al., 2006). They also indicate that PKS2 is found in the same complex than phot1 and phot2 *in vivo*. However, these data do not show whether PKS2, PKS1 and NPH3 are present in a same complex with phot1 and/or phot2. It is relevant to point out that phot1-GFP and phot2-GFP were expressed under the control of their respective promoters and at similar levels than the endogenous protein, supporting the notion that the protein-protein associations reporter are physiologically meaningful (Sakamoto and Briggs, 2002; Kong et al., 2006).

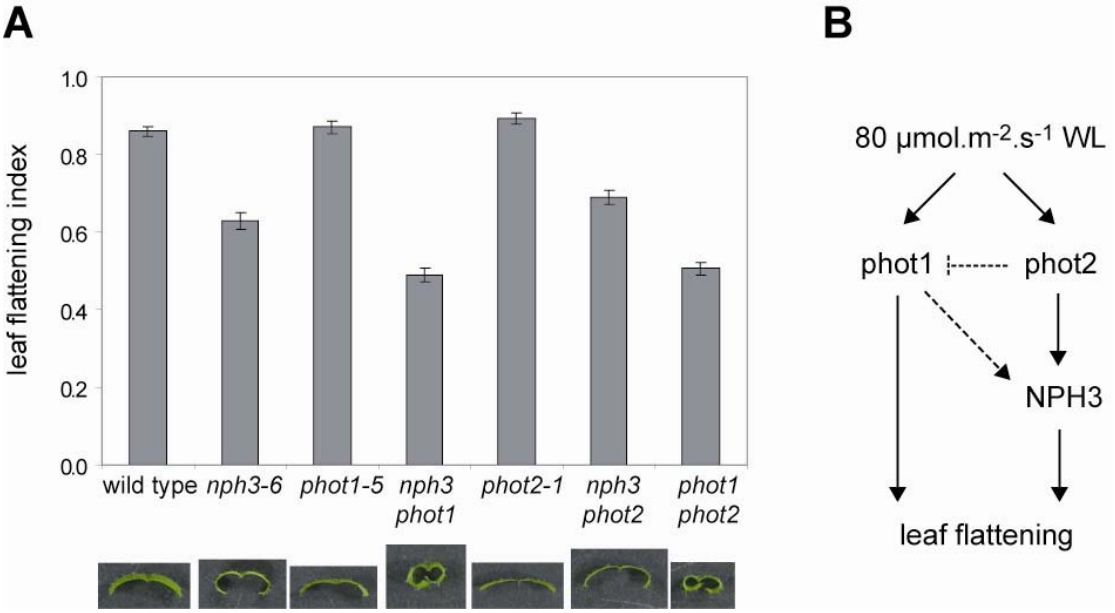


Figure 9: Epistasis between *NPH3*, *PHOT1* and *PHOT2* during leaf flattening

(A) Plants were grown and leaf flattening index of leaf number 5 was calculated as described in figure 1. Graph shows average ± 95% confidence intervals for 17 or 18 plants.

(B) Epistasis data position *NPH3* within the phot2 pathway. Phot1 and phot2 pathways both trigger leaf flattening under 80 µmol.m^{-2}.s^{-1} WL. *NPH3* loss-of-function in the *phot1* background leads to *phot1phot2*-like phenotype, indicating that NPH3 plays an important role in the phot2 pathway. *PHOT2* loss-of-function in wild type or *nph3* backgrounds generates flatter leaves, suggesting that phot2 slightly inhibits the phot1 pathway (dashed line). The intermediate phenotype of *nph3* indicates that NPH3 has a significant role in the phot1 pathway (dashed arrow).

Important contribution of NPH3 during phot2-mediated leaf flattening

In a recent study, NPH3 was reported to act predominantly in the phot1 pathway during leaf flattening (Inoue et al., 2008a). However, several results in our present work also point towards a role for NPH3 in the phot2 pathway. First, *nph3* showed clear signs of leaf curvature under conditions where phot1 and phot2 acted redundantly (Figure 1). Second, *nph3pks1pks2pks4* leaves were much more epinastic than *phot1pks1pks2pks4* leaves (Figure 1). Third, NPH3 co-immunoprecipitated with phot2-GFP (Figure 8). Thus, we analyzed epistasis between *NPH3*, *PHOT1* and *PHOT2* to understand in more detail the position of NPH3 in phot signaling.

In the leaf-flattening assay, *phot1nph3* "phenocopied" *phot1phot2* providing again strong genetic evidence that NPH3 acts in the phot2 pathway (Figure 9). Interestingly, *phot2nph3* showed no increase in leaf curling (Figure 9). This result is striking because we would have expected leaves to resemble *phot1phot2* since NPH3 was previously shown to act predominantly in the phot1 pathway during leaf flattening (Pedmale and Liscum, 2007; Inoue et al., 2008a). Moveover, the *phot2nph3* mutant revealed an additive phenotype between *nph3* and *phot2* (i.e. more flat than *nph3*) (Figure 9; p-value<0.02). Indeed, we previously observed that leaves from *phot2* plants were slightly more flat than wild type leaves (Figure 1; p-value<0,02). This result suggests that NPH3 and phot2 may also have independent roles in leaf flattening. In summary, the most parsimonious interpretation of these genetic data is that, under our conditions, NPH3 can act downstream of both phototropins, with a strong effect in phot2-mediate light responses.

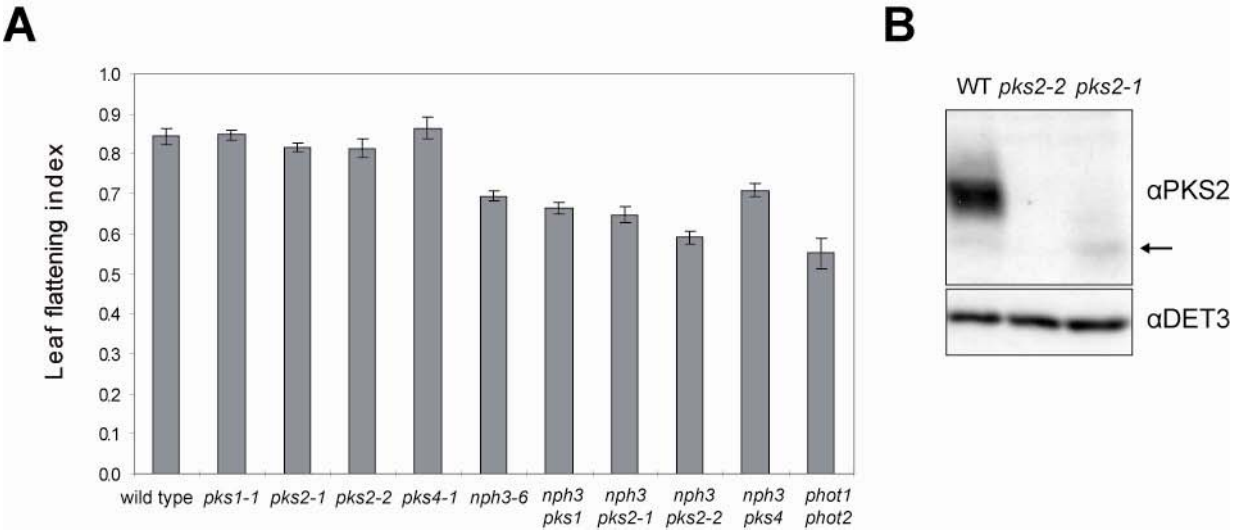


Figure 10: Leaf flattening in *pks* single mutants.

(A) Plants were grown and leaf flattening index of leaf number 5 was calculated as described in Figure 1. Graph shows average ± 95% confidence intervals for 12 or 13 plants.

(B) Western blot of protein extracts from wild type (WT), *pks2-1* and *pks2-2* plants probed with anti-PKS2 and anti-DET3 (loading control) antibodies. A truncated form of PKS2 appears to be present in low amounts in the *pks2-1* allele (arrow). No signal could be detected in *pks2-2* plants.

PKS2 and to a lesser extent PKS1 act in leaf flattening

To investigate in more detail the function of individual PKS proteins in leaf flattening, we first determined which family member(s) played the main role(s) in this process. We analyzed leaf curling in *nph3pks* double mutants because the effect of PKS1/2/4 loss of function was most clearly seen in the *nph3* sensitized background (Figure 1). We observed a weak enhancement of curling in *nph3pks1* and a stronger enhancement in a *nph3pks2-2* mutant (Figure 10A). *nph3pks2-1* had a weaker phenotype than *nph3pks2-1* that resembled *nph3pks1-1* presumably because some functional PKS2 protein was still present in the *pks2-1* allele (Figure 10B). However, no significant differences between *nph3* and *nph3pks4* could be seen (Figure 10A). Consistent with these data, we noticed in higher-order mutants (in the *nph3* sensitized background) an increase in leaf curling in plants lacking PKS1 and PKS2 but not PKS4 (Figure 12 at the end of this Chapter). *pks2-2* and *pks1-1pks2-1pks4-1* also had significantly reduced LFI compared to wild type (Figures 1 and 10; $p<0.05$) but the effect of PKS loss-of-function was better seen in the *nph3* background. The fact that PKS2, PKS1 and NPH3 are associated with phot1 and phot2 is also consistent with these genetic data (Figure 8).

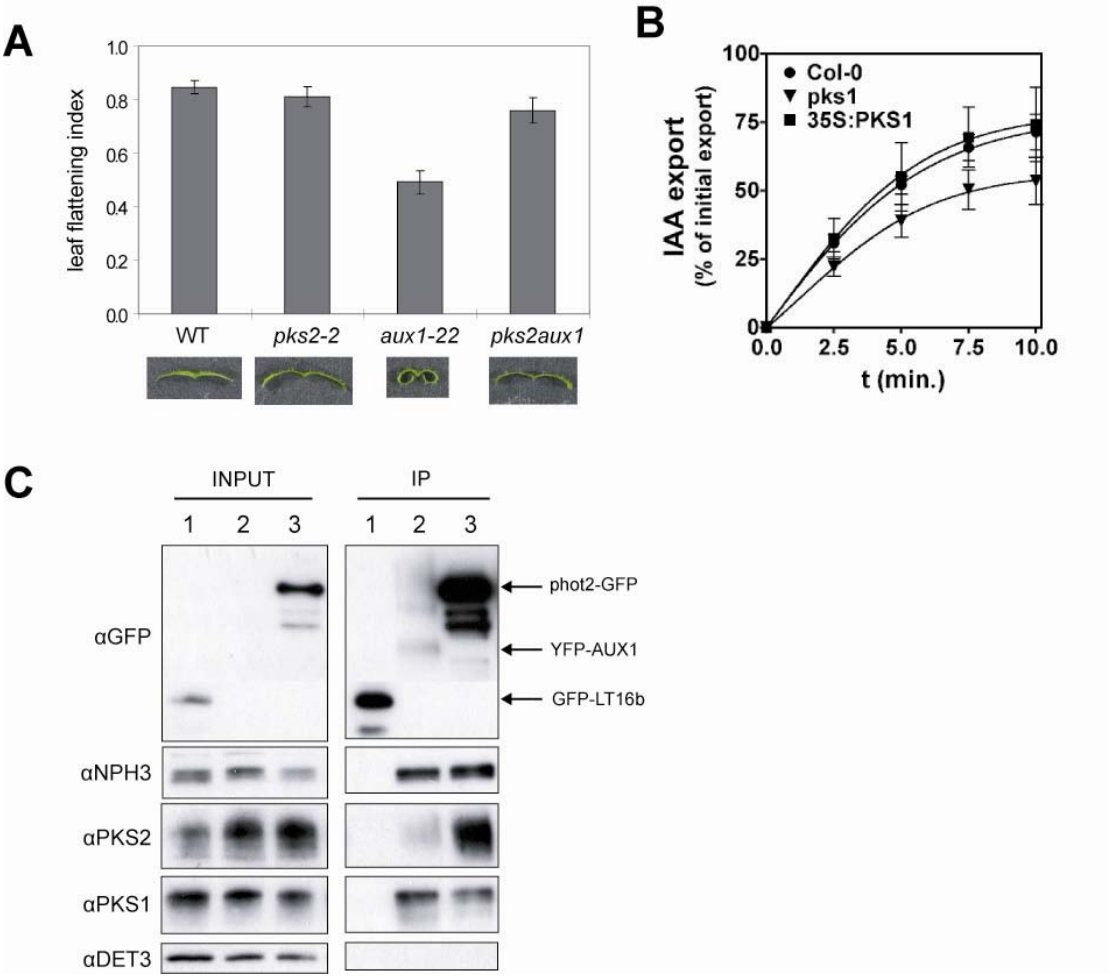


Figure 11: PKS2 is involved in regulation of auxin transport.

A/ *PKS2* loss-of-function rescues the *aux1* leaf phenotype. Plants were grown and leaf flattening index of leaf number 5 was calculated as described in Figure 1. The graph shows average ± standard deviation for 12<n<15 plants per genotype.

B/ Cellular IAA export is significantly reduced in *pks1* compared to wild type (paired *t* test, $p < 0.05$) (Col Wt). Shown are means with standard errors of three individual experiments, n = 4.

C/ PKS2, PKS1 and NPH3 are associated with AUX1 *in vivo*. The following genotypes were analyses as in Figure 8B: *35S:GFP-LT16b* (plasma-membrane associated protein, lane 1), *AUX1:YFP-AUX1 aux1-22 (lane 2) PHOT2:PHOT2-GFP phot2-2* (lanes 3). The latter was used as a positive control for co-immunoprecipitation of PKS2, PKS1 and NPH3 (Figure 8). DET3 was used as a loading control.

^

A link between PKS function and auxin transport

NPH3 is an important regulator of phototropism and leaf flattening (Pedmale and Liscum, 2007; Inoue et al., 2008a). Several elements suggest that NPH3 may achieve these roles by acting on auxin transport in these organs (Haga et al., 2005; Furutani et al., 2007) of the strong molecular and genetic interactions between PKS1/PKS2 and NPH3, we decided to further analyze the possible effects of *PKS* loss-of-function in leaves of mutants impaired in auxin transport (Figure 1, 5 and 7). We focused our genetic analysis on *pks2* because it had the strongest effect on leaf flattening among the *pks* mutants tested (Figure 7). We chose the *aux1* auxin import carrier mutant because it displayed epinastic leaves (Pickett et al., 1990; Bennett et al., 1996; Bainbridge et al., 2008). Interestingly, *PKS2* loss-of-function restored leaf flattening in the epinastic *aux1* mutant (Figure 8A). The roots of the *aux1pks2* mutant however did not recover normal gravitropism (data not shown). This observation is consistent with the absence of *PKS2* expression in the roots (Lariguet et al., 2003). To test whether the *pks2* mutation affected leaf flattening at the level of auxin transport, we analysed auxin efflux in protoplasts obtained from *pks2* leaves. We observed a reduction of auxin efflux in the pks1 mutant as compared with the wild type (Figure 8B). Given that PKS1, PKS2 and AUX1 are all associated with membranes we then tested whether they associate *in vivo*. We used plants expressing YFP-AUX1 to test this hypothesis by co-immunoprecipitation (Swarup et al., 2004; Lariguet et al., 2006). Interestingly PKS1, PKS2 and NPH3 were found to co-immunoprecipitate with YFP-AUX1 but not with GFP-LTi6b (phot2-GFP was used as a positive control) (Figure 8C). Although low quantities of YFP-AUX1 were immunoprecipitated (as compared with phot2-GFP), PKS1 and NPH3 co-immunoprecipitated in large amounts with YFP-AUX1.

Discussion

Using a systematic genetic approach we found that PKS1/2/4 are not required for blue-light-regulated chloroplast movements or stomatal opening, but that PKS1 and PKS2 act with NPH3 as important regulators of leaf flattening. PKS1 is a phot1-associated protein that plays important roles in phot1-mediated tropisms (Lariguet et al., 2006; Boccalandro et al., 2008). Our epsistasis and immunoprecipitation results expand the role of PKS1 and PKS2 to the phot2 pathway during leaf flattening (Figures 1 and 8). NPH3 is required both for phot1 and phot2-mediated phototropism (Motchoulski and Liscum, 1999). NPH3 was recently shown to be involved in phot1-mediated leaf flattening, and our results now show that NPH3 also acts in the phot2 branch regulating this light response (Figures 9; Inoue et al., 2007). The PKS thus specifically act in phot-controlled processes of developmental nature,
as is the case for the important phot signalling element NPH3.

It is possible that phototropins utilize different protein families with distinct biochemical properties to control different types of processes. However, it is surprising that RPT2 (a member of the NPH3 family) is also required for stomatal opening (Inada et al., 2004). Thus, while PKS function seems restricted to asymmetric growth processes, the NRL family may have more versatile functions during phototropin signalling (Inada et al., 2004).

The two AGC kinases phot1 and phot2 represent the initial step in phototropin signaling because blue light induced processes in tissues where phototropins are expressed are abolished in the *phot1phot2* double mutant (Briggs and Christie, 2002). It is not clear yet whether the four PKS proteins play an essential role in the pathway controlling leaf flattening because the *pks1pks2pks3pks4* mutant is not yet available. The fact that root phototropism is abolished in the *pks1* mutant (*pks1* phenocopied the *phot1* mutant) indicates that PKS proteins might accomplish key functions during phot signaling (Boccalandro et al., 2008). Two basic models can explain the synergistic interactions observed between *pks* mutants and *nph3* during leaf flattening. In the first one, both gene products act in parallel pathways controlling leaf flattening. In the second model, partial knock-out of different steps of the same pathway can also

result in synergistic aggravation of leaf flattening. Analysis of the *pks* quadruple mutant will allow us to determine whether the PKS control a key step in this signalling pathway.

At present we notice a good correlation between the expression pattern of *PKS* genes and the organ in which they play the most predominant function. For instance, *PKS1* is highly expressed in roots and is essential for root phototropism while *PKS2* is expressed in leaves and controls leaf flattening (Figure 10; Lariguet et al., 2003; Boccalandro et al., 2008). This may represent an example of functional specialization of *PKS1* and *PKS2*, which have arisen during the last Arabidopsis whole gene duplication (WGD). Distinct gene expression pattern is a common phenomenon for paralogous pairs having arisen from WGD (Duarte et al., 2006).

Several findings connect NPH3 and PKS proteins with auxin signaling. There is a growing body of literature that functionally link phototropin-mediated asymmetric growth processes with auxin signaling (Esmon et al., 2006; Whippo and Hangarter, 2006). For instance, phot1 has been shown to control blue light induced PIN1 relocalisation in response to lateral blue light (Blakeslee et al., 2004). Auxin transport by PIN3 and AUX1, as well as auxin-dependant transcription are also required for phototropism (Friml et al., 2002; Tatematsu et al., 2004; Stone et al., 2008). Although in the case of leaf flattening a direct connection between phototropin and auxin signaling has not been yet established, several genetic and pharmacological experiments provide evidence that leaf flattening is also regulated by auxin homeostasis (Keller and Van Volkenburgh, 1997; Li et al., 2007). Analogous scenarios can be envisaged where in hypocotyls the phototropins coordinate asymmetric growth while in the leaves the same photoreceptors coordinate symmetric growth of the lamina to ensure its flatness (Poethig, 1997; Whippo and Hangarter, 2006). In rice the *cpt1* mutant (disrupted in the *NPH3* orthologous gene), auxin relocalization no longer occurs in response to unilateral blue light indicating that CPT1 acts upstream of asymmetric auxin distribution (Haga et al., 2005). Several other NRLs are involved in auxin-regulated organogenesis (Cheng et al., 2007; Furutani et al., 2007; Cheng et al., 2008). Taken together these studies suggest that NRL proteins play a central role in auxin-mediated growth processes. Phenotypic analyses of *pks* mutants in phytochrome and phototropin-mediated responses indicate

that these genes are primarily required for asymmetric growth responses (gravitropism and phototropism) (Lariguet et al., 2006; Boccalandro et al., 2008; Molas and Kiss, 2008; Schepens et al., 2008). The function of PKSs and NPH3 in the same subset of phot-mediated responses, their presence in the same complex *in vivo* and the synergistic genetic interaction between *pks* and *nph3* during leaf flattening support the notion that these proteins are required for a subset of auxin-mediated growth responses (Figures 1-3 and 8). This model is supported by the striking genetic interaction between *PKS2* and *AUX1* during leaf flattening (Figure 11A).

It is interesting to point out that *phot1* loss of function generated a similar effect than *pks* loss of function in the *nph3* sensitized background (Figures 1 and 9). A similar genetic interaction was observed between *NPY1* and *PID1*, both homologs of *NPH3* and *PHOT1* (Cheng et al., 2007, 2008). Taken together, these results bring the PKS family forward as novel players in a genetic framework including NRLs and AGC kinases.

Our data suggest that PKS may act in this framework at the level of auxin transport (Figure 11). In the *aux1* mutant, impaired auxin import may affect the balance of auxin signaling in specific cells responsible for coordinating tissue growth and lamina flatness. Reversal of the *aux1* phenotype by *PKS2* loss of function suggests that auxin signaling may be restored in these specific cells. One mechanistic possibility is that PKS2 regulates auxin transport, either by negatively regulating auxin import or positively regulating auxin export. Impaired auxin efflux from leaf protoplasts points towards the second possibility. However, the molecular association we observe between PKS1, PKS2 and AUX1 indicate that PKS may also be involved in auxin import mechanisms.

An important question is whether PKS and NPH3 strictly dependent on photoreceptor-induced stimuli or whether these proteins play roles beyond photoreceptor signaling. The fact that the *nph3pks1pks2pks4* mutant has slightly more curled leaves than the *phot1phot2* mutant illustrates this point (Figure 1; p-value<0.05). One possible interpretation of our physiological studies is that the reduced growth of the mutant is due to reduced photosynthetic activity caused by defective phot signaling (Figure 6). The significant aggravation with time of the

nph3pks1pks2pks4 phenotype is consistent with this model (Figure 4). Alternatively, this quadruple mutant may have a developmental growth defect which may contribute to reduced leaf growth and may not be directly related to phot signalling. The abnormal gravitropism observed in the dark-grown *PKS4*-overexpressing hypocotyls is consistent with a possible function of PKSs independent of photoreceptor signaling (Schepens et al., 2008).

Finally, to address this question more directly we looked for developmental defects in the epidermis of curled *nph3pks1pks2pks4* leaves (the epidermis is a critical tissue that restricts and coordinates organ growth; (Savaldi-Goldstein and Chory, 2008). At present, the similar cellular phenotypes of *nph3pks1pks2pks4* and *phot1phot2* point towards a role for NPH3 and the PKSs in phot signaling rather than phot-independent basal development during leaf flattening. Further studies are planned to understand in more detail the molecular functions of these protein families at the level of both photoreceptor- and auxin-mediated signalling.

Materials and methods

Plant material

The following mutants used in this study were described elsewhere: *pks1-1*, *pks2-1*, *pks4-1* single and triple mutants (Lariguet et al., 2006), *phot1-5* (Huala et al., 1997) , *phot2-1* (Kagawa et al., 2001), *nph3-6* (Motchoulski and Liscum, 1999), *gl1-1* (Oppenheimer et al., 1991), *aux1-22* (Roman et al., 1995). The *pks2-2* allele has a T-DNA insertion in the 113th codon and *pks2-2* plants showed no *PKS2* transcript on a northern or western blot. To genotype *pks2-2* plants we used CF338 [5'-CAT TTG GAC GTG AAT GTA GAC AC-3'] and AH022 [5'-CCC AAA GCC CAT TAA CGA CC-3']) to detect the T-DNA and a second pair (CF359 [5'-TCG AAC ACA CGC ATC TGC AG-3'] and AH022) to test for homozygosity. *phot1-5pks1-1pks2-1pks4-1*, *phot2-1pks1-1pks2-1pks4-1*, *nph3-6/pks1-1/pks2-1/pks2-2/pks4-1*, *nph3-6phot1-5*, *nph3-6phot2-1* and *aux1-22pks2-2* mutants were obtained by crossing. *phot1-5phot2-1GL1+/+* was obtained by crossing *phot1-5phot2-1gl1-1* with *phot2-1*. All alleles used in this study are in the *Arabidopsis thaliana* (L.) Columbia-O background. We tried to obtain mutants with trichomes (outcross the *gl1-1* mutation) to exclude possible effects of lack of trichome on the leaf phenotypes analyzed. Conditions of plant growth varied depending on the physiology experiment. Before plating on ½ MS pH 5.7 0.7% (w/v) agar (Sigma), seeds were surface-sterilized (3 mins 70% (v/v) ethanol + 0.05% (v/v) Triton X-100, then 10 mins 100% (v/v) ethanol, then resuspended in distilled sterile water. In all conditions, plants were stratified at 4°C/dark for 3 days before incubation. Light intensities were determined with an International Light IL1400A photometer (Newburyport, MA) equipped with an SEL033 probe with appropriate light filters.

Leaf flattening index experiments

Approximately 50 seeds were plated on agar in 90mm × 15mm Petri dishes and placed under 100±10 µmol.m^{-2}.s^{-1} BL continuous WL in a 22°C phytotron. After 10 days of incubation (dai) when wild-type plants reached growth stage 1.4 (Boyes et al.,

2001) plants were transplanted onto soil. Plants were then grown for 15-16 more days in a growth chamber under 80±8 µmol.m^{-2}.s^{-1} with a 16/8hrs light/dark cycle at 20.5±0.5°C and 55-75% relative humidity until wild-type plants reached growth stage 1.10-1.11 (Boyes et al., 2001). White light source was provided by a combination of Coolwhite (L36W/20) and Limilux ® Warmwhite (L36W/830) Osram fluorescent tubes. Plants were shuffled around to minimize the influence of microclimates in growth chamber. We chose this transplantation method because we notice that it yielded plants with more homogenous growth. However, it is important to note that the leaf phenotype of some mutants (e.g. *phot1phot2*) was not the same if grown on agar or directly on soil (see *Results*). We expected these effects to have minimal consequences on our leaf flattening analyses because the major part of plant growth and leaf development occurred after transplantation onto soil, including leaf number 5. Lamina of the 5th rosette leaf was detached from the petiole, placed on its abaxial side on wet white whatman paper, and photographed from above using a PowerShot A640 digital camera (representing curled leaf projections area). The lamina was then artificially flattened by uncurling the edges, gently pressed onto wet whatman paper under transparent plastic sheet to keep lamina flat by capillarity, and photographed again from above (representing total projection area). Projection areas were selected using the Photoshop elements 4.0 magic wand tool and measured using imageJ software (http://rsb.info.nih.gov/ij/). Leaf flattening index is the ratio of curled to total projection areas.

Stomatal aperture experiments

Fully expanded rosette leaves were harvested from 4-week-old plants in the dark. The leaves were blended in a Waring blender (Waring Commercial) for 15 sec in 35 ml of distilled water. The epidermal tissues were collected on a 58-µm nylon mesh and rinsed with distilled water. The epidermal fragments were kept in 2ml of basal reaction mixture (5mM mesbistrispropane (BTP), 50mM KCl, and 0.1mMCaCl2, pH6.5) and were irradiated with red light at 50 µmol.m^{-2}.s^{-1} BL superimposed by blue light at 10 µmol.m^{-2}.s^{-1} BL, for 3h at room temperature. Stomatal apertures were measured in the abaxial epidermis by focusing on the inner lips of stomata. The abaxial epidermises were easily distinguished from the adaxial ones by the shape of

their epidermal cells. In each line, the apertures of 45 stomata were determined. All measurements were done between 8:00am and 11:00am.

Stomatal conductance experiments

Plants were grown in climate cabinets for 8 to 10 weeks, with a day/night cycle of 8/16 h, the temperature cycling between 22/16 °C and illuminated with fluorescent tubes (Osram L36W/25, Munich, Germany) at a photon flux density of 200 µmol.m^{-2}.s^{-1} BL. Relative humidity was not controlled. Plants were transferred to the laboratory the night before measurements, on the next morning (8.00 am), a leaf was excised, its petiole was cut again under water to avoid embolism and kept in water thereafter. A section of the leaf was enclosed in a sandwich-type cuvette (diameter 2.1cm) with glass windows on the upper and lower side. The abaxial side of the leaf was directed upwards and exposed to a gas stream of 0.5liter/min. The relative humidity of the air was 46%, the temperature was 24°C and the CO_2 concentration was 350µl/l. Light was provided by halogen lamps (HLX 64657, Osram, Munich, Germany) to the adaxial side of the leaf and passed through infra red filers (Calflex C, Balzers, Lichtenstein) in combination with colour glass filters; blue short pass $\lambda_{1/2}$ 487 nm (5030, Corning Glass Works, Corning, NY) and red long pass $\lambda_{1/2}$ 630 nm (Schott, Mainz, Germany). The photon flux densities were: 25 µmol.m^{-2}.s^{-1} for blue light and 500 µmol.m^{-2}.s^{-1} BL for red light. Transpiration rates were measured by infrared gas analysis technique (Binos, Heraeus, Hanau, Germany).

Chloroplast movement experiments

Chloroplast movement was assessed photometrically by measuring changes in red light transmittance of leaves through time (Walczak and Gabrys, 1980; Jarillo et al 2001, DeBlasio 2003, 2005) using a microprocessor controlled system based on the design of Berg et al (2005). Plants were grown under 12h light /12h dark photoperiod and 100-120 µmol.m^{-2}.s^{-1} BL of white light (Cool white fluorescent tubes, GE,ect). Temperature was 24°C and humidity was not controlled. When plants reached c.a. 45 days old, one adult leaf per plant was detached, its petiole placed between two wet Whatman strips, and a region of the lamina between the midvein and the margin was

positioned over a light sensor. Epinastic leaf laminas were gently uncurled by making a small section at the edge. A black plastic cover containing built-in red-blue LEDs was positioned above the lamina. Red light transmittance was measured every 5 mins with a 100 µs pulse of RL. Leaves were dark-adapted overnight. Red light transmittance was monitored for one hour in the absence of BL before chloroplast relocalisation was triggered by ten increments of BL (0.1-120 $\mu mol.m^{-2}.s^{-1}$), two hours per fluence rate. BL-induced chloroplast movement was determined by calculating the percentage change in RL transmittance relative to the dark position. Percentage change in red light transmittance ($\%\Delta t$) was determined as $\%\Delta t = (T_t - T_D)/I*100$, where T_t was the transmitted red light at time t, T_D was the mean transmitted red light in dark acclimated leaves (mean value over the first hour of measurement) and I was the incident red light. To account for differences in leaf transmittance all data were scaled to have and initial transmittance of 10%.

Growth experiments

Approximately 15 seeds were sown directly on moist soil (ref of soil) on aracon pots. After vernalization, seeds were placed in a growth chamber under 70±8 $\mu mol.m^{-2}.s^{-1}$ BL or 150±15 $\mu mol.m^{-2}.s^{-1}$ BL WL under transparent plastic lids. Dark/light photoperiod was 8/16hours, temperature was 20±1°C and relative humidity was between 55 and 75%. Fourty-eight hours after incubation (when the hypocotyl was starting to emerge) lids were taken off to reduce temperature and/or humidity effects on hypocotyl agravitropism of epinastic mutants (otherwise this caused heterogeneity in plant establishment). Trays were shuffled every 2 days and plants were regularly watered from below. At three different time points between day 14 and 31 after incubation, hypocotyls were sectioned and the green tissue fresh weight of plants was measured using a precision balance.

Determination of epidermal cell size and stomata numbers

Abaxial and adaxial sides of leaves were gently pressed onto a glass slide containing a layer of nail polish. After drying out, peels of nail polish were pulled off using fine forceps and mounted in a drop of water on a glass slide. To maintain lamina of epinastic mutants flat, the leaves were sectioned at apex and artificially flattened on double adhesive tape. Regions of the lamina analyzed were located between 25 and 75% of the distance between the tip and the base of the leaf and halfway between midrib and margin. Bright field digital photographs were taken from one focal plane view using a plan neofluore 0.3 10× objective (100-fold magnification) on a Leica inverted confocal microscope. Micrographs of prints and of a micrometric ruler were printed onto paper. Outlines of 40 to 130 cells were drawn then scanned, and total area was determined by ImageJ software (http://rsb.info.nih.gov/ij/) and number of epidermal cells and stomata were counted within that area. From these measurements the following parameters were determined: average cell area, number of cells per leaf, stomatal density, stomata:epidermal cell ratio (stomatal index; fraction of guard cells in the total population of epidermal cells). Five leaves were analyzed and mean ±SD calculated.

Immunoprecipitation experiments

Plants were grown on 1/2MS agar under 120 µmol/m^2/sec WL at 22°C for 15 days (growth stage 1.05; Boyes et al., 2001). About 300 mg of aerial parts of plants were harvested and grinded in 1 ml of cold extraction buffer EB (300 mM sucrose, 150 mM NaCl, 10 mM K-acetate, 5 mM EDTA, 100 mM AEBSF (prefabloc), 1% of protease inhibitor mixture for plant extracts (Sigma P9599), 50 mM Hepes pH 7.9) using a pestle and mortar. After separating debris (5 mins at 1000 *g*, 4°C), microsomes were isolated by ultracentrifugation (75 mins at 75'000 *g*, 4°C) and resuspended in 750 µl of EB plus 0.5% (v/v) Triton X-100 to solubilize membrane-associated proteins. Suspension was centrifuged 5 mins at 16'000 *g*, 4°C and GFP-fused proteins present in the supernatant (INPUT) were immunoprecipitated by gentle mixing with 60 µl of magnetic beads coupled to monoclonal anti-GFP (Miletenyi Biotec, Product number

130-091-125) for 1 h at 4°C. Immunoprecipition solution was then passed onto a magnetic column and washed extensively with 20 column volumes of EB plus 0.5% (v/v) Triton X-100. Immunoprecipitated protein complexes (IP) were collected by adding 50 µl of 95°C 2× Laemmli buffer onto the column.

Western blotting

Proteins were separated on 10% SDS/PAGE gels and transferred onto nitrocellulose with 100mM CAPS pH11 + 10% (v/v) methanol. The blots were probed with anti-DET3, anti-NPH3, anti-PKS1 and anti-GFP antisera as described (Lariguet et al., 2006). Polyclonal anti-PKS2 antibodies were raised as follow: a *PKS2* cDNA sequence encoding the first 155 amino acids was fused to the C-terminus of glutathione-S-transferase (GST) coding sequence using the BamH1 site in the pGEX-4T-1 vector (to generate pMC30). GST-PKS2(aa1-155) recombinant proteins were produced in *E. coli* by inducing gene expression with 0.1mM IPTG for 3hrs at 20°C. Purified soluble GST-PKS(aa1-155) proteins were used to immunize rabbits. After six boosts the serum of one rabbit was retrieved and polyclonal antibodies specific to PKS2 were obtained by negative (using protein extracts from *pks2-2* plants) and positive (using purified GST-PKS2(aa1-155) proteins) purifications. Anti-PKS2 antibodies were used at a 1/300 dilution in PBS, 0.1% Tween 20, and 5% nonfat milk.

Protoplast auxin efflux experiments

Intact *Arabidopsis* mesophyll protoplasts were prepared from rosette leaves of plants grown on soil under white light (100 µmol m^{-2} s^{-1}, 8 h light/16 h dark, 21°C) and auxin efflux experiments were performed as described in Bouchard et al. (2006). In short, intact protoplasts were isolated as described (16), and loaded by incubation with 1µl/ml ^{3}H-IAA (specific activity 20 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO) on ice. External radioactivity was removed by percoll gradient centrifugation. Effluxed radioactivity was determined by scintillation counting of aqueous phases and is presented as relative efflux of initial efflux (efflux prior to incubation), which was set to zero.

2. Additional results and observations

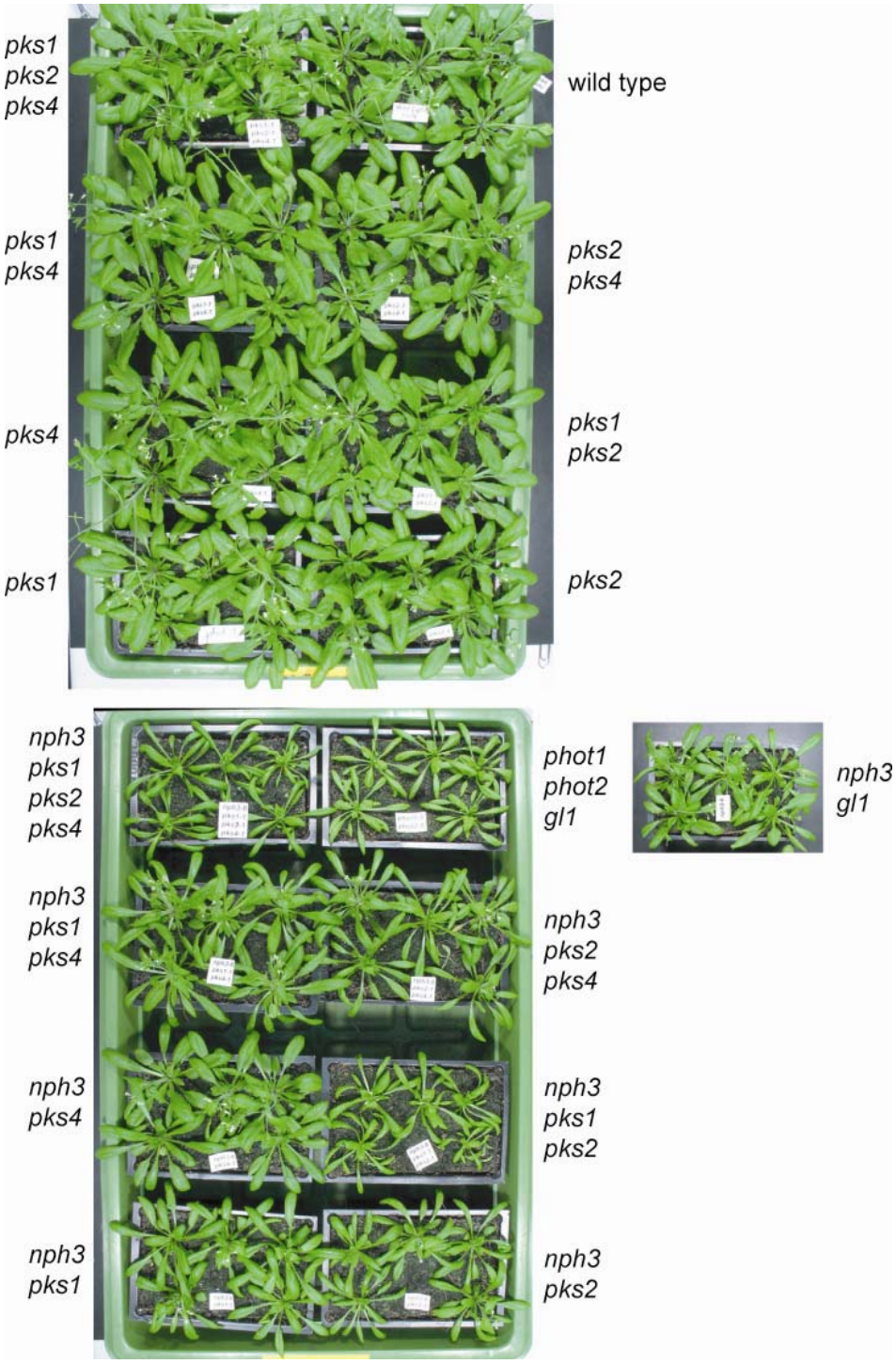


Figure 12: Leaf curling phenotypes of *nph3/pks* multiple mutants indicate that *PKS* genes play redundant roles in leaf flattening.

Plants were grown in parallel for 44 days under our standard growth room conditions (150 µmol.m^{-2}.s^{-1} at 20°C, 16 hrs light/8 hrs dark photoperiod).

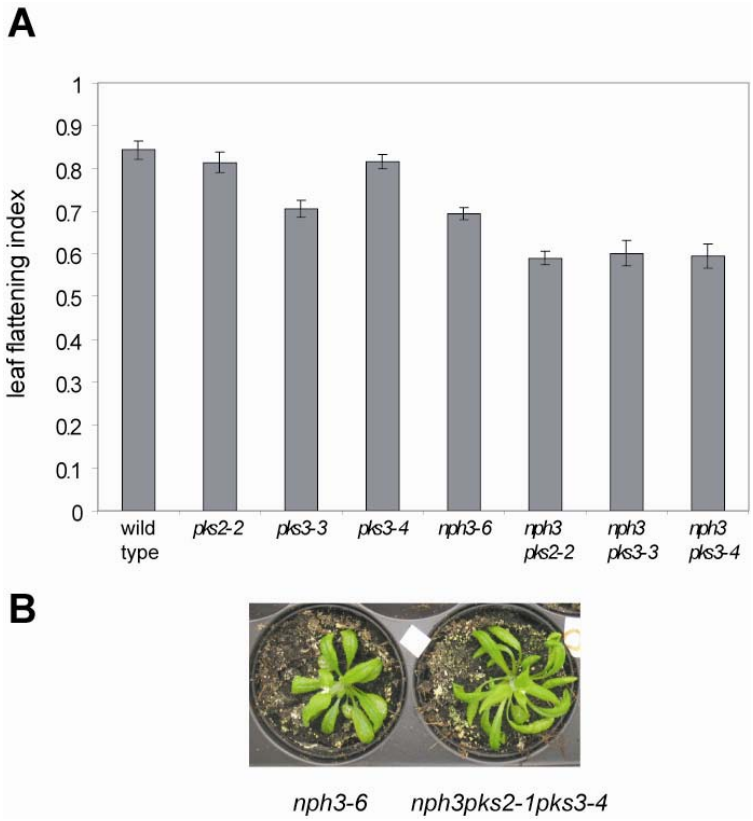


Figure 13: Leaf flattening phenotypes of *pks3* mutants.

(A) Leaf flattening phenotypes of *pks3* single and *nph3pks3* double mutants. Leaf flattening data was analysed as previously described (Figure 1). Data shows mean ± 95% confidence intervals for 12<n<13 plants, one experiment. Note that the *pks3-3* single mutant already had curled leaves, unlike the *pks2-2* null mutant. Curling in *pks3-3* occurs mainly at the lamina margin (data not shown), unlike *nph3* which has more regular concave-shaped laminas (Figure1).

(B) The *nph3pks2-1pks3-4* mutant has very epinastic leaves. Plants were grown under standard conditions for approximately 33 days. Control pictures of wild type and *pks2-1pks3-4* are not available but leaf flatness between these two genotypes was not visually distinguishable. Control pictures of *nph3pks2-1* and *nph3pks3-4* are neither available but leaves of the *nph3pks2-1pks3-4* triple mutant were clearly more epinastic.

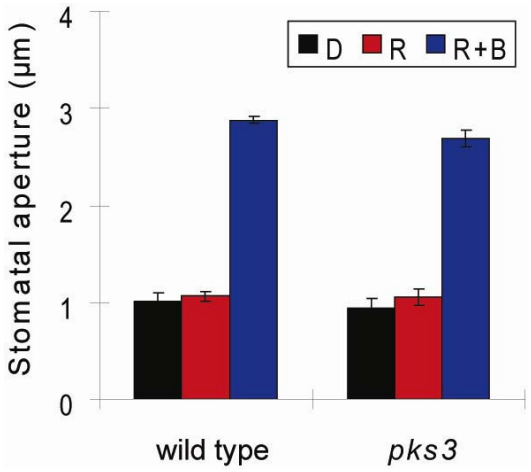


Figure 14: ***pks3-3*** **shows no impairment in BL-induced stomatal opening – data of Shin-ichiro Inoue**

Stomatal opening was measured as in Figure 3.

3. Brief summary of results

The main results from this chapter are as follow:

- The PKS are not global regulators of phot signalling

- The PKS act in the same subset of phot-mediation processes as NPH3 (i.e. phototropism and leaf flattening but not chloroplast movements and stomatal opening)

- Leaf epinasty hinders plant growth, even under favourable light conditions

- Impaired plant growth may be a consequence of the combination of: (i) reduced light capture, (ii) reduced gas exchange, and (iii) a developmental defect of leaf expansion in curled leaves

- The *PKS* act redundantly during leaf flattening in the phot2 pathway, and their relative contributions are as follow: PKS2 ~ PKS3 > PKS1 > PKS4

- In addition to its reported role in the phot1 pathway, NPH3 also has an important function in the phot2 pathway

- *nph3* and *pks1pks2pks4* or *pks2pks3* mutants interact synergistically

- PKS1, PKS2 and NPH3 can associate with phot1 and phot2 *in vivo* in aerial parts of 2-week-old plants. However, it is not known whether they are found in the same protein complex

- PKS2 and PKS1 may contribute to leaf flattening by acting on auxin transport

Questions that are raised by this study are:

1/ Do the PKS also regulate leaf positioning – another NPH3-controlled BL response?

2/ How – at the molecular level - does PKS2 act with NPH3 during phot signalling in leaves? Do PKS2 and phots interact directly?

3/ If PKS2 is indeed a regulator of auxin transport then what is its molecular function in that process?

In the next chapter I addressed these questions using genetic and molecular approaches.

CHAPTER 3 – STUDY OF PKS ROLE IN LEAF POSITIONING

1. Brief introduction and significance of this work in the overall project

Adequate expansion and flattening of the leaf lamina is certainly an important adaptive process for optimization of light capture (Takemiya et al., 2005; Inoue et al., 2007; Chapter 2). Once the leaf lamina (acting as "solar panels") has reached an optimal shape it orientates itself according to the direction and intensity of incident light (Koller, 1990; Inoue et al., 2007).

Although *Arabidopsis* does not have pulvini to provide high flexibility in lamina movements, it's petioles and blades still retain a strong solar tracking potential. Blue light direction has a dramatic influence on these leaf positioning events (Inoue et al., 2008a). In addition to directional BL, leaf orientation in rosette plants like *Arabidopsis* is also influenced by the photoperiod, light intensity, the gravity set-point angle and the ratio or RL to FRL (shade of competing neighbours). For instance, nictinastic sleep movements have been postulated to protect the plant from chilling (Enright, 1982). Under increasing light intensities, *Arabidopsis* leaves become more horizontal, while under shade they become more erect as to overtake neighbouring leaves (Pierik et al., 2004; Millenaar et al., 2005; Mullen et al., 2006). As during hypocotyl phototropism, the leaf's response to the gravity vector also determines its final orientation, and this process is modulated by different signals (changing the gravity set-point angle) (Mano et al., 2006). Thus, leaf positioning / orientation are highly modular processes that are continuously fine-tuned under changing light environments.

Phototropins and the signalling element NPH3 were recently shown to play essential roles in leaf positioning in response to BL. As for the leaf blade, the petioles of *phot1phot2* and *nph3* mutants are highly epinastic and this leads to reduced light capture by the lamina (Inoue et al., 2007). Early observations of the *pks* mutants, in

particular in the *nph3* sensitized background, clearly indicated that the PKS are also involved in this distinct leaf process (i.e. petiole positioning).

Thus, this Chapter extends the analysis of PKS function in phot signalling to leaf positioning. Analysis of leaf positioning was meaningful for several reasons. First, it expanded the analysis to another important leaf process that is complementary with leaf flattening (leaf flattening and positioning act in concert to obtain optimal light utilization). Second, leaf positioning (unlike leaf flattening) experiments could be performed using more precise light treatments that allowed selective activation of phot1 or phot2 pathways. This enabled us to perform more detailed epistasis experiments and better characterise *PKS* function in phot signalling. Third, the effect of the *PKS* genes was more visible and significant than during leaf flattening, allowing us to dissect in more detail the roles of the different *PKS* genes.

As for leaf flattening, *PKS2* had an important role during leaf positioning. Molecular and biochemical experiments were performed in an attempt to further characterise the function of PKS2 during phot signalling. Results of these experiments are also presented in this Chapter.

Finally, when results / observations were described and discussed in the text but were not shown, this was indicated as “(data not shown)”. An appendix of these results has been created but was not included in this report (see Christian Fankhauser).

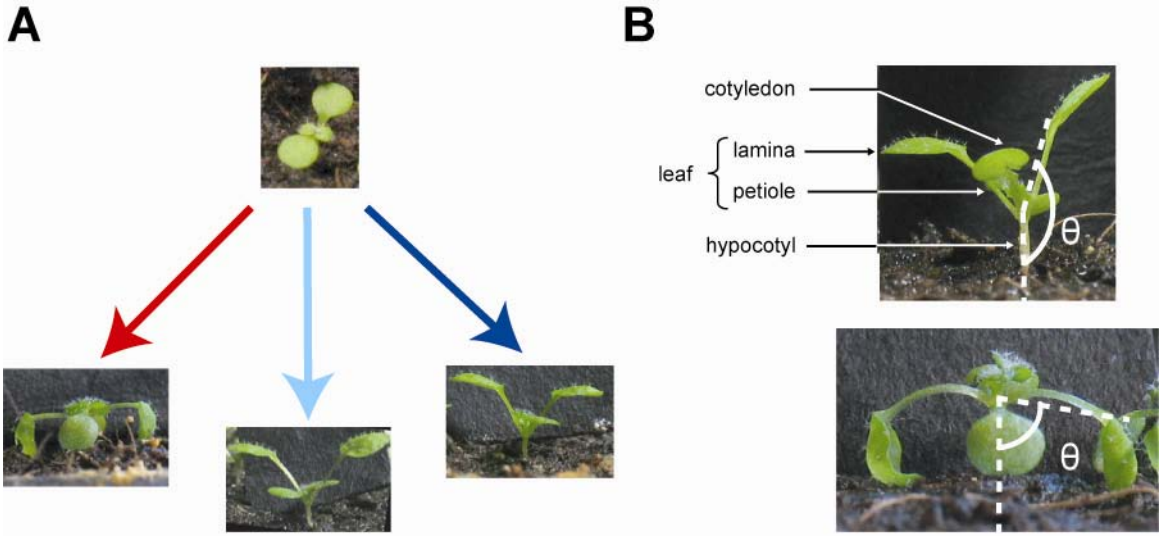


Figure 1: Method for leaf position analysis.

(A) Method adapted from the protocol of Inoue et al., (2007) with many modifications. Seeds were germinated on soil and plants grown for 9 days under 130 µmol.m^{-2}.s^{-1} white light (16 hrs light/8 hrs dark photoperiod) at 20°C until first pair of true leaves reached c.a. 1mm in length (growth stage 1.01; Boyes et al., 2001). Plants were then transferred to different incubators containing light emitting diodes and illuminated with 50 µmol.m^{-2}.s^{-1} red light (RL, red arrow), RL plus 0.3 µmol.m^{-2}.s^{-1} blue light (LBL, light blue arrow) or RL plus 5.0 µmol.m^{-2}.s^{-1} blue light (HBL, dark blue arrow), unless indicated otherwise. First true leaves were allowed to develop under these light treatments for 5 days 8 hours (until growth stage c.a. 1.04) and plants were photographed from the side between 5.30pm and 8pm.

(B) Measurement of leaf position.
Upper panel: schematic representation of a plant for which leaf position was measured. Leaf position was determined as the angle (θ) between the hypocotyl and the leaf's petiole (white dashed lines) to which 90° was subtracted to obtain an indicative angle relative to horizontal.
Lower panel: for petioles that are strongly curled downwards (epinastic (Kang, 1979)), a line between the shoot apex and the petiole-lamina connection was drawn.

2. Genetic and physiological analysis of PKS function during leaf positioning

2.1. Cotyledon and leaf positioning are controlled by phot1, phot2 and NPH3 - Method and confirmation of published results

The group of Ken-ichiro Shimizaki has shown that wild-type plants grown under RL display highly epinastic leaves. However, when superimposing BL, the petioles become erect and the laminas are positioned so to capture light from above (Takemiya et al., 2005; Inoue et al., 2007). The direction of BL illumination seems to be important for this process because lateral BL caused re-orientation of the petioles and the laminas towards the light source (a behaviour reminiscent of solar tracking) (Inoue et al., 2008a). Thus, BL has a critical influence on how plants position their leaves and leaf positioning can be analysed by applying light from above and measuring the angle of the petioles (Inoue et al., 2008a).

A method was designed based on the work of Inoue and co-workers to study the role of the *PKS* in leaf positioning (Inoue et al., 2007; Figure 1). Please note two main differences: (i) the red light fluence rate used was 50 $\mu mol.m^{-2}.s^{-1}$ instead of 25 $\mu mol.m^{-2}.s^{-1}$ and (ii) the blade-petiole intersection instead of the petiole tangent was used as a reference point to measure petiole angle (Figure 1B). Interestingly *phot1*, *phot1phot2* and *nph3* mutants (but not *phot2*) displayed agravitropic hypocotyls in early stages of development when grown under high temperature and/or humidity (when kept too long under a transparent plastic lid) (data not shown). In addition, under conditions where Arabidopsis were essentially blind to directional BL from above (e.g. *phot1* and *nph3* under LBL – Inoue et al., 2007), randomized growth orientation was probably enhanced by the action of phys (Hangarter, 1997; Lariguet and Fankhauser, 2004; Schepens et al., 2008). Thus, for these two reasons, the angle of the petiole relative to the hypocotyl was preferred to the angle between horizontal and the petiole.

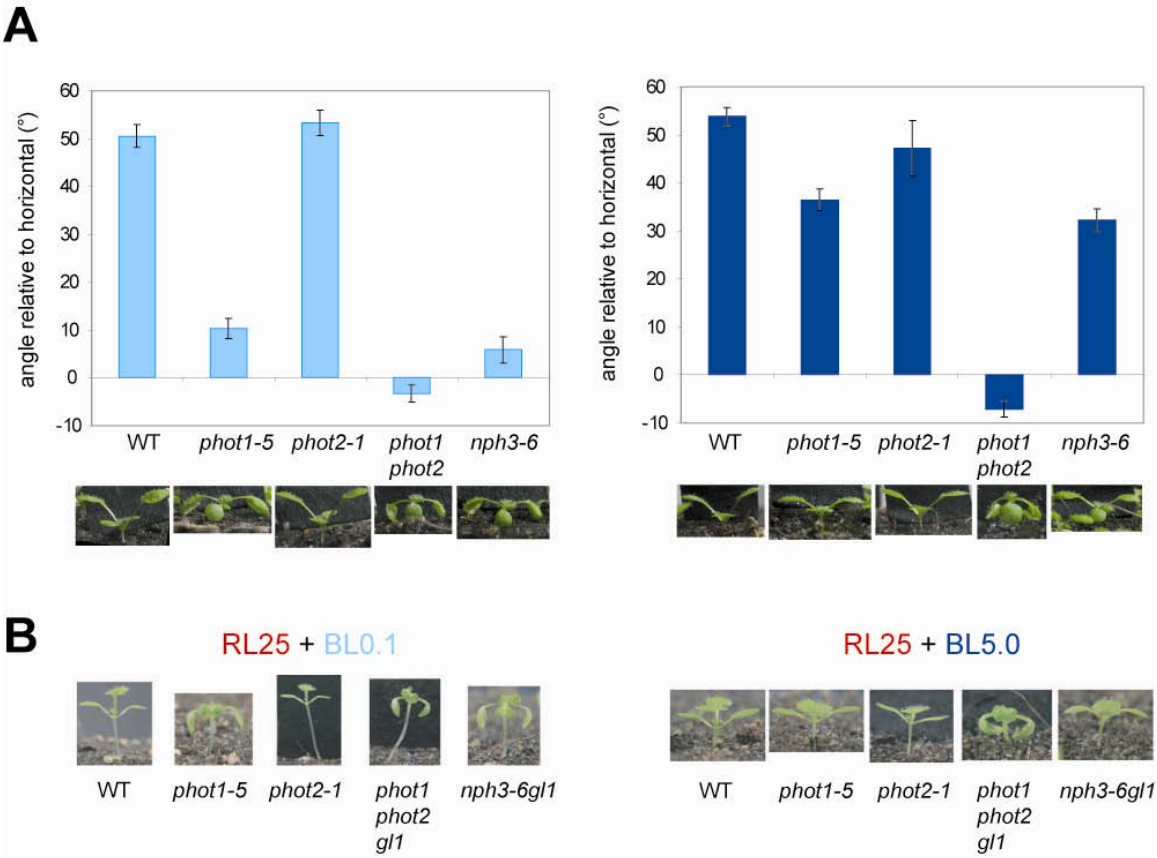


Figure 2: Cotyledon and leaf positioning is controlled by phototropin signalling – control experiments reproduced based on Inoue et al. 2007.

(A) Leaf positioning in *phototropin* and *nph3* mutants. Light blue bars: RL + LBL. Dark blue bars: RL + HBL. Bars indicate mean ± 95% confidence intervals for 32<n<52 plants (64<n<104 measured petioles). Graphs show representative results for one of two independent experiments that gave similar data.

(B) Cotyledon positioning in *phototropin* and *nph3* mutants. RL and BL fluence rates are indicated because they were different in early setup preliminary experiments during which these observations were made. Pictures of *phot2* and *phot1phot2gl1* were obtained in a different experiment. Their hypocotyls appear very long under RL25 + BL0.1 because a small amount of FRL (0.4 µmol.m^{-2}.s^{-1}) was also added my mistake (presumably causing a shade-avoidance responses).

However, it is important to note that the angle between the petiole and the lamina also changed depending on light treatments and genotypes (Figures 1 and 2). In highly epinastic leaves, the lamina often touched the soil and this limited the extent of petiole epinasty (Figure 1B lower picture). Since in epinastic leaves the lamina also curled, this provided more space for the petiole to bend downwards. Thus, the measured angles describe a combination of petiole bending and lamina curling. Attempts were made to dissociate these different aspects of leaf epinasty (by adding hypocotyl-petiole and petiole-lamina angles for e.g.) but were not followed up because of over-complexity (data not shown).

In summary, leaf positioning is determined by the shapes of laminas and petioles and by the relative angle between these different organs. In this study only hypocotyl-petiole angles were quantified and observations on other aspects of leaf positioning were only mentioned.

The group of Ken-ichiro Shimazaki showed that phot1 and NPH3 control leaf positioning under LBL and phot2 acted redundantly with phot1 under HBL (Inoue et al., 2008a). Similar results were obtained for both leaves and cotyledons (Figures 2A and B). In fact, cotyledon positioning defects could be seen during early seedling development (3-4 dai) suggesting that this process may also be important during early stages of establishment (data not shown). The similarities in leaf epinasty between *nph3* and *phot1* and the fact that NPH3 is a well-characterised phot1 signalling component led to the conclusion by Inoue and co-workers that NPH3 acts mainly in the phot1 pathway. However in our assays *nph3* leaves were consistently more epinastic than *phot1* suggesting that this component may act in an additional pathway than phot1 (Figure 2A).

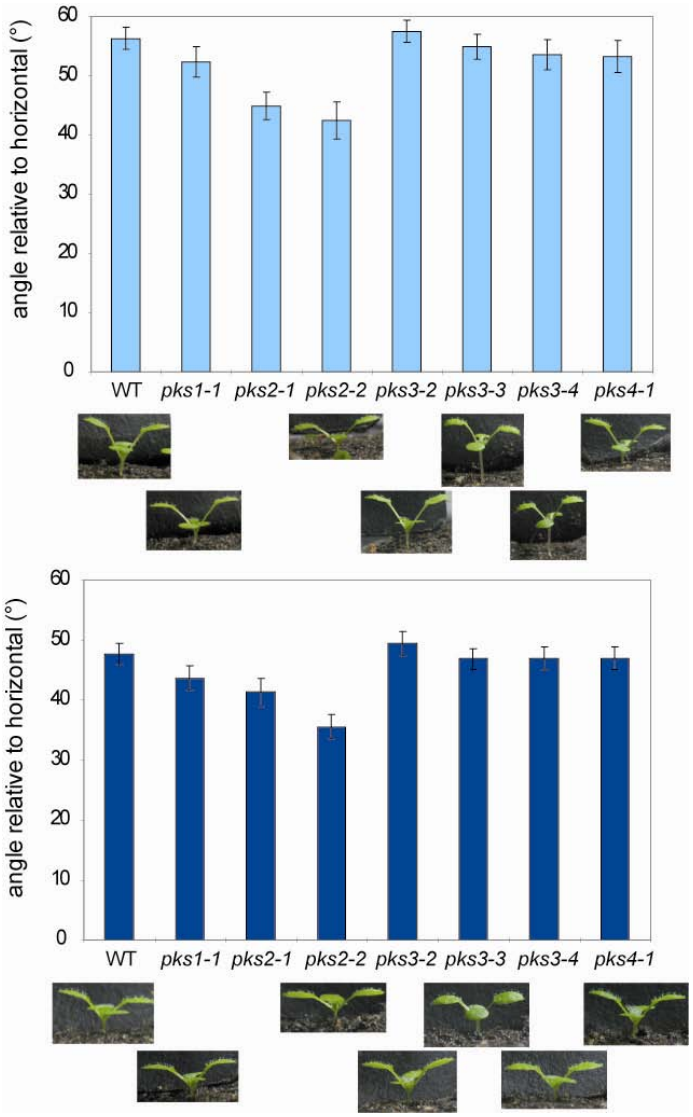


Figure 3: Systematic analysis of leaf positioning in plants mutated in each *PKS* gene.

Light and dark blue histogram bars correspond to LBL and HBL, respectively. *pks1-1*, *pks2-1*, *pks2-2* and *pks4-1* alleles correspond to T-DNA insertion mutants (Lariguet et al., 2006; Chapter 2 Figure 10; Ariane Honsberger). *pks3-2*, *pks3-3* and *pks3-4* alleles are tilling alleles that were isolated, genotyped and backcrossed by Martine Trevisan and Christian Fankhauser. See the Materials and Methods for details. Note that *pks3-3* leaves are concave in shape, as shown by the apparent thicker lamina in the lower side pictures. Bars indicate mean ± 95% confidence intervals for 21<n<31 plants (42<n<62measured petioles). Graphs show representative results for one experiment.

2.2. PKS2 *is involved in leaf positioning*

To determine whether the *PKS* controlled leaf positioning, *pks1, pks2* and *pks4* null mutants were analysed (Lariguet et al., 2006; Schepens et al., 2008). Three *pks3* missense mutants containing potentially deleterious amino acids substitutions in the PKS3 protein were also included in this analysis (Martine Trevisan and Christian Fankhauser, unpublished). These mutants were obtained from an *Arabidopsis* TILLING collection (Till et al., 2006) The strongest *pks2* allele - *pks2-2* which resulted in gene knock-out (Chapter2 - Figure 10) – generated mild but significant defects under both LBL and HBL. A weaker *pks2* allele - *pks2-1* in which a truncated form of PKS2 protein is present (Chapter2 - Figure 10B) showed weaker but still significantly defects in leaf positioning compared to WT (Figure 3). However, *pks3* and *pks4* mutants displayed wild type-like leaves. Although very weak, the *pks1* phenotype in leaf positioning is reminiscent of the weak phenotype of *nph3pks1* in leaf flattening (Chapter2 Figures 10A and 12).

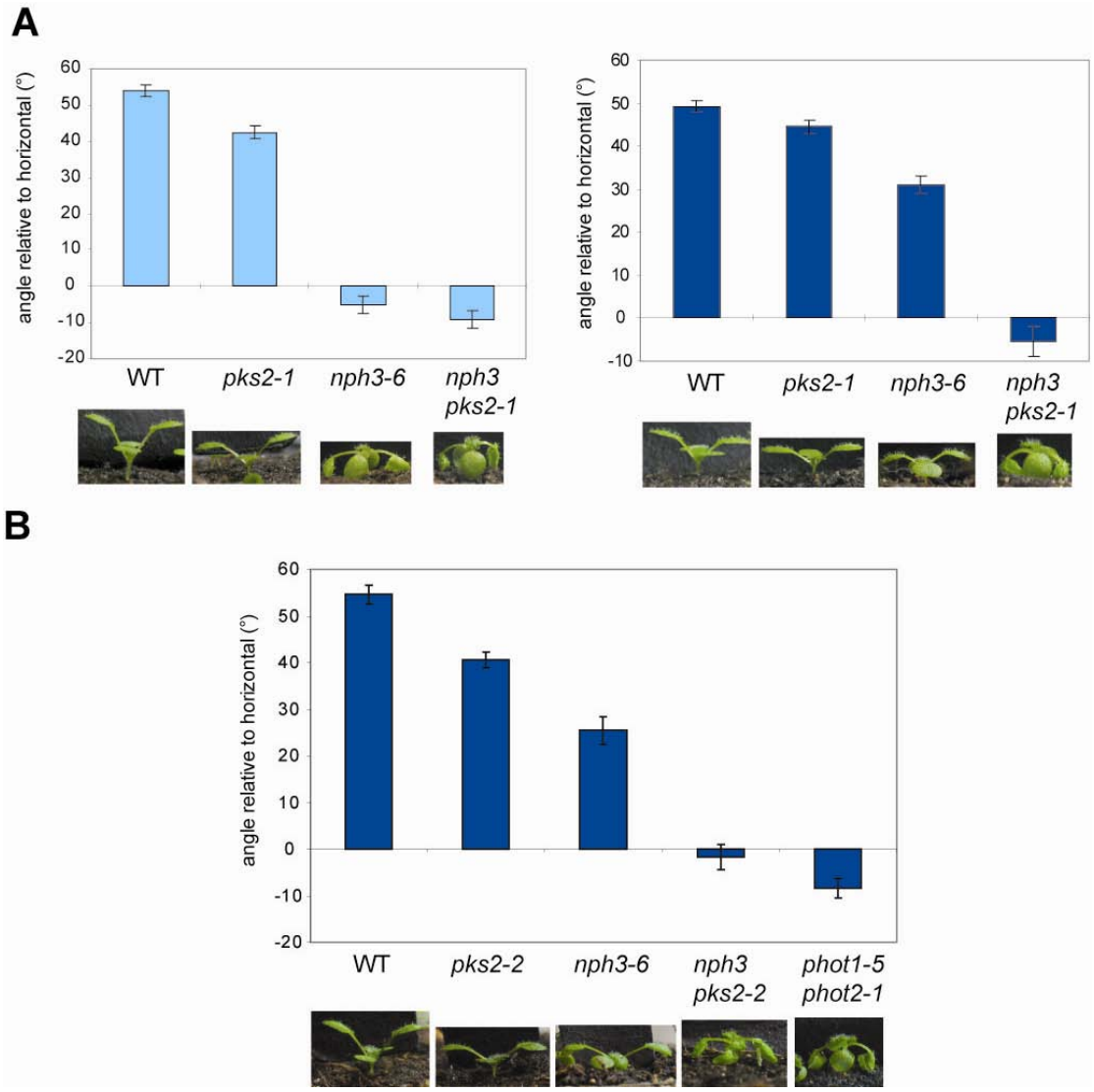


Figure 4: *PKS2* loss-of-function generates highly epinastic plants in the *nph3-6* sensitized background.

(A) Leaf epinasty in the *pks2-1* mutant background. Bars indicate mean ± 95% confidence intervals for 32<n<55 plants (64<n<110 measured petioles). Graphs show representative results for one experiment.

(B) *nph3pks2* resembles *phot1phot2* under HBL. Bars indicate mean ± 95% confidence intervals for 27<n<51 plants (54<n<102 measured petioles). Graphs show representative results for one experiment.

2.3. Synergy in leaf epinasty between PKS2 *and* NPH3 *mutants*

Because the *pks2* mutant was previously shown to strongly interact with *nph3* in leaf flattening (Chapter 2, Figure 10A) the *nph3pks2* mutant was analysed in leaf positioning assays. Under LBL *nph3* was epistatic over *pks2* which is not surprising given that *nph3* fully controls leaf positioning under this condition (Inoue et al., 2007; Figure 2A). Interestingly under HBL the *nph3pks2* mutant was extremely epinastic and essentially resembled *phot1phot2* while *pks2* and *nph3* displayed intermediate phenotypes (Figure 4B). This indicated that the combination of both mutations had a synergistic effect.

As previously discussed, the similarities between *nph3* and *phot1* under LBL and HBL suggest that NPH3 acts in the phot1 pathway controlling leaf flattening (Inoue et al., 2007; Figure 2). The mild phenotype of *pks2* under both HBL and LBL indicates that PKS2 may also act in the phot1 pathway but not in an essential manner (unlike NPH3). One element consistent with this possibility is the fact that laminas of *pks1pks2pks4* displayed an intermediate epinasty phenotype under LBL conditions (data not shown). In addition, the highly epinastic *nph3pks2* phenotype suggests that PKS2 may also act in the phot2 pathway. Indeed if this were the case, disruption of the phot2 pathway in a sensitized *nph3* background would accentuate leaf epinasty and lead to phenotypes resembling *phot1phot2* (Figure 2). To test these hypotheses and to position with more accuracy both *PKS2* and *NPH3* in phototropin signalling pathways, epistasis between *nph3*, *pks2* and *phot1/phot2* mutants was studied.

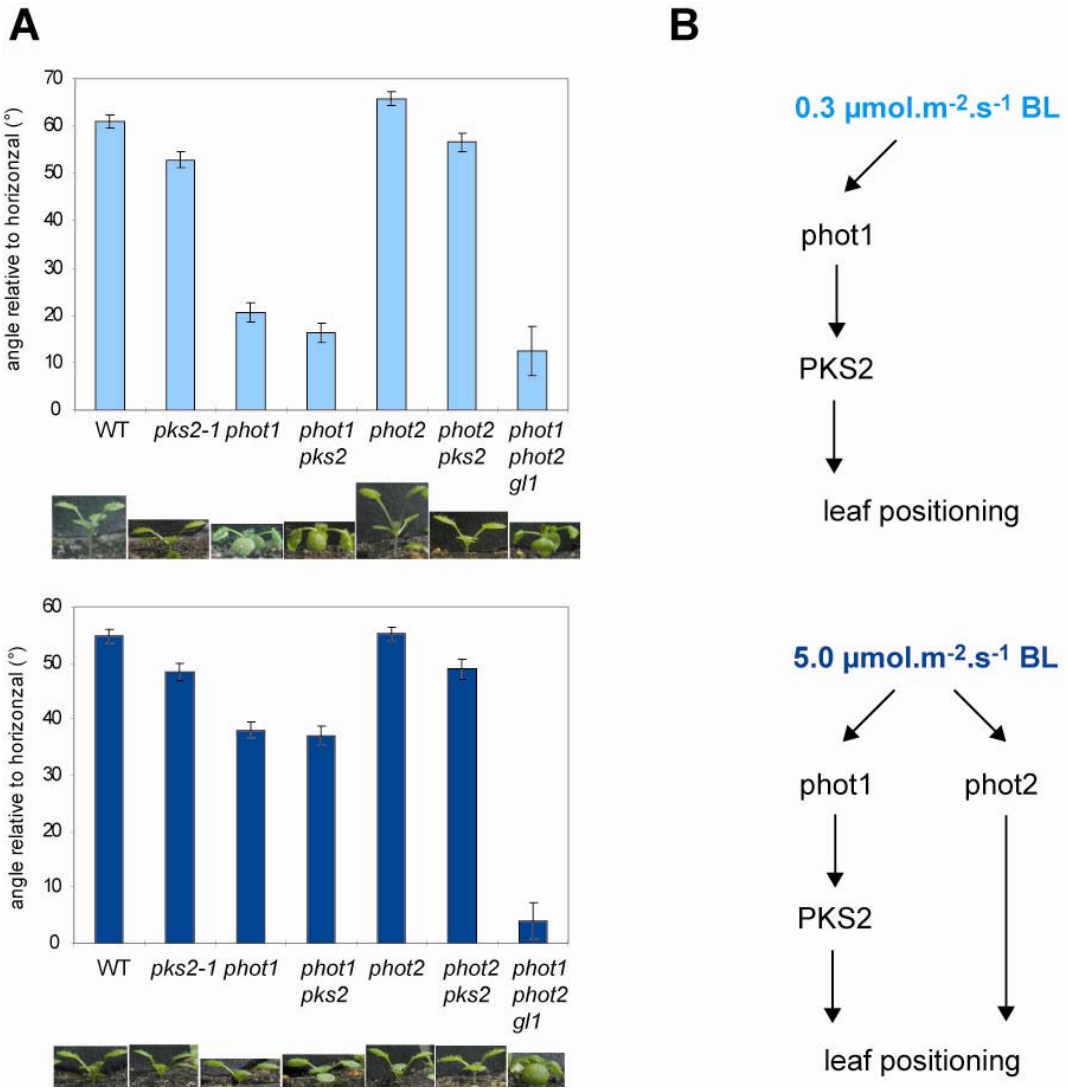


Figure 5: Epistasis between *PKS2* and the *PHOTs*.

(A) Phenotype of *pks2phot* mutants under LBL and HBL. Note that under HBL, the *phot1pks2* mutant has slightly concave laminas compared to *phot1* or *pks2* mutants. This phenotype is consistent with the leaf flattening phenotype of *phot1pks1pks2pks4* described in the previous chapter, and supports a role for PKS2 in the phot2 pathway controlling leaf flattening. Bars indicate mean ± 95% confidence intervals for 22<n<61 plants (44<n<122 measured petioles). Graphs show representative results for one of two independent experiments that gave similar results.

(B) Epistasis data position *PKS2* within the phot1 pathway. LBL activates predominantly the phot1 pathway. phot1 and phot2 pathways are activated in under HLB. In both light conditions the *pks2* phenotype is intermediate between WT and *phot1*, and *phot1pks2* resembles *phot1*.

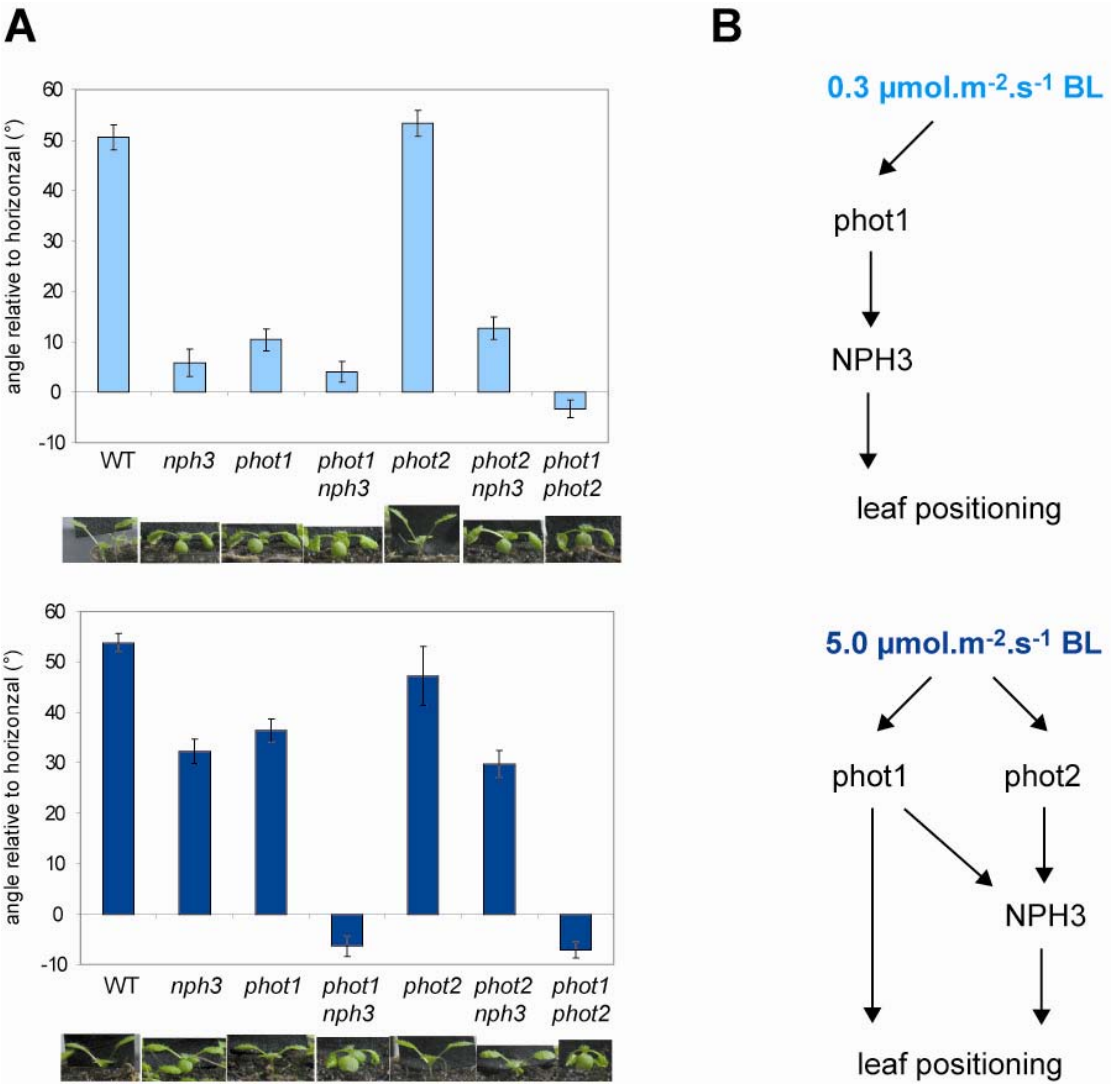


Figure 6: Epistasis between *NPH3* and the *PHOTs*.

(A) Phenotype of *nph3phot* mutants under LBL and HBL. Note that under HBL, *nph3* laminas are slightly concave compared to wild type of *phot1*. Bars indicate mean ± 95% confidence intervals for 32<n<52 plants (64<n<102 measured petioles). Graphs show representative results for one experiment.

(B) *NPH3* plays an important role within the phot2 pathway under high BL fluence rate. Low BL (0.3 µmol.m^{-2}.s^{-1}) activates predominantly the phot1 pathway (in this experiment *phot1phot2* was clearly more epinastic than *phot1*, indicating that phot2 may also be slightly activated). *nph3* is slightly more epinastic than *phot1* indicating an important role for NPH3 in the phot1 pathway and a weak contribution in the phot2 pathway. Under higher BL (5.0 µmol.m^{-2}.s^{-1}), both phot1 and phot2 pathways are activated. *nph3phot1* resembles *phot1phot2* indicating a strong role for NPH3 in the phot2 pathway. *nph3* has an intermediate suggesting that NPH3 also plays a partial role in the phot1 pathway.

2.4. Genetic analysis of NPH3 *and* PKS2 *roles in the phot1 and phot2 pathways*

Under both HBL and LBL *phot1* appeared epistatic over *pks2*, while *pks2* was epistatic over *phot2* (Figure 5A). These data indicate that PKS2 acts predominantly in the phot1 pathway during leaf positioning (Figure 5B). It is interesting to note that in these leaf positioning assays, *phot1pks2* and *phot1pks1pks2pks4* laminas appeared more concave in shape under HBL, which is consistent with the previously reported role for PKS2 in the phot2 pathways during leaf flattening (Figure 5A, data not shown; Chapter 2 – Figure 1).

Under LBL *nph3* globally appeared epistatic over both *phot1* and *phot2*. This results is consistent with NPH3 acting as an essential player of the phot1 pathway (Figure 6). However, this hypothesis is strongly challenged by the phenotypes of *nph3phot1* and *nph3phot2* mutants under HBL (Figure 6). Indeed, one would have expected *nph3phot2* – not *nph3phot1* - to resemble *phot1phot2* if NPH3 acted downstream phot1. This result indicates that NPH3 is also an important component of the phot2 pathway (Figure 6B).

Under LBL, *PHOT2* loss-of-function had a hyponasty effect on the petioles. This effect was observed in wild type, *pks2* and *nph3* backgrounds (Figures 5 and 6). One possible interpretation is that phot2, even under a low fluence rate of 0.3 µmol.m^{-2}.s^{-1} BL (Sakai et al., 2001), is slightly activated and negatively regulates the phot1 pathway. The phot1phot2 phenotype appears more severe than the phot1 phenotype under LBL, supporting the view that the phot2 may be partially activated. As a remark, I would like to add that *phot1phot2* mutants (especially *phot1-5phot2-101*, where *phot2-101* is a complete knock-out while *phot2-1* is a leaky allele (Jarillo et al., 2001; Cho et al., 2007)), display very severe phenotypes such as short petioles and small blades (as compared with *phot1* or *nph3* under LBL). I believe that PHOT loss-of-function leads to basal defects in plant development. It would be interesting to compare the wild type and *phot1-5phot2-101* phenotypes under 50 µmol.m^{-2}.s^{-1} red light (early experiments that I did not follow up).

The summary of epistasis studies is as follow. Under LBL, NPH3 is positioned mainly in the phot1 pathway. This is consistent with previous reports (Inoue et al., 2008a). Under HBL, NPH3 acts predominantly in the phot2 pathway and retains a slight role in the phot1 pathway (as indicated by the intermediate phenotype of *nph3*). The data positions PKS2 in the phot1 pathway under both HBL and LBL. The strong enhancement of leaf epinasty in the *nph3pks2* mutant is consistent with the interpretation of epistasis data obtained for *PKS2* and *NPH3* independently under HBL: since *NPH3* has a partial role in the phot1 pathway (and is crucial for the phot2 pathway), loss of *PKS2* function may aggravate the disruption of the phot1 patway in the *nph3* sensitized background, and lead to leaf positioning defects that are similar to the *phot1phot2* mutant (Figure 4B).

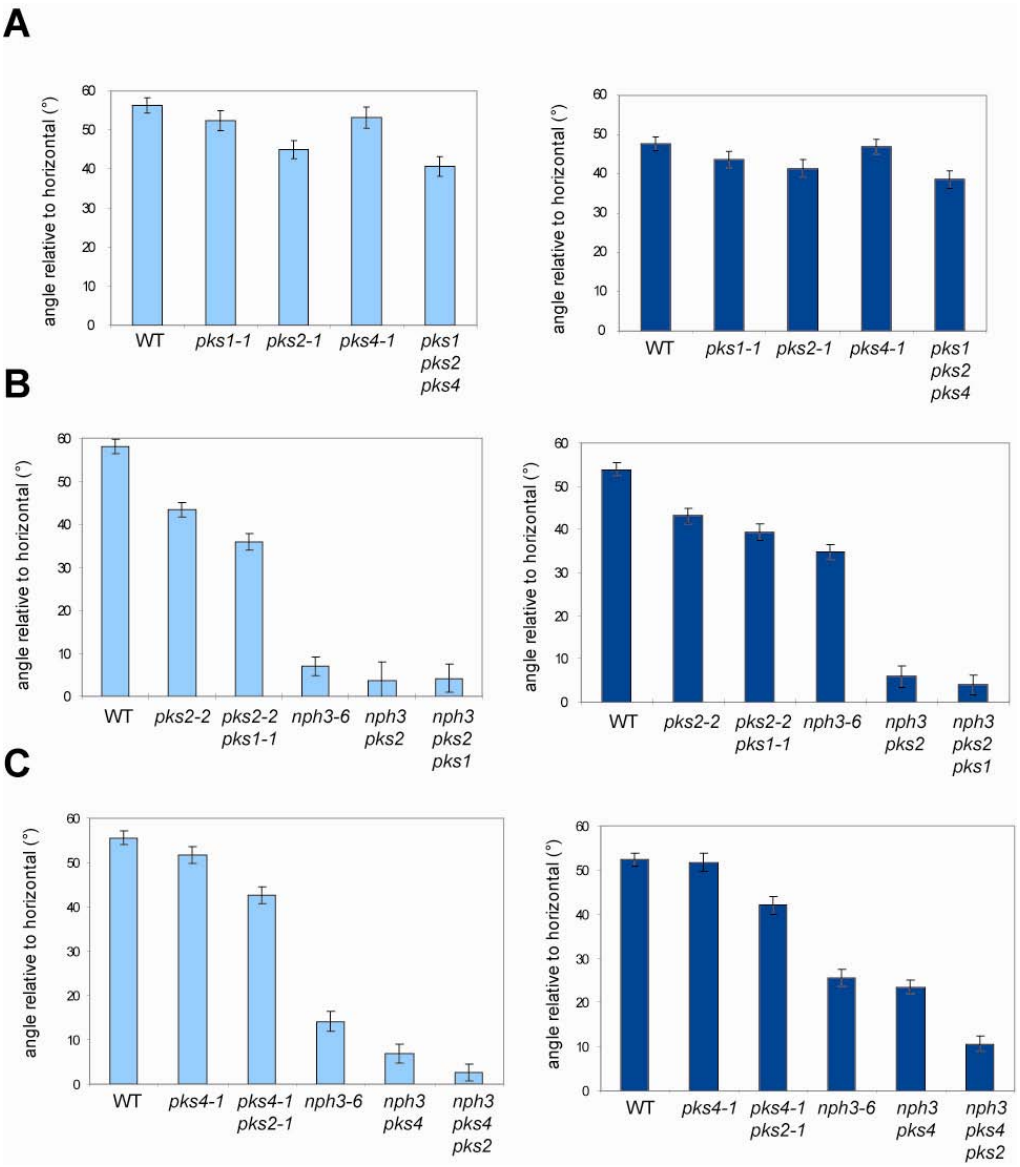


Figure 7: Analysis of potential functional redundancy in the *PKS* genes family.

(A) Phenotype of the *pks2pks1pks4* mutant. Bars indicate mean ± 95% confidence intervals for 21<n<32 plants (42<n<64 measured petioles). Data was obtained during the same experiment as in shown Figure 3A.

(B) Redundancy between *PKS1* and *PKS2*. Bars indicate mean ± 95% confidence intervals for 24<n<70 plants (48<n<140 measured petioles). Data was obtained during the same experiment as in shown Figure 4B.

(C) Redundancy between *PKS4* and *PKS2*. Bars indicate mean ± 95% confidence intervals for 51<n<73 plants (102<n<146 measured petioles). Graphs show representative results for one experiment.

2.5. Contribution of the PKS *family to leaf positioning: analysis of higher-order* PKS *mutants*

Results of early experiments aimed at studying redundancy among the *PKS* during leaf positioning are presented in Figure 7. Although incomplete, these results provide some information about genetic interactions between the *PKS*s. In a *pks2* mutant background, *PKS1* loss-of-function clearly accentuated leaf positioning defects (Figure 7A and B), supporting the previous result that *PKS1* has a mild role in this process (Figure 3). The mild phenotype of *pks4* under LBL, especially in the *nph3* sensitized background, suggests that PKS4 may also play a slight role in leaf positioning (Figure 7C). However, *PKS4* loss-of-function did not accentuate leaf epinasty in wild type or *nph3* background under HBL (Figure 7C). Finally, the *pks1pks2pks4* mutant is only slightly more epinastic than *pks2* indicating that *PKS2* is indeed the predominant player in this blue light response (Figure 7A).

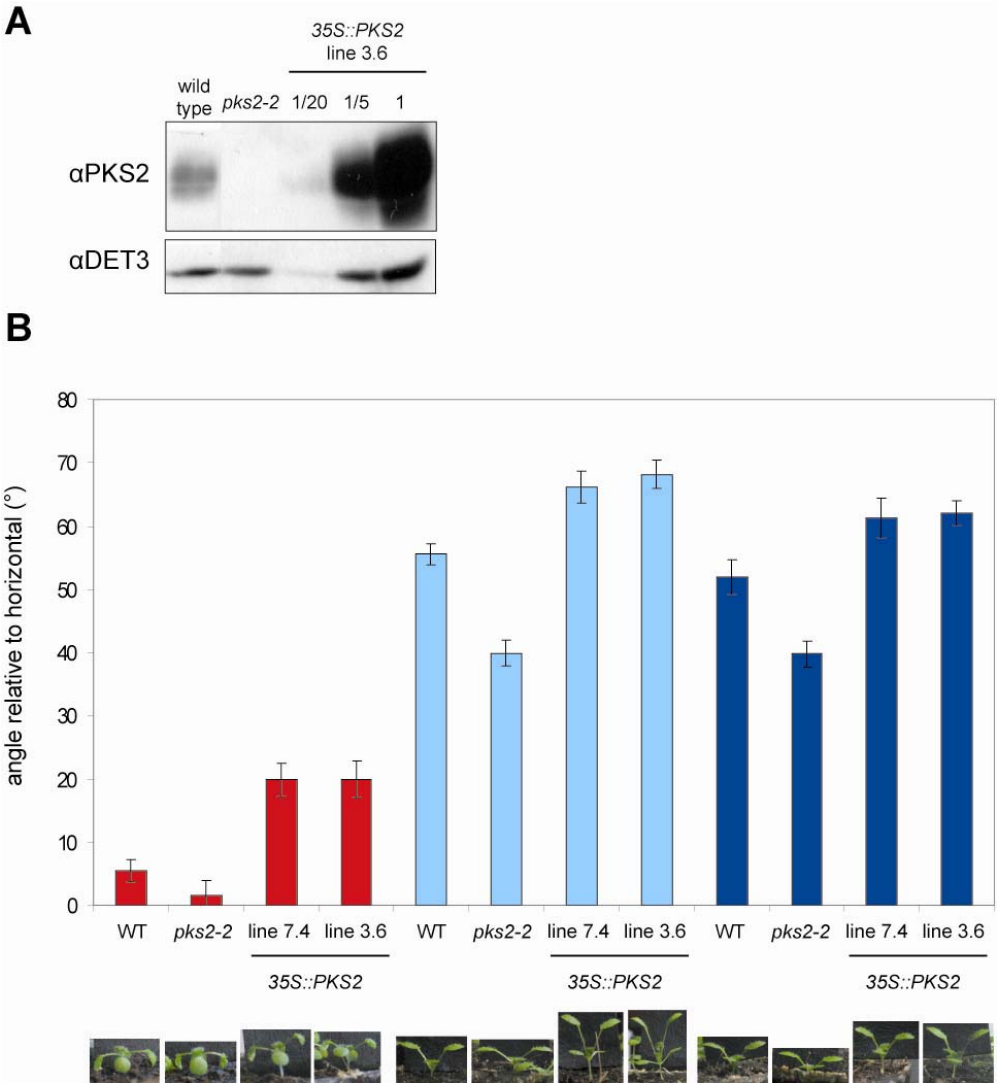


Figure 8: Leaf positioning phenotype in *PKS2* gain-of-function plants.

(A) PKS2 amounts in *PKS2* over-expressing plants (*35S::PKS2*). Proteins were extracted from wild type, *pks2-2* null and PKS2-OX line 3.6 (WT background) plants grown in parallel under RL + LBL. Protein extracts from PKS2-OX line 3.6 were diluted 5 and 20 times and were separated on 10% SDS-PAGE, Western-blotted and probed with anti-PKS2 (upper lane) or anti-DET3 (lower lane – loading control). *PKS2* silencing in *PKS2* over-expressing plants occurred with low frequency, and PKS2 levels in line3.6 were slightly higher than in line 7.4 (data not shown).

(B) Leaf positioning in wild type, *pks2-2* null and two *PKS2* over-expressing lines (lines 7.4 and 3.6) under RL (red bars), RL+LBL (light blue bars) and RL+HBL (dark blue bars). The two *35S::PKS2* lines were generated by Patricia Lariguet (Lariguet et al., 2003). Note that the over-expressed PKS2 proteins have two amino acid substitutions compared to the Col-O primary sequence because of polymorphism in the cloned EST (see Materials and Methods for details). Bars indicate mean ± 95% confidence intervals for $34<n<57$ plants ($68<n<114$ measured petioles). Graphs show representative results for one experiment.

2.6. Elements suggesting that PKS2 may act independently of phot signalling pathways to control leaf positioning.

Loss-of-function studies showed that PKS2 is the predominant PKS member acting in leaf positioning (Figure 3). *PKS2* gain-of-function plants expressing approximately 10-times more PKS2 proteins were further analysed to gain more insight into PKS2 function (Figure 8A; Lariguet et al., 2003). Under both LBL and HBL, PKS2-OX plants had the opposite phenotype than *pks2* mutants. Indeed, the two PKS2-OX lines studied displayed highly hyponastic leaves and cotyledons (Figure 8B). The gain-of-function phenotype confirmed that PKS2 plays a significant role in leaf positioning.

One important question then was whether PKS2-OX affected leaf positioning by enhancing phototropin signaling (causing exaggerated solar tracking for instance) or by another mechanisms. To address this question, PKS2-OX plants were grown in conditions where phototropins are not directly activated (i.e. under RL). Interestingly, PKS2-OX plants also presented hyponastic (or less epinastic) leaves under these conditions (Figure 8B) suggesting that PKS2 may act beyond phot signaling.

In addition to hyponastic petioles, PKS2-OX plants also displayed longer petioles and longer hypocotyls (not quantified, but visible in Figure 8B). It is interesting to note that this phenotype is strikingly similar to plants displaying the shade avoidance syndrome regulated by phytochromes (Franklin and Whitelam, 2005). However, because of the ectopic expression of PKS2 in the plant (controlled by the constitutive CaMV 35S promoter) caution must be taken when interpreting these data. For instance, high amounts of PKS2 in tissues where it is normally not present may affect the plant's development and a fashion that is not representative of the true physiological role of PKS2.

Finally, *nph3pks1pks2* petioles appeared more epinatic than wild type petioles under RL (Figure 9). Given that leaf epinasty in wild type plants grown under RL is believed to be a consequence of the absence of phot activation (Takemiya et al., 2005), this observation supports the hypothesis that NPH3 and PKS2 may have a role in leaf positioning that is independent of phot signaling. To test this hypothesis

further, it will be important to analyse the leaf positioning phenotype of the *phot1phot2* mutant under RL. Comparison of *phot1phot2* with *phot1phot2nph3* and *phot1phot2pks2* under HBL may also provide a good indication about this possibility.

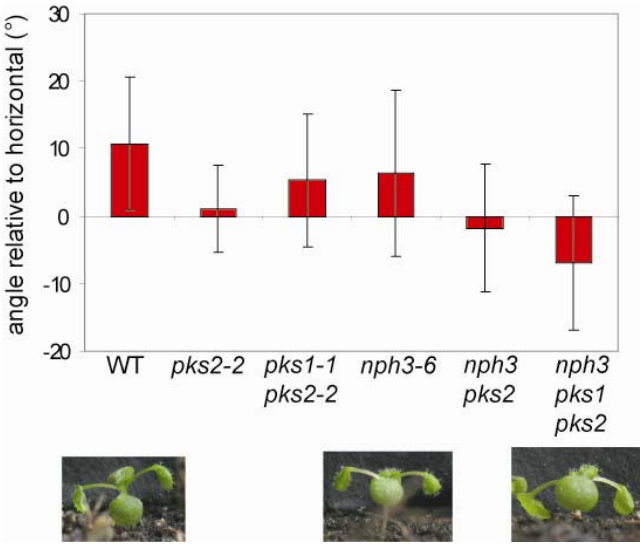


Figure 9: Increased epinasty of *nph3pks* mutants compared to WT under RL.

Plants were grown in parallel with those analysed in Figure 4B but under RL only. Bars indicate mean ± standard deviation for 34<n<57 plants (68<n<114 measured petioles). Graphs show representative results for one experiment.

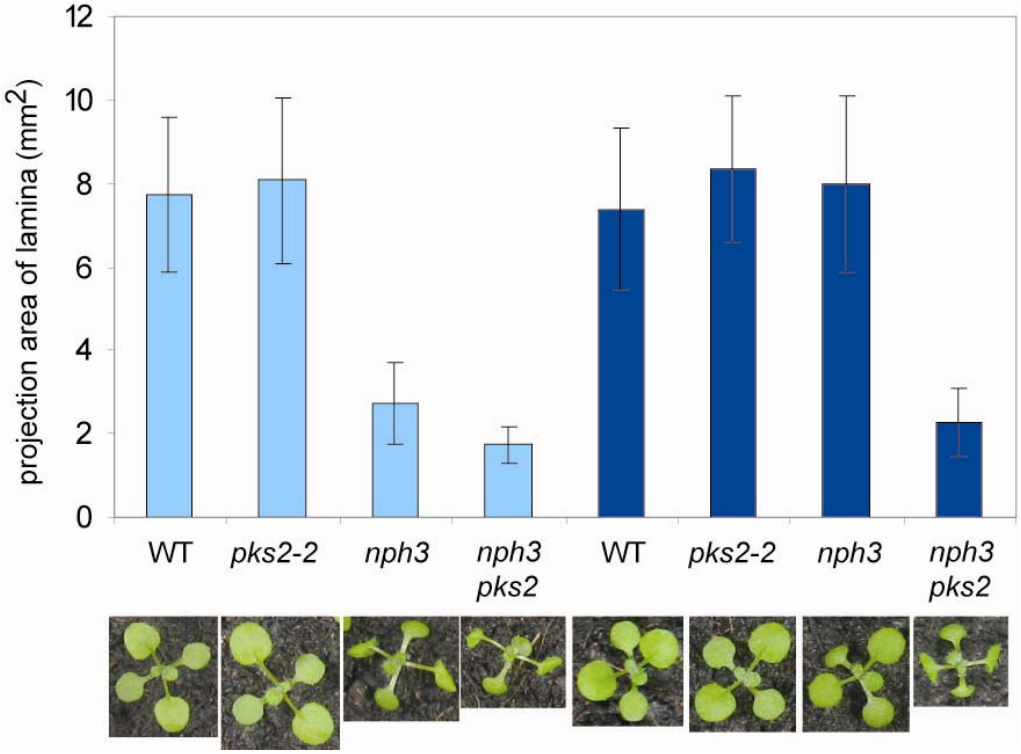


Figure 10: Reduction of light capture by leaves of epinastic mutants

Similar plants than those analysed in Figure 4B were photographed from above. Data for only four representative genotypes is shown. Projection area of the laminas of the first true leaves was measured. This area serves as an indicator of light capture by the leaf (see Materials and Methods). Bars indicate mean ± standard deviation for $31<n<41$ plants ($62<n<82$ measured laminas). Graph show results for one experiment.

2.7. Reduced light capture is a consequence of strong leaf epinasty

To study the possible consequences of leaf positioning in overall plant growth, the amount of light captured by epinastic and wild type leaves was compared. As shown in Figure 10, weekly epinastic leaves (or less hyponastic leaves – e.g. *nph3* under HBL) still captured similar amounts of light than wild type when illuminated from above. However, highly epinastic leaves suffered great reduction in light capture. It is important to point out that reduced light capture in these epinastic mutants was not solely the consequence of petiole positioning defects. Indeed, strong epinasty in the lamina also certainly contributed to reduced light capture, as shown in the plant pictures of Figure 10.

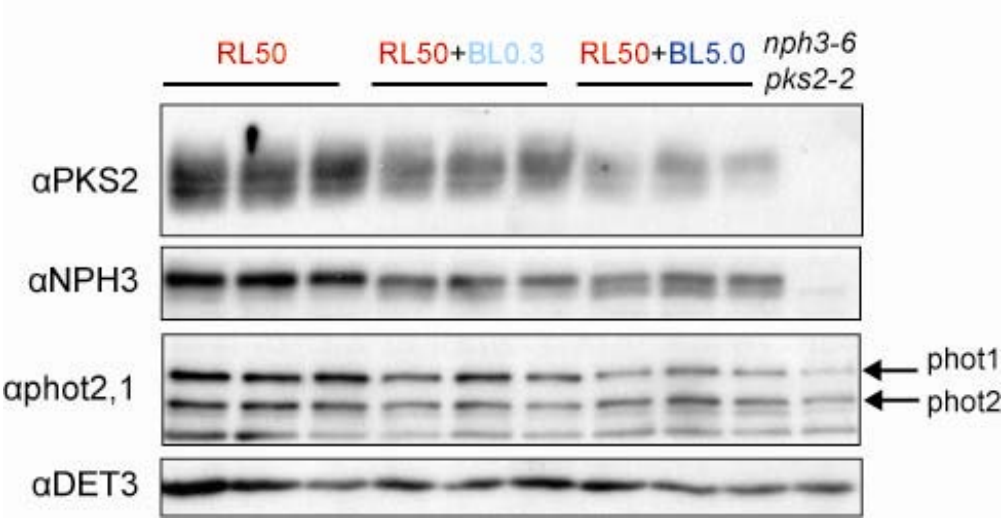


Figure 11: Amounts of PKS2, NPH3, phot2 and phot1 proteins in wild type plants grown under different light treatments.

Proteins were extracted from the aerial parts of plants of similar growth stage than in petiole positioning assays (three plants per light treatment). Extraction was performed directly under the light incubator in a dark room to avoid possible influence of other lights. Proteins were separated on 8% SDS-PAGE, western-blotted and probed with the indicated antibodies (see Materials and Methods). αDET3 served as a loading control.

3. Analysis of PKS2 molecular mode of action

3.1. Preparation of PKS2-specific antibodies

To investigate how PKS2 may regulate leaf flattening and positioning at the molecular level, PKS2-specific antibodies were prepared. See the *Materials and Methods* for a detailed description on how these antibodies were prepared.

3.2. PKS2 protein amounts in plants grown under different light treatments

Results from genetic experiments revealed an important role for PKS2 in the phot1 pathway during leaf positioning under BL (Figures 2, 4 and 5). In addition, some lines of evidence indicate that PKS2 may also act independently of phot under RL (Figures 8 and 9). As described in the introduction, leaf positioning is the combined result of many different light responses (e.g. BL direction, BL intensity, RL:FRL ratio) (Pierik et al., 2004; Millenaar et al., 2005; Vandenbussche et al., 2005; Mano et al., 2006; Mullen et al., 2006). Thus, to gain more insight into PKS2 roles in light-regulated leaf positioning, a simple question was addressed: are the effects of light treatments on leaf positioning correlated with different amounts of PKS2? Since PKS2 acts in concert with NPH3 in the phot signalling, levels for NPH3, phot1 and phot2 were also assayed.

No strong differences in PKS2, NPH3 and phot protein levels under the different light treatments were detected. However BL seemed to lead to reduction in PKS2 levels. In addition, dephosphorylation of NPH3 was also visible under BL as previously reported (Pedmale and Liscum, 2007). Interestingly, reduction of PKS2 amounts and the proportion of dephosphorylated : phosphorylated NPH3 both appeared proportional to the BL intensity (Figure 10). These results indicate that epinasty in RL-treated plants is not a consequence of low amounts of PKS2, NPH3 or phototropins. This observation is consistent with the notion that these signaling proteins (NPH3 and phot1/phot2) control BL-induced responses mainly by post-

translational mechanisms (Christie, 2007; Pedmale and Liscum, 2007; Sullivan et al., 2008; Inoue et al., 2008b).

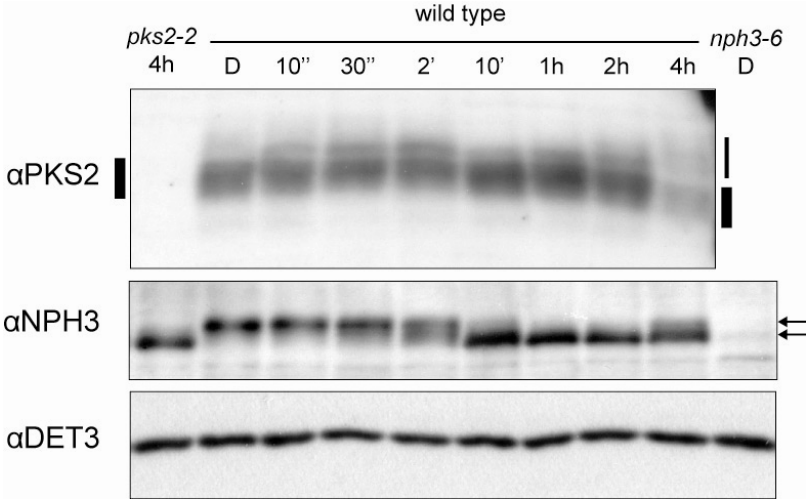


Figure 12: Rapid appearance of new PKS2 isoforms under white light.

Three-day-old dark-grown seedlings were illuminated with 80µmol.m^{-2}.s^{-1} WL for the indicated duration (10 seconds → 4 hours). Proteins were quickly extracted, separated on 8% SDS-PAGE, western-blotted and probed with the indicated antibodies. αDET3 serves as loading control. Thin and thick bars indicate slower- and faster-migrating forms of PKS2, respectively. Both arrows point towards phosphorylated (upper band) and de-phosophorylated (lower band) isoforms of NPH3 (Pedmale and Liscum, 2007).

3.3. PKS2 undergoes rapid light-induced post-translational modifications

PKS2 plays a significant role the phot1 pathway during leaf positioning. However, PKS2 levels appear lower in RL + BL compared to RL alone. This suggests that PKS2 – as for NPH3 and phots – may regulate light responses via post-translational mechanisms. To address this possibility, 3-day-old dark-grown plants were illuminated with WL for periods of 10 seconds to 4 hours. Since the WL used contained high proportions of RL and BL (fluorescence lamp tubes), phot and phy photoreceptors were activated.

In dark-grown seedlings, the pattern of protein migration of PKS2 was very different from that of NPH3 and showed a "smear" (Figure 12). This pattern suggested that several isoforms of PKS2 might be present in the dark. Interestingly, one main slowly-migrating isoform appeared in seedlings exposed to 10 seconds of WL. Between 30 seconds and 2 minutes of illumination, more isoforms appeared indicating the presence of other putative slower migrating forms. After 10 minutes, only one main slowly-migrating isoform was again visible. Between 1 hour and 2 and 4 hours of illumination, more slowly-migrating isoforms appeared again. At the 4 hours time point, the intensity and pattern of the upper and lower signals (smears) appeared symmetrical. Thus, PKS2 underwent dynamic and rapid modifications upon WL illumination.

It is interesting to note that NPH3 underwent light-induced dephosphorylation within similar time-scale than PKS2 (Figure 12; Pedmale and Liscum, 2007). A slight re-phosphorylation of NPH3 after long light exposure (from four hours) was observed (Pedmale and Liscum, 2007).

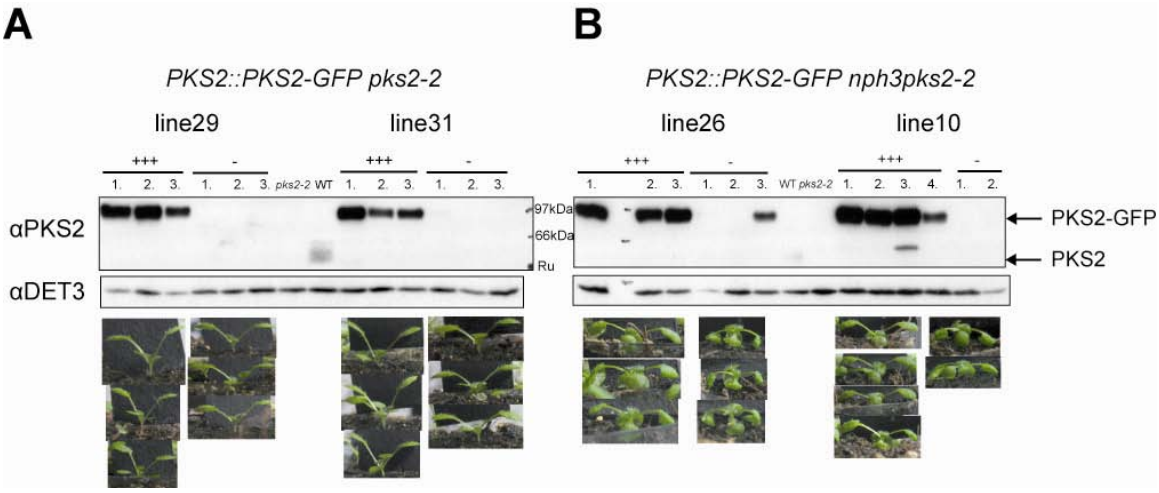


Figure 13: PKS2-GFP is partially functional *in planta*.

Leaf positioning in heterozygous T2 plants containing single T-DNA insertions were assayed in plants expressing *PKS2::PKS2-GFP* in *pks2-2* (A) or *nph3pks2-2* (B) backgrounds. Plants from T2 generation were analysed because strong silencing of *PKS2-GFP* expression was observed in the T3 generation. Proteins from plants that showed good leaf positioning complementation (+++) or no complementation (-) were separated on 10% SDS-PAGE, Western blotted and probed using antibodies specific to PKS2. αDET3 served as a loading control.

3.4. PKS2-GFP cell localization

phot1 and phot2 are plasma-membrane associated proteins that undergo dynamic cellular redistribution events upon light activation (Kong et al., 2006; Aihara et al., 2008; Wan et al., 2008). NPH3 and PKS1 are also known to be tightly associated with the plasma membrane, but no sub-cellular relocalisation for these two proteins has been yet described (Lariguet et al., 2006). PKS2 (and PKS1 and NPH3) co-immunoprecipitated with both phot1-GFP and phot2-GFP in protein extracts obtained from 2-week-old de-etiolated plants (Chapter 2, Figure 8B). PKS1 interacts with both NPH3 and PKS2 *in vitro* (Lariguet et al., 2003; Lariguet et al., 2006). Thus, PKS2 may be present in similar protein complexes than PKS1 and NPH3 associated with phot1 and/or phot2. However, as briefly discussed previously, such interactions may be transient and these *in vitro* interaction data do not prove that these proteins are indeed associated *in vivo* (they may still be present in distinct phot complexes).

Thus, to address these possibilities and to better understand the function of PKS2 during phot signaling, it is key to describe its localization (and possible intracellular dynamics) in the cell. For this, transgenic plants expressing GFP-tagged PKS2 proteins under the control of a 500 bp promoter regulatory region were constructed (a short promoter because of the presence of a gene higher ORF upstream). The GFP protein was fused to the C-terminus of PKS2. PKS2-GFP was expressed in *pks2* and *nph3pks2* mutant backgrounds. Taking advantage of the phenotype of *PKS2* loss-of-function in both wild type and *nph3* sensitized backgrounds (Figures 3 and 4), complementation assays were done to test the functionality of PKS2-GFP recombinant proteins *in planta*.

As shown in Figure 13, PKS2-GFP recombinant proteins could rescue the loss-of function phenotypes. However, two elements indicate that PKS2-GFP is only partially functional: (i) PKS2-GFP levels in transgenic plants were much higher than in WT plants (similar to PKS2-OX levels; Figure 8B) but the plants did not show hyponastic leaves (Figure 13A), and (ii) judging from petiole angle measurements, PKS2-GFP recovered 50-90% of petiole positioning in transgenic lines at best (data not shown). Heterogeneous levels of PKS2-GFP (other than *PKS2-GFP* $^{-/-}$ plants in the segregating

population) were also observed in plants in the T2 population, indicating the presence of silencing effects on expression of the transgene (data not shown). This may also have contributed to the partial complementation observed in the T2 population.

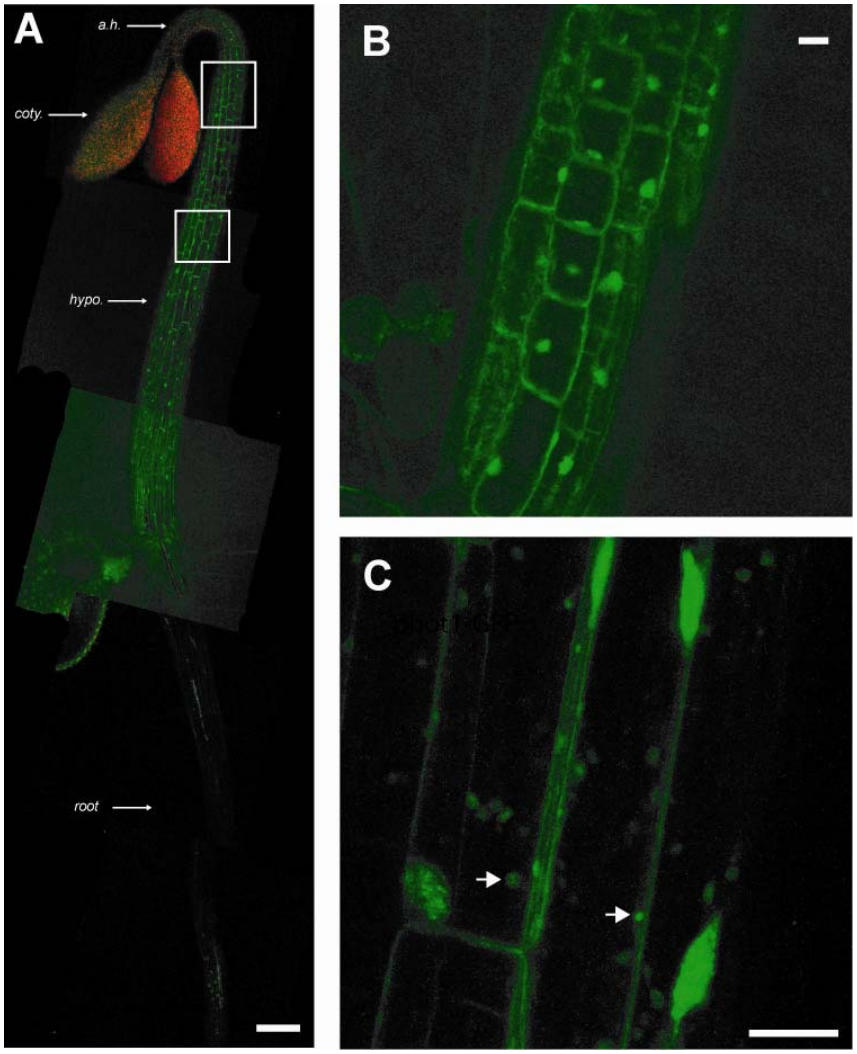


Figure 14: Analysis of PKS2-GFP cell localization in dark-grown seedlings.

(A) Localisation of PKS2-GFP in a whole 68-hour-old dark-grown seedling. Montage was made from micrographs of one seedling (T3 generation, *PKS2::PKS2-GFP nph3pks2-2* line10) obtained using a Plan-Neofluar 10× objective on a confocal microscope. GFP and plastids were excited using $\lambda_{excitation}$=488nm and signals were distinguished using a 505-530nm BP filter for GFP signal and a 650nm LB filter plastid signal. Picture shows merged signals. a.h., apical hook; coty., cotyledon; hypo., hypocotyls. Rectangular and square white boxes indicate the seedling areas from which pictures in panels B and C were taken (different seedlings). bar=100µm.

(B) PKS2-GFP localization in cells in the elongation zone (rectangular box, panel A). Micrograph of a T2 seedling from *PKS2::PKS2-GFP pks2-2* line 29 using a Plan-Neofluar 20× objective on a confocal microscope. Bar=25µm

(C) PKS2-GFP localization in cells below the elongation zone (square box, panel A). Micrograph of a T3 seedling from *PKS2::PKS2-GFP nph3pks2-2* line 10 using a Plan-Apochromat 63× objective Picture shows the maximum signal projections of 8 optical slices (Z-stack). Distance between each optical slice was 3.72µm. White arrows indicate intracellular compartments of unknown nature. Bar=25µm

On western blots probed with PKS2-specific antibodies, the appearance of PKS2-GFP signal was different than that of PKS2. Indeed, PKS2-GFP had rather a “band”-like appearance compared to the smear characteristic of PKS2 (e.g. Figure 13A). This is certainly due to the larger size of PKS2-GFP which reduced the separation range of putative isoforms on a 10% SDS-PAGE. However, another possibility is that the recombinant PKS2-GFP protein did not undergo similar post-translational modifications than PKS2. This possibility would be consistent with reduced physiological activity of PKS2-GFP.

Using a fluorescence confocal microscope, PKS2-GFP signal in etiolated seedlings was seen along the whole hypocotyl and in the cotyledons, but was much weaker in the apical hook and root (Figure 14A). When focusing on the elongation zone of the hypocotyl, the PKS2-GFP signal appeared clearly different from the plasma membrane-associated signal observed for PKS1-GFP (Lariguet et al., 2006). Indeed, strong signal was emitted from what appeared to be the nuclei and the signal along cell periphery was more diffuse (Figure 14B). In an attempt to visualize the sub-cellular localization in more detail, higher magnification was used and a Z stack of maximum intensity projections was constructed. The signal appeared clearly associated with the plasma membrane and nucleus regions. In some optical slices the nuclear signal was concentrated in punctuate patterns (data not shown). Interestingly, GFP signal was also localized in cytosolic bodies of unknown nature (Figure 14C).

The presence of PKS2-GFP in the hypocotyl coincided with PKS2 being involved in hypocotyl phototropism (Lariguet et al., 2006). PKS2-GFP signal was also observed in plasma-membrane region and nuclei of leaf and cotyledon petioles, which is consistent with the role of PKS2 in leaf positioning (Figures 15 A-B). It was difficult to determine whether PKS2-GFP also localized in the chloroplasts because plastid fluorescence was not totally filtered by the GFP filter.

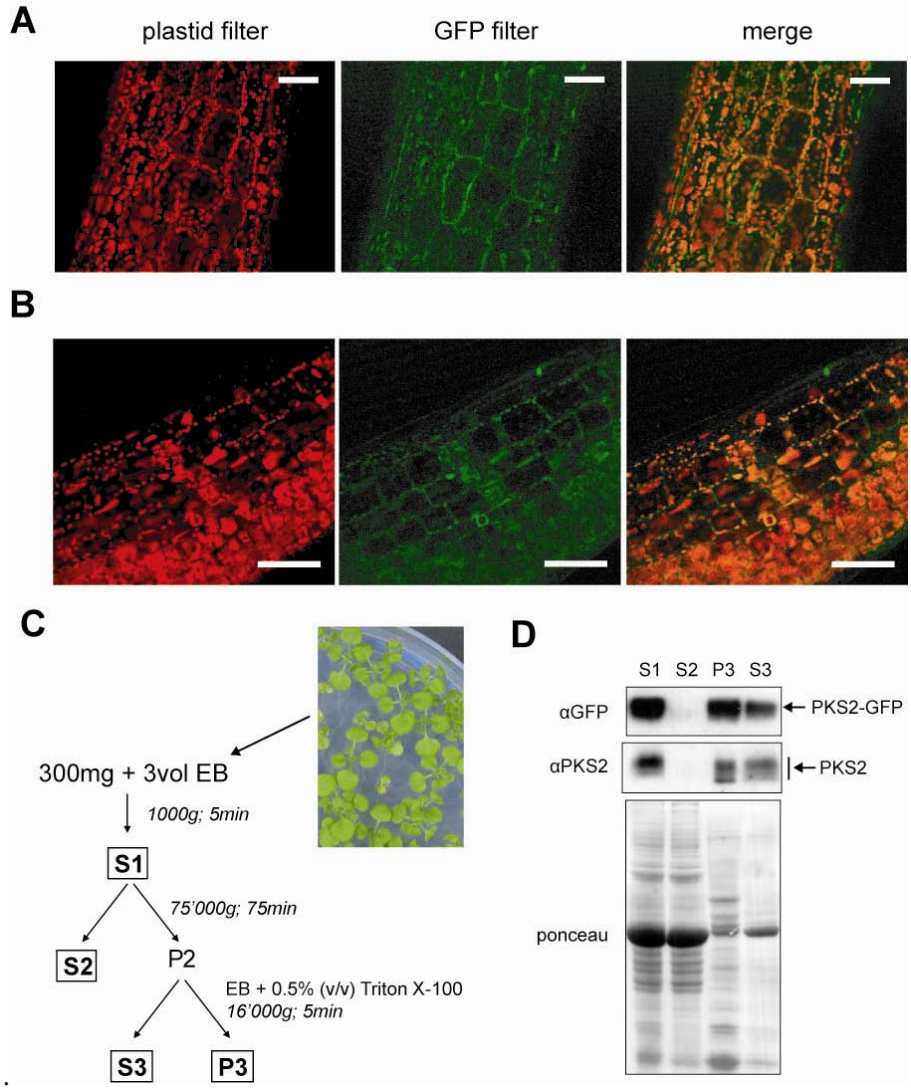


Figure 15: Analysis of PKS2-GFP localization in 2-week-old plants.

(A-B) PKS2-GFP in cotyledon petiole (A) and in leaf petiole (B) (*PKS2::PKS2-GFP nph3pks2-2* line 10). Plants were grown for 11 days under 100 µmol.m^{-2}.s^{-1} white light. Plants showing best visual epinasty complementation (i.e. resembling *nph3* – see Figure 12B) were analysed using a Plan-Neofluar 20× objective on a confocal microscope. Red signal corresponds to plastid signal collected with a 650nm LB filter. Green signal corresponds to GFP signal collected with a 505-530nm BP filter. Both were excited using $\lambda_{excitation}$=488nm. Bar=100µm

(C) Protein fractionation protocol. Protein extracts from aerial parts of 2-week-old plants (grown on 1/2MS-supplemented agar under 100 µmol.m^{-2}.s^{-1} WL -growth stage c.a. 1.05; Boyes et al., 2001) were fractionated as described. P2 pellet (microsomal fraction) was resuspended gently using 0.5% (v/v) Triton X-100 detergent and solubilised proteins in S3 were collected after centrifugation. See Materials and Methods for details.

(D) PKS2 and PKS2-GFP co-localise in the insoluble fraction. Proteins prepared from WT and PKS2-GFP expressing plants (*PKS2::PKS2-GFP pks2-2* line 29) were separated on 10% SDS-PAGE, western-blotted and probed with GFP-specific and PKS2-specific antibodies, respectively. Ponceau staining illustrates the pattern of proteins recovered in each fraction.

Conclusions of these preliminary microscopy studies are as follow: (i) PKS2-GFP differed from PKS1-GFP and NPH3 localizations (ii) in addition to the plasma membrane region, PKS2-GFP localized in the nucleus and in unknown intracellular compartments (iii) PKS2-GFP signal was found in organs in which PKS2 plays a role.

Finally, it is interesting to point out that PKS2-GFP recombinant proteins - even if present in much larger amounts - co-fractioned with endogenous PKS2 in the microsomal fraction (Figure 15D). The fact that PKS2-GFP proteins were (i) (partially) functional *in vivo* and (ii) co-fractioned with endogenous PKS2, indicates that the intracellular PKS2-GFP signal observed may indeed represent the localization of endogenous PKS2. It is noteworthy to precise that, in good protein extraction samples, very little PKS2-GFP degradation was observed when probing western blots with GFP-specific antibodies. Thus, the GFP signal observed in the nucleus and cytosol was probably not due to free GFP proteins cleaved off from PKS2-GFP (Lariguet et al., 2006; data not shown).

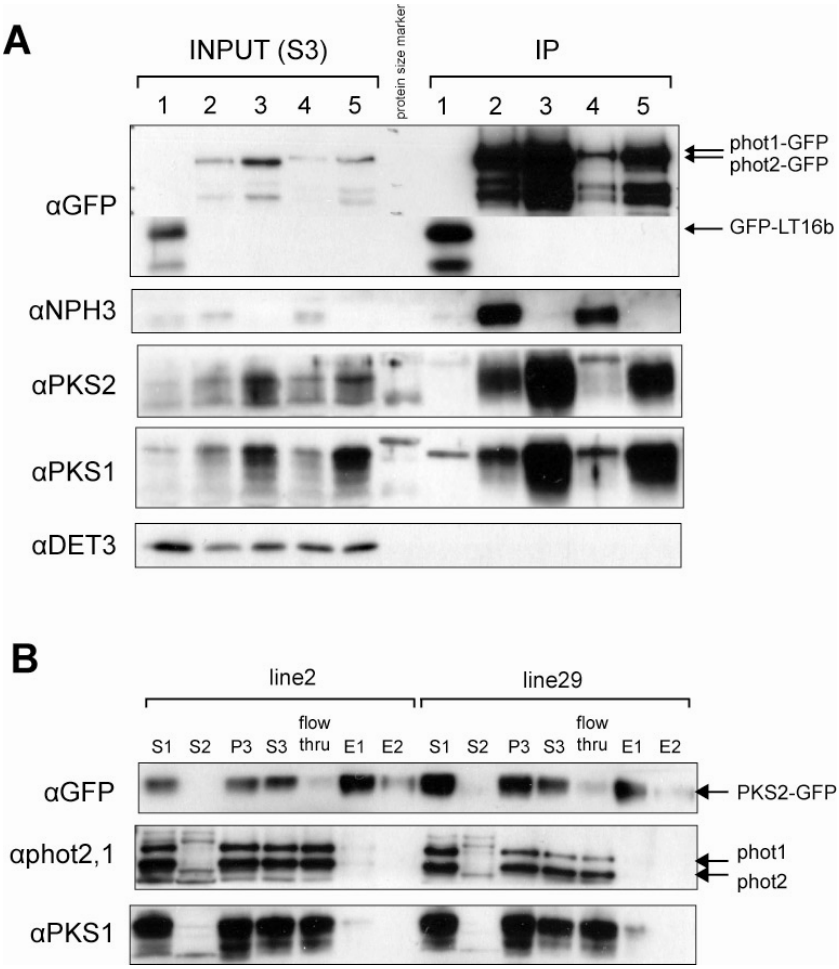


Figure 16: Analysis of phot/NPH3/PKS1/PKS2 protein complexes by immunoprecipitation

(A) PKS2 and PKS1 association with phot1 and phot2 does not require NPH3. INPUT and IP samples were prepared from 2-week-old plants grown on 1/2MS agar plates under 100 µmol.m^{-2}.s^{-1} continuous WL (as in Figure 14C). Proteins were separated in 10% SDS-PAGE, Western-blotted and probed with the indicated proteins (as in Chapter 2 Figure 8). The following genotypes were used: lane 1: *35S::GFP-LT16b*; lane2: *PHOT2::PHOT2-GFP phot1-5phot2-2*; lane3: *PHOT2::PHOT2-GFP nph3-6 PHOT2-/?*; lane 4: *PHOT1::PHOT1-GFP phot1-5*; lane 5: *PHOT1::PHOT1-GFP nph3-6 phot1-5*.

(B) phot2, phot1 and PKS1 association with PKS2-GFP. Protein fractions were prepared as described (Figure 14C) from two PKS2-GFP expressing lines (*PKS2::PKS2-GFP pks2* line2 and line29). Reduced signal in the flow-through (flow thru) indicates that immunoprecipitation efficiency was high. E1 and E2 correspond to two IP elution fractions obtained by boiling GFP-coupled magnetic beads with Laemmli buffer. Proteins were separated on 8% SDS-PAGE, blotted and probed with indicated antibodies as described in the *Materials and Methods*.

3.5. Analysis of PKS2/PKS1/NPH3/phot1/phot2 protein complexes

PKS1 and NPH3 co-immunoprecipitated (co-IPed) with phot1-GFP in etiolated seedlings extracts. In addition, NPH3 and phot1 co-IPed with PKS1-GFP, and PKS1 can interact *in vitro* with NPH3 and phot1 (Lariguet et al., 2006). Together, these data indicate that PKS1, NPH3 and phot1 are present in a same complex *in vivo*. As previously discussed, PKS2 may also be present in a same complex because it interacts with PKS1 *in vitro*. Since PKS1, PKS2 and NPH3 all co-IPed with phot2-GFP, a similar complex may be present with phot2 (Chapter 2 Figure 8). However, these interactions have not been confirmed *in vivo* so far.

Phot1 and phot2 undergo dynamic cellular relocalisation suggesting that they may associate with different protein complexes to carry out their cellular functions (Kong et al., 2006; Wan et al., 2008). PKS1 and NPH3 are likely to interact with plasma membrane-associated phot1 because microscopy and biochemical fractionation indicate they are strongly associated with the plasma membrane (Motchoulski and Liscum, 1999; Lariguet et al., 2006). However, PKS2 sub-cellular localisation appeared different in PKS2-GFP expression lines, suggesting that PKS2 may associate with different pools of phot1 (Figure 14).

Thus, to investigate PKS2/PKS1/NPH3/phot protein complexes in more detail, two sets of experiments were designed. First, the dependency on NPH3 for PKS1 and PKS2 association with phot1 and phot2 was tested. Second, phot1, phot2 and PKS1 associations with PKS2-GFP were analysed.

Results clearly showed that PKS1 and PKS2 co-immunoprecipitated with the phots with high efficiency in the absence of NPH3, indicating that NPH3 was not required for PKS-phot associations (Figure 16A). Probing of the IP samples with NPH3-specific antibodies confirmed the presence or absence of NPH3 in plants expressing phot1-GFP and phot2-GFP, and also confirmed high co-immunoprecipitation efficiency between NPH3 and phot1-GFP and phot2-GFP in *NPH3* +/+ plants (Figure 16A).

It is interesting to note that PKS1 and PKS2 amounts in *nph3* IP samples were also much higher than the amounts obtained from *NPH3 +/+* plants. This is due in part to higher phot1-GFP and phot2-GFP amounts and also higher PKS1 and PKS2 amounts in these samples. However, one other possible reason could be that PKS1 and PKS2 are no longer targeted for proteolysis, or are relieved from NPH3-inhibited phot-association. Finally, PKS1 and PKS2 may compete with NPH3 for phot1/phot2 binding. Further experimentation is required to test these hypotheses.

Preliminary IP assays using PKS2-GFP as bait were also performed. PKS2-GFP IP by antiGFP antibodies coupled to magnetic beads was highly efficient (as shown by the reduced PKS2-GFP amounts in the flow through and large PKS2-GFP amounts recovered in elutions) (Figure 16B). However, very small amounts of phot1 and phot2 were co-immunoprecipitated compared to IPs using phot1-GFP and phot2-GFP as baits (Figure 16A; Chapter 2 Figure 8B).

Two reasons may explain this result. First, PKS2-GFP may be much less abundant than phot1 and phot2 and only a small fraction of the phots could be recovered during the IP (compare S3 with E1 fractions). Conversely, most PKS2 and PKS1 proteins in the input may have been immunoprecipitated by phot1-GFP and phot2-GFP explaining the strong signal obtained in the elution (Figure 16A). Second, the GFP moiety fused in C-terminus of PKS2 may reduce binding affinity of phots to PKS2. Consistent with this possibility is the partial functionality of PKS2-GFP *in vivo*, as shown by the incomplete complementation of *pks2* and *nph3pks2* plants (Figure 13).

Similarly, only small amounts of PKS1 co-IPed with PKS2-GFP. Since PKS1 is certainly less abundant than phot1 and phot2, one would have expected a large proportion of PKS1 in the INPUT to be bound to PKS2-GFP if these proteins were indeed associated. This result indicates that either PKS1 association with PKS2 is weak *in vivo*, or the GFP moiety of PKS2-GFP may also hinder the interaction.

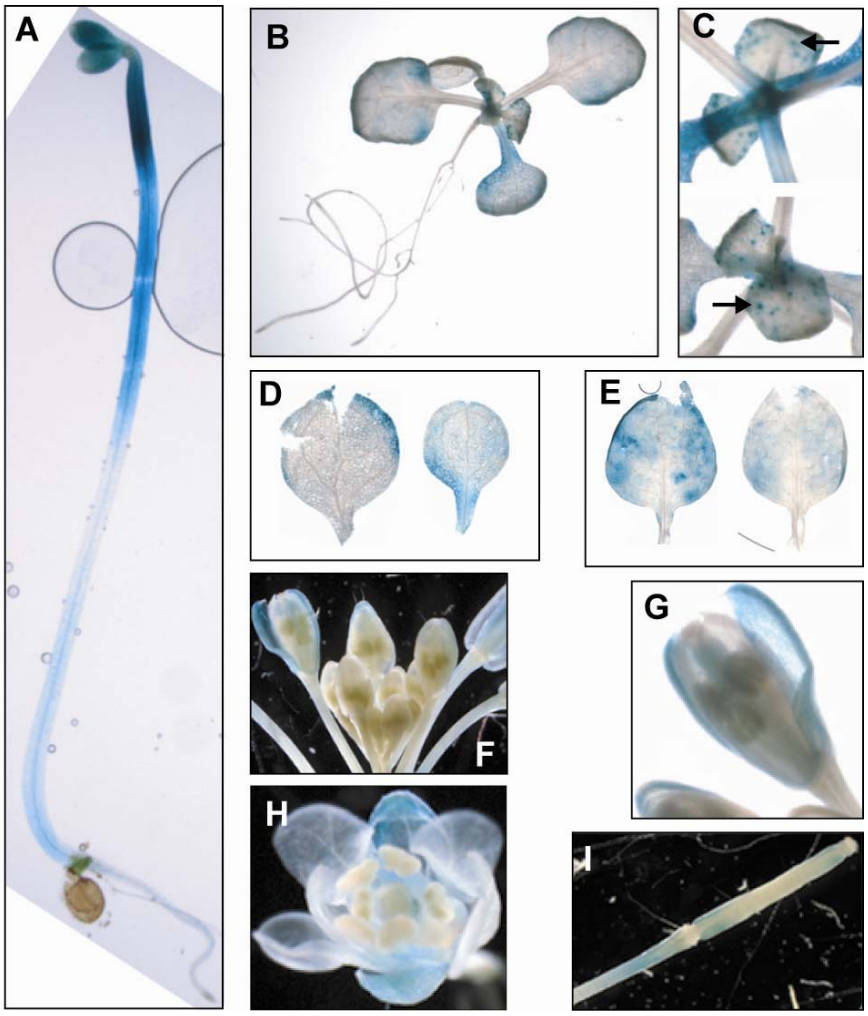


Figure 17: Analysis of *PKS2::GUS* expression patterns.

(A) *PKS2::GUS* expression in 84 hour-old dark-grown seedlings. Seedlings were incubated with X-GLUC substrate at 37°C for 6 hours. Similar patterns were found in 7 out of 8 seedlings.

(B-E) *PKS2-GUS* expression in 10-day-old plants (similar growth stage than plants analyzed in petiole positioning assays) grown under 100 µmol.m^{-2}.s^{-1} WL. (B) whole plant; (C) leaf number 3 and 4; arrows indicate a punctuate pattern of staining; (D) two cotyledons showing different staining patterns (E); two first true leaves showing different staining patterns. Out of 10 plants analyzed, none showed staining in roots, all showed a punctuate staining pattern in young leaves 3 and 4, and different patterns were observed in cotyledons and leaf 1&2. Seedlings were incubated with X-GLUC substrate at 37°C for 24 hours.

(F-I) *PKS2::GUS* expression in inflorescence apex (F), flower organs (G and H) and developing seed pod (I). Two analyzed inflorescences showed similar patterns. Seedlings were incubated with X-GLUC substrate at 37°C for 48 hours.

3.6. Analysis of PKS2::GUS *expression patterns in the plant*

Knowledge about the organ and tissue localisation of PKS2 is also essential to fully understand the physiological role of the protein. For instance, PKS2 may be restricted to specific leaf tissues such as the epidermis and directly regulate cell elongation in this tissue, thus controlling leaf flatness. Another possibility is that PKS2 is localised in specialised regions distant from where its physiological effect is actually seen, for instance in long-distance auxin transport or biosynthesis.

Thus, to understand better how PKS2 might act during phototropism, leaf flattening and leaf positioning, *PKS2::GUS* expression patterns were analysed in etiolated seedlings and 2-week-old light-grown plants. Caution must be taken when interpreting these results because one single line has been analyzed (Lariguet et al., 2003). More independent transgenic lines have been constructed and a detailed analysis of the expression pattern in the leaves and cotyledons (alongside *35S::GUS* and *DR5::GUS*) was recently performed during the last review of this manuscript (see Christian Fankhauser).

Interestingly, GUS staining was very strong in the elongation zone of young seedlings which is consistent with the role of PKS2 during phototropism (Figure 17A; Lariguet et al., 2003; Lariguet et al., 2006). Expression was much weaker in the apical hook and roots as previously seen in *PKS2-GFP* lines (Figures 14A and 17A-B). *PKS2::GUS* expression was found in cotyledons and leaves (Figures 17B-E). However, the pattern of expression was very heterogeneous within each leaf and cotyledon and among the plants analyzed (Figures 17D-E). Nonetheless, it is interesting to note that one consistent pattern found among all plants studied: a punctuated coloration in young leaves (Figure 17C).

In addition, sections of unexpanded cotyledons from etiolated seedlings (Figure 17A) showed staining mainly in the adaxial epidermis (preliminary result, data not shown). Phot1-GFP was observed in the cotyledon abaxial epidermis (Wan et al., 2008).

Finally, *PKS2::GUS* expression patterns were also explored in the flowers because gene-expression databases indicated massive and highly localized *PKS2* expression (particularly petals) suggesting a role for *PKS2* in flowers. In addition, NRL genes are well known regulators of flower organogenesis (Lalanne et al., 2004; Cheng et al., 2008). Interestingly, GUS staining appeared restricted to one of the two pairs of sepals in mature flowers (Figure 17 F-H). Weak expression was also found in the style but not in the stigma or stamen. Finally, weak expression was observed in the maturing seedpod and the pedicel (Figure 17 I). The physiological significance of these floral expression patterns is not yet known. Some *PKS2-GFP* high expressing lines displayed defects in flower distribution along the inflorescence stem and in silique size and shape (data not shown). However, *pks2-2* sepals did not display obvious morphological defects (data not shown).

4. Brief summary of results

The main results from this chapter are as follow:

- The *PKS* act redundantly during leaf positioning in the phot1 pathway, and their relative contributions are as follow: PKS2 > PKS1 > PKS3/PKS4

- As previously reported NPH3 is essential for the LBL response (mediated by phot1). In addition, as similarly shown for leaf flattening, NPH3 plays an important role in the phot2 pathway (*phot1nph3* phenocopies *phot1phot2*)

- *nph3* and *pks2* mutants interact synergistically, and *nph3pks2* phenocopies *phot1phot2*, indicating that PKS2 plays a crucial role with NPH3 in this process

- PKS2 probably acts in additional pathways than phot signalling to control leaf positioning (red light effects)

- PKS2 undergoes rapid light-induced post-translational modifications

- PKS2-GFP, like PKS1 and NPH3, localises at the plasma membrane, but also in the nucleus and intracellular compartments

- PKS2 and PKS1 associations with phot1 and phot2 in leaves do not required NPH3

- PKS2 is highly expressed in the elongation zone of etiolated hypocotyls. PKS2 expression may be concentrated in specific regions of young developing leaves (punctuate pattern).

5. Discussion

5.1. Leaf positioning and leaf orientation

Leaf lamina flattening may be seen as a long-term developmental process. In contrary, leaf positioning is a reversible movement response. There are two main types of leaf movements: (i) those associated with non-directional light signals (nastic movements determined by endogenous signals – e.g. sleep movements) and (ii) others related to directional light signals (e.g. tropic or solar tracking movements; avoidance of competing neighbours). All these different movement responses have adaptive values for the plant and are integrated to determine an end-position /end-orientation of the leaf (Koller, 1990).

Thus, it is very difficult to study in an isolated manner just one light response. Shin-ichiro Inoue and co-workers proposed that the leaf position of plants illuminated with BL from above is mainly the result of tropic movements for the following reasons: (i) under RL the petioles were arch-shaped but under superimposed BL the petioles became erect (ii) phototropism mutants *nph3* and *phots* were impaired in this BL-induced response and (iii) leaf positioning was reversible in response to BL (Inoue et al., 2008a).

In addition to the BL direction, two additional parameters affected leaf positioning: nictinastic movements and BL fluence rate. During our leaf positioning assay, all plants were analysed between 5.30 pm and 8 pm. According to Mullen and co-workers, leaf inclination may change by a maximum of 2-3 degrees over this time period (Mullen et al., 2006). Moreover, although *PKS2* expression appears to be under circadian control (Lariguet et al., 2003) we could not detect any phase-change in *pks2* or PKS2-OX plants (Laurie Vuillet, unpublished). In rosette plants, low photosynthetically active radiation (BL in particular) also influences leaf inclination, and this response is viewed as a process associated with the shade avoidance response (Pierik et al., 2004; Mullen et al., 2006). Under our conditions, low BL (0.3 $\mu mol.m^{-2}.s^{-1}$) and high BL (5.0 $\mu mol.m^{-2}.s^{-1}$ – which should be viewed rather as moderate BL)

had only weak effects on petiole angles in wild type. Interestingly, the phot2 mutant appeared slightly more hyponastic that WT under LBL.

In conclusion, although the phenotypes of *pks* mutants were mild, they are likely to be mainly the result of defects in petiole tropism. The striking genetic interactions with *NPH3* support this hypothesis. It is also interesting to note that the PKS act in the phot1 pathway during both hypocotyl phototropism and leaf positioning (but in the phot2 pathway during leaf flattening).

To confirm the important roles of NPH3 and PKS2 during leaf tropism, one interesting experiment would be to apply light to the plants laterally and observe their re-orientation potential under HBL. These assays require more advanced equipment (e.g. infrared CCD cameras) and the quantification of leaf positioning defect would be much more complex (data extraction from time-course experiments using sophisticated software such as metamorph).

5.2. Interpretation of genetic results and future prospects

Epistasis data indicate that PKS2 acts in the phot1 pathway during leaf positioning (Figure 5) and in the phot2 pathway during leaf flattening (Chapter 2 – Figure 1). This apparent specificity in PKS function in different aspects of leaf development supports the notion that leaf positioning is a complex process that involves different types of tissues in which different PKS may fulfil specific roles. It is also interesting to point out that the PKS act in the phot1 pathway during phototropism (their role in the phot2 pathway is not yet reported) (Lariguet et al., 2006). Since petiole positioning is believed to be a tropic response like hypocotyl bending (Inoue et al., 2008a), it is perhaps not surprising that the PKS act in similar pathways in those two processes.

The functional specificity among *PKS1, PKS2* and *PKS4* is similar to that observed in leaf flattening (Chapter2 - Figures 10 and 12). Interestingly, this is not the case for *PKS3*. Indeed, *pks3-3* had curled leaves (similar to *nph3*) and *nph3pks3-3* and *nph3pks3-4* leaves were as epinastic as *nph3pks2-2* leaves (Chapter 2 - Figure 12A).

In that respect, note that *pks3-3* laminas also appeared concave (curled downwards) in these leaf positioning assays (Figure 3). This suggests that different PKS members may act in different leaf tissues. These differences may be due to different expression patterns in leaf tissues, as observed for root and hyocotyl phototropism (Boccalandro et al., 2008).

PKS2 and *PKS3* acted redundantly in leaf flattening in the *nph3* backgound, and *nph3pks2pks3* had very epinastic leaves that resembled *phot1phot2* (Chapter 2 - Figure 13). Interestingly, loss of function of only *PKS2* in the *nph3* sensitized background generated *phot1phot2*-like plants in petiole positioning assays under HBL (Figure 4B). This indicates that PKS2, respective to other PKS members, played a more predominant role in leaf positioning compared to leaf flattening. Consistent with the hypothesis is that *PKS3* did not appear to play an important role during leaf positioning (Figure 3).

Epistasis experiments between *NPH3* and the *PHOTs* yielded surprising data. Under both LBL and HBL, the *nph3* mutant was slightly more epinastic than phot1. This suggests that NPH3 is an essential component of the phot1 pathway and has a minor role in the phot2 pathway (Chapter 2 – Figure1; Figure 2A). However, the fact that *phot1nph3* phenocopied *phot1phot2* and that *nph3phot2* did not show increased epinasty compared to *nph3* suggests that NPH3 only acts in the phot2 pathway (Figure 6). Thus, two incompatible interpretations exist: (i) NPH3 acts 100% in the phot1 pathway and a fraction of phot2 pathway; (ii) NPH3 acts 0% in phot1 pathway and 100% of phot2 pathway. Epistasis data indicate that PKS2 plays a partial role in the phot1 pathway (Figure 5). Thus, according to interpretation (i), *nph3pks2* should resemble *nph3*, and according to interpretation (ii) *nph3pks2* should be slightly more epinastic than *nph3*. However, it is not the case for either possibility (i.e. *nph3pks2* phenocopied *phot1phot2*; Figure 4B).

The identical phenotypes of *nph3phot1*, *nph3pks2* and *phot1phot2* may help us clarify this issue (Figures 4 and 6). The *nph3* mutant may be viewed as a highly sensitized background where a large proportion of signals controlling leaf positioning is “shut down”. *npy1* (a homolog of NPH3) and *pid1* (a homolog of phot1 from the AGC VIII subfamily of kinases) also interact synergistically in the YUCCA-mediated auxin

signalling pathway controlling organogenesis (Furutani et al., 2007; Cheng et al., 2008). Thus, *PHOT1* and *PKS2* loss-of-function may disrupt the remainder of functional signalling in the *nph3* background. In addition, the similar effects of *PHOT1* and *PKS2* loss-of-function in the *nph3* background combined with epistasis data between *PKS2*, *PHOT1* and *PHOT2* suggest that PKS2 and phot1 may act in the same pathway. This is also consistent with the important role of the PKSs during phot1-mediated phototropism.

In conclusion, interpretation of the available genetic data does not yield a clear picture of *PKS2* and particularly *NPH3* roles in the phot signalling pathways controlling leaf positioning. Complementary molecular and biochemical data are required to clarify this issue.

One interesting question is whether the PKS represent a key step in phot signalling during leaf positioning. To answer this question, the *pks1pks2pks3pks4* full knockout is required. Two *pks3* alleles (*pks3-3* and *pks3-4*) yielded clear leaf flattening phenotypes indicating that some signalling is compromised in these mutants. However, these same alleles did not cause visible defects in leaf positioning. Since the *pks1pks2pks4* phenotype is mild and *pks3* phenotype is not significant, it is unlikely that the PKS represent a key step in linear phot signalling pathways controlling leaf positioning. The synergistic interactions observed with *nph3* rather support a scenario where the PKS family would act in parallel with the NRL family during this developmental process.

5.3. Interpretation of molecular results and future prospects

PKS2 and NPH3 can associate with phot1 and phot2 *in vivo* (Chapter 2 Figure 8B). This suggests the following possibilities: (i) PKS2 and NPH3 are present in a same complex with phot1 and/or phot2, (ii) PKS2 and NPH3 are present in different phot-associated complexes (e.g. in different pools of phot1 and phot2 in the cell), and (iii) a combination of the first two possibilities (e.g. as a consequence of dynamic relocalisation events). Because *pks2* and *nph3* mutants interact synergistically in leaf

positioning assays, PKS2 may fulfil complementary biochemical roles with NPH3 during phot signalling. In principle, this genetic interaction could correlate with the different scenarios of molecular associations stated above.

Several experiments were performed to explore the role of PKS2 during phot signalling. PKS2 underwent rapid post-translational modifications within similar time-scale than NPH3 (Figure 12). Interestingly, no obvious differences in PKS2 migration patterns were detected in 2-week-old plants grown under RL and RL + BL indicating that both RL and BL may potentially induce PKS2 modifications (Figure 11). Further experiments under different light treatments and in *phot* and *phy* mutants backgrounds are required to determine which photoreceptor may be regulating PKS2 post-translational modifications.

It is interesting to note that PKS2 levels were lower under BL compared to RL. Turnover of signalling components is a well known mechanism for signal regulation (e.g. attenuation and de-sensitization) (Figure 11). This suggests that PKS2 may act in BL signalling. The levels of PKS2 in the *nph3* background should be compared with wild type background.

As for NPH3 and PKS1-GFP, PKS2-GFP appeared associated with the plasma membrane (Pedmale and Liscum, pers. com.; Lariguet et al., 2006; Figure 14). However, PKS2-GFP signal was also found in the nucleus and intracellular compartments (Figure 14). Further samples from independent lines should be analysed to confirm the observed patterns. In addition, dyes such as DAPI (nuclear staining) and FM4-64 (plasma membrane localization) should be used to confirm PKS2-GFP localization. PKS2-GFP did not localise in the supernatant after ultracentrifugation, suggesting that it was predominantly associated with membranes (Figure 15D). However, it is surprising that the nuclear signal did not correspond to soluble proteins. Perhaps the nuclear bodies in which PKS2-GFP appeared to be concentrated in the nucleus were also pelletted during ultracentrifugation. Probing the protein fractions with a CRY2-specific antibody may help solve this issue (CRY2 is also present in nuclear bodies (Chen et al., 2004)). Finally, according to these first results, it is possible that PKS2 associates with intracellular vesicles. If this were the case, ARA6-mRFP, mRFP-ARA7, Venus-SYP31 and VenusSYP41 which are

intracellular markers of late endosome, early endosome, cis-Golgi and trans-Golgi network, respectively, may provide useful to identify the nature of these compartments (via co-expression with PKS2-GFP in protoplasts for instance) (Furutani et al., 2007).

The precise subcellular localisation of NPH3 has not been yet reported. Immunostaining of hypocotyls showed a clear signal at the periphery of the cells (Lariguet et al., 2006). However, no published work has reported detailed intracellular localisation of NPH3. In fact, according to Ullas Pedmale (Emmanuel Liscum lab) NPH3-mCherry appears restricted to the plasma membrane, similarly to PKS1-GFP (personal communication).

PKS2 and NPH3 co-localisation at the plasma membrane and the discrete subcellular localisation of PKS2 in other cellular regions than NPH3 suggests three possible scenarios: (i) PKS2 acts in concert with NPH3 at the plasma membrane (possibly in a same linear pathway), (ii) PKS2 may act independently of NPH3 in other cell compartments (possibly in parallel signalling pathways) and (iii) a combination of both possibilities. In the first scenario, PKS2 and NPH3 may be present in similar complexes with the plasma membrane-associated phot1 and phot2 pools. In the second scenario, PKS2 may be associated with the intracellular phot1 and phot2 pools (in the Golgi or endosomes for instance). To test this second hypothesis, it will be interesting to see whether PKS2, as for phot1 and phot2, is translocated from the plasma membrane into the cell upon light treatments. Preliminary experiments with Chitose Kami showed that 68-hour-old etiolated seedlings exposed to minimum light (using safe green filter over confocal laser for instance) still showed strong nuclear signal (data not shown).

Two molecular results are consistent with PKS2 acting in a parallel pathway than NPH3. First, PKS2-GFP localisation appeared similar in *pks2-2* and *nph3pks2-2* backgrounds, suggesting that NPH3 did not (at first sight in these first analyses) greatly influence PKS2-GFP localisation (Figure 14 A and B). Second, PKS2 associations with phot1 and phot2 was independent of NPH3 (Figure 16A). If *nph3* were completely epistatic over *pks2*, then such effects of NPH3 on PKS2 would make

sense. However, the synergistic interaction combined with these molecular results point more towards independent roles.

Several experiments should be performed to further study the molecular associations of NPH3 and PKS2. First, PKS2-GFP and NPH3-mCherry could be used as baits to test NPH3 and PKS2 co-immunoprecipitations, respectively. Second, bi-molecular fluorescence complementation (BiFC) assays would prove very useful to better understand the *in vivo* dynamics of putative NPH3-PKS2 interactions (and also NPH3 and PKS2 associations with phot1 and phot2). Third, *in vitro* interaction assays should be performed to test direct physical interactions between these proteins.

During phototropism, leaf flattening and leaf positioning, *PKS1* and *PKS2* acted redundantly (Lariguet et al., 2006; this work). However, their roles are different during FRL-induced de-etiolation. Indeed, PKS1 and PKS2 appeared to antagonise each other's roles during the phyA VLFR and this was proposed to provide regulatory loop for phyA signalling (Lariguet et al., 2003). Thus, it is possible that the molecular functions of PKS1 and PKS2 are different under BL or FRL. Data presented here showed that PKS1 and PKS2 were both associated with phot1 and phot2 *in vivo*. Genetic redundancy suggests that the PKS may act in one common biochemical step in linear phot signalling pathways. In the case of root phototropism, PKS1 appears to play an essential role in this signalling step because *pks1* roots were completely aphototropic (Boccalandro et al., 2008). However, as previously discussed, the PKS do not appear to represent a key step during leaf positioning. As for leaf flattening, the question remains open since the *pks3-3* single mutant diplayed partially epinastic leaves (similar to nph3). The quadruple knock-out will be required to solve this issue.

Finally, the punctuate pattern of *PKS2::GUS* expression in young leaves number 3 and 4 was intriguing (Figure 17C). Young leaves have a high capacity to synthesize auxin and contribute to plant auxin homeostasis. Highest auxin levels are contained within the basal region of young leaves – a region where cell division is intense (Ljung et al., 2001). Leaf morphogenesis is controlled by cell cycling in marginal meristems at the base of leaves (Donnelly et al., 1999). Interestingly, the *PKS2::GUS* pattern appeared rather concentrated in this same basal area of the young leaves number 3 and 4. Epinastic *nph3pks1pks2pks4* leaves contained fewer cells (in the

epidermis) than wild type leaves, suggesting that the quadruple mutant may show defects in cell division (Chapter 2 - Figure 7). Although purely speculative, when taken together these data raise the possibility that the PKS may act on auxin-regulated cell division to control leaf flattening. (this point of discussion should be reviewed using my newest data on *PKS2pro::GUS* expression pattern in the leaves).

GENERAL DISCUSSION

Table 1: Summary of genetic experiments.

The font size indicates the relative contributions of each PKS.

	phot1 pathway	phot2 pathway	reference
Stomatal opening	no	no	Chapter 2
Chloroplast relocation	No (PKS3?)	No (PKS3?)	Chapter 2
Root phototropism	PKS1 (PKS3?)	?	Boccalandro et al., 2008
Hypocotyl phototropism	PKS4, PKS1, PKS2, (PKS3?)	?	Lariguet et al., 2006
Leaf positioning	PKS2, PKS1		Chapter 3
Leaf flattening		PKS2, PKS3, PKS1	Chapter 2
Inhibition of hypocotyl elongation	?	?	

1. Comparing PKS roles in phototropism, leaf flattening and leaf positioning

These three processes are achieved by coordinated asymmetric growth processes that are likely to be controlled by auxin redistribution. Phototropism is the best-characterized response and knowledge about the tissues involved as well as the underlying auxin transport mechanisms is becoming available. Although leaf positioning via petiole bending (as opposed to pulvini swelling) is less well described, it may be seen as an analogous process than phototropism (Lyon, 1963; Inoue et al., 2008a). On the contrary, leaf flattening is poorly understood (Poethig, 1997; Inoue et al., 2008a) Lin et al., 2007). Leaf flattening is likely to be achieved via (a)symmetric growth processes within different tissues of the lamina. However, the critical tissues controlling this process are not well defined. For instance, the epidermis may play a predominant role since it restricts growth (Savaldi-Goldstein and Chory, 2008). The vasculature may also serve as a physical structure for flatness (Scarpella et al., 2006). Inner palisade and spongy mesophyll tissues are also known to change shape and structure in response to light, but how these inner morphological changes lead to flatness and curvature is unknown (Van Volkenburgh, 1999; Lopez-Juez et al., 2007).

Genetic and physiological experiments clearly showed that the *PKS* control the three processes. At least three interesting observations emerge from these genetic studies: (i) different PKSs appear to play specific roles in each of these processes (Table 1); the PKSs act in either phot1 or phot2 pathways and (iii) PKS role may be essential (key step) or partial (redundancy with another protein family - e.g. NPH3). Interestingly, PKS homologs stem from whole-genome duplication events. Differential expression patterns may confer each PKS its specific physiological function (Blanc and Wolfe, 2004) as previously proposed for root phototropism (Boccalandro et al., 2008). The PKS proteins may also have distinct biochemical functions.

It is interesting to note that the *aux1* mutant displayed curled leaves but normal petiole positioning (Chapter 2 - Figure 11A; data not shown). Thus, auxin control of these different processes may involve different sets of regulators. In the lamina, we

proposed that PKS2 modulates auxin homeostasis by acting on auxin import (possibly via mediation of the phot2 pathway). It is possible that the PKS act differently in the phot signalling pathways during phototropism and leaf positioning, and control the activity of different targets in these other tissues (as discussed for the differences between *pks2* and *pks3* leaf phenotypes: e.g.perhaps PKS2 and PKS3 act on different AUX / LAX proteins which are expressed in different places).

2. PKS2 – a link between phot signalling and auxin transport?

The main message of this thesis is that the PKS act in phot-signalling pathways controlling developmental processes. First, the PKS are not involved in chloroplast movement or stomatal opening. Second, epistasis studies clearly showed that the PKS act in either phot1 or phot2 pathways (or both) during lamina flattening and petiole positioning. As presented in the introduction, the phot signalling pathways controlling each of these BL responses are starting to be elucidated. They may be of different nature (e.g. different protein families). The targets of the distinct phot signalling pathways appear to be different (e.g. cytoskeleton-associated proteins in chloroplast movements, and auxin transporters during phototropism). Thus, the fact that the PKS act in a subset of phot responses already provides information about their possible molecular mode of action.

An important aim of this thesis was to explore the molecular function of PKS2 during phot signalling. Although the precise mode of action is still unclear, I hope that these first results will generate more precise hypotheses and will provide a basis for future experimentation (as proposed in the discussion of Chapter 3). In my view the main findings are the following: (i) PKS2 is associated with phot1 and phot2 *in vivo,* (ii) PKS2 localises at the plasma membrane and in putative intracellular compartments (iii) *nph3* and *pks2* mutants interact synergistically. As previously discussed, an important aim will be to precise the respective biochemical functions of PKS2 and NPH3 during phot signalling (e.g. co-localisation, interactions…). At present it is still unclear whether PKS2 and NPH3 act in a same phot complex, and whether they function in the same linear pathway or in parallel pathways.

The synergistic interactions between *nph3/ pks2*, *nph3 / phot1* and *phot1 / phot2* under HBL (in petiole positioning experiments) are particularly interesting because a similar scenario was observed between *npy1*, *pid1* and *yuc* mutants (Furutani et al., 2007; Cheng et al., 2008). This comparison suggests that the PKSs may act as novel players (representing a discrete biochemical step) in important signalling pathways controlling auxin-regulated development.

Several lines of evidence indicate that the PKS are likely to regulate auxin homeostasis in the organs they act in. First, they are important regulators of growth processes. It is noteworthy to point out that ectopic and high expression of *PKS4* and *PKS2* also had strong consequences on plant development in the dark and under RL, respectively (Schepens et al., 2008; Chapter 3 - Figure 7). Second, *PKS2* loss-of-function restored flat leaves in the epinastic *aux1* mutant ***(!!! Genetic interaction not true!!! See details in results section of Chapter 2)***. Third, PKS1 is involved in auxin export in protoplasts. Together, these results suggest that the PKS regulate development and may act on auxin homeostasis at the level of auxin transport. It would be interesting to test the effects of auxin transport inhibitors on leaf flattening and positioning. If a strong effect is seen, then it would be interesting to check whether th effects of such treatments are altered in *pks* and *nph3* mutants.

Finally, PKS2 and NPH3 associations with YFP-AUX1 *in vivo* indicate a possible molecular link with auxin transport. As discussed for phot1 and phot2, it is not currently known whether they are present in identical or distinct protein complexes with YFP-AUX1 in the cell. For instance, NPH3 may associate with plasma membrane AUX1 while PKS2 may localise with the intracellular pool of AUX1 (in the Golgi for instance) (Kleine-Vehn et al., 2006). It is interesting to note that phot2 also localises to the Golgi and PKS2 can be found in the same complex as phot2 (Kong et al., 2006; Chapter 2 Figure 8). Because PKS2 appears to localise in intracellular compartments, it is possible that PKS2 plays a role with phot2 and AUX1 in the Golgi. It will also be important to analyse the possible interactions with PIN and PGP proteins. Indeed, the rescue of the aux1 phenotype by *pks2* points towards a possible role for PKS2 in auxin efflux (Chapter 2 – Figure 11A).

In summary, PKSs and NPH3 may provide a direct link between phot signalling and auxin transport. How these proteins mediate (either in concert or independently) such connection is still elusive. In general, signalling processes involve multi-protein complexes that undergo dynamic changes in composition and localisation (Alberts et al., 2002). Dissecting the cellular localisation and molecular function of PKS2 and NPH3 in relation with the phots and auxin carriers, as well as the identification of additional players, will allow us to elucidate these important signalling processes.

MATERIALS AND METHODS

1. Plant growth

Plant growth on soil

Standard soil was a blend of weakly decomposed white sphagnum peat and clay and other additives (type GS90; FAI11) from Einheitserde company (Germany). Some batches of soil were sometimes contaminated with fungi that caused strong heterogeneous plants growth during the physiology experiments. When so, another soil prepared by the gardener of the DBMV (Philippe Reymond lab, University of Lausanne) (4% peat, 1% sand, 0.6% vermiculite, 0.6% organic soil) was used. Plants were grown either in 132mm x 86mm x 55mm pots (for 6 plants) or standard Aracon pots (for 1 plant) or Petri dishes with punched holes. In all cases, soil was compacted into pots and imbibed with water from below. Seeds were sown on moist surface, covered with a transparent plastic lid in a tray, and stratified at 4°C/dark for 3-4 days to obtain homogenous germination. One-and-a-half days after transfer to growth chamber the lids were taken off. It was noticed that some mutants displayed strong agravitropism (non-vertical growth) when the lid was kept too long over the developing seedlings (possible effect of temperature and/or humidity). The zero reference time point of growth was considered as the time when trays were transferred from the cold room to the growth chamber.

Plant growth on agar

Seeds were surface-sterilized for 3min in 70% ethanol + 0.05% Triton X-100 then for 10min in 100% ethanol then resuspended in sterile water. Seeds were plated on 0.7% phytagar containing half-strength Murashige and Skoog (Murashige and Skoog, 1962) medium (Gibeo Invitrogen Corporation, Paisley, UK) in clear plastic Petri dishes (42 x 35 mm^2 x 20 mm) and stratified for three days at 4°C / dark. For growth of etiolated seedlings, germination was induced by 6-8hrs white light (80 $\mu mol \cdot m^{-2} \cdot s^{-1}$), and Petri dishes were wrapped with aluminium foil and placed in phytotron (20°C).

Growth under white light

For leaf flattening and overall plant growth experiments, plants were grown on soil in growth chamber with following parameters: 20°C; 55-75% relative humidity (RH), 16hrs light/8hrs dark photoperiod. White light source was provided by a combination of Coolwhite (L36W/20) and Limilux ® Warmwhite (L36W/830) Osram fluorescent tubes. For immunoprecipitation, GFP microscopy, GUS staining experiments, plants were grown in petri dished on agar in a phytotron with following parameters: 22°C, RH not controlled; mixture of 2 fluorescent tubes (phytotron)..

Growth under red and blue light

Blue light LED panels were constructed by Adlos AG (Schaan, Lichtenstein) with Vishay LEDs (TLCB5800 lambda max 462 nm, half band width 24 nm; Malvern, PA, USA). Light intensities were determined with an International light IL1400A photometer equipped with an SEL033 probe with appropriate light filters.

Light intensities were determined with an International Light IL1400A photometer (Newburyport, MA) equipped with an SEL033 probe with appropriate light filters.

2. Plant material: mutants and transgenic plants

2.1. Genes and mutant alleles

All mutants analyzed in this study are in the *Arabidopsis thaliana* (L.) Columbia-O background. The details for each allele are presented in Table 2.

Table 2: List of genes studied in this project and information on the different mutant alleles used.

Gene	AGI code	Mutant allele	Reference	Mutation type	Null
PKS1	At2g02950	*pks1-1*	(Lariguet et al., 2003)	Salk T-DNA (KanaR) insertion after 67th codon	yes
PKS2	At1g14280	*pks2-1*	(Lariguet et al., 2003)	Salk T-DNA (KanaR) insertion at the 359th codon	Some traces of transcript
		pks2-2	Ariane Honsberger	GABI T-DNA (SulfaR) insertion in 113th codon	Yes (Ariane Honsberger and this study)
PKS3	At1g18810	*pks3-2*	Martine Trevisan and Christian Fankhauser ABRC stock CS91606	EMS mutagenesis; C642T substituting serine 167 with phenylalanine	?
		pks3-3	Martine Trevisan and Christian Fankhauser ABRC stock CS93404	EMS mutagenesis; G1185A substituting cysteine 343 with tyrosine	?
		pks3-4	Martine Trevisan and Christian Fankhauser ABRC stock CS93887	EMS mutagenesis; G953A substituting glutamate 266 with lysine	?
PKS4	At5g04190	*pks4-1*	(Lariguet et al., 2006)	Salk T-DNA (KanaR) insertion after the 114th codon	yes
PHOT1	At3g45780	*phot1-5*	(Huala et al., 1997)	Fast neutron mutagenesis; breakpoint after residue 556	yes
PHOT2	At5g58140	*phot2-1*	(Kagawa et al., 2001)	EMS mutagenesis; base 3121 from the start codon, the last nucleotide in intron 11 changed from G to A	Possibly a few percent WT protein (Achard et al., 2006)
		phot2-101	Jarillo *et al.* (2001)	T-DNA insertion	yes
NPH3	At5g64330	*nph3-6*	(Motchoulski and Liscum, 1999)	Fast neutron mutagenesis; 2nd codon (W) replaced by stop	yes
AUX1	AT2G38120	*aux1-22*	(Swarup et al., 2004)	Salk T-DNA, where?	yes
PHYA	At1g09570	*phyA-211*	(Nagatani et al., 1993)	EMS mutagenesis	yes
GL1	At1g18750	*gl1-1*	(Oppenheimer et al., 1991)	???	yes

pks2-2 mutant– work of Ariane Honsberger and Christian Fankhauser

Ariane Honsberger isolated homozygous T-DNA insertion mutants and confirmed absence of *PKS2* transcript by northern blotting. The presence of a single T-DNA insertion was verified by checking the 1:4 ratio of whitening seedlings on Sulfadiazine-containing MS medium. Homozygous seeds at the T2 generation were identified by genotyping the *PKS2* locus (PCR). To genotype the *pks2-2* allele we used one pair of primers to detect the presence of the transgene (CF338 [5'-CAT TTG GAC GTG AAT GTA GAC AC-3'] / AH022 [5'-CCC AAA GCC CAT TAA CGA CC-3']) and a second pair to test for homozygosity (CF359 [5'-TCG AAC ACA CGC ATC TGC AG-3'] / AH022).

pks3 mutants – work of Martine Trevisan and Christian Fankhauser

pks3 EMS-generated mutants (point-mutations) were ordered from ABRC stocks by Martine Trevisan and Christian Fankhauser. Mutant alleles were chosen based on their potential to disrupt the protein's biochemical function (highly conserved residues among PKS primary sequences; SIFT score indicating potential deleterious effects) (Till et al., 2006). Three to four backcrosses with wild type plants were done by Martine Trevisan. Genotyping strategies for the *pks3* alleles were designed by Martine Trevisan and Christian Fankhauser. Briefly, for *pks3-2*: PCR on genomic DNA with CF405 [5'ggt tta gat gtt acg gtc cct gat3'] and CF410 [5'tgg tat tgc gtc cca tgt aag3'] generated a 256bp product that was digested with MboI to yield 177bp+56bp+23bp fragments in wild type or 177bp+79bp in mutated sequence. For *pks3-3*: PCR on genomic DNA with CF407 [5'tcg gct ggg ttt ttg tcg gga t3'] and CF408 [5'tca agt ctt gaa tcc taa tct cag3'] generated a 146bp product that was digested with FokI to yield 115bp+31bp fragments in wild type or 146bp in mutated sequence. For *pks3-4*: PCR on genomic DNA with CF409 [5' tgc tta cga cgc aag aaa gca t3'] and CF408 [5'tca agt ctt gaa tcc taa tct cag3'] generated a 600bp product that was digested with BsmAI to yield 249bp+243bp+64bp+42bp fragments in wild type or 492bp+64bp+42bp in mutated sequence. Martine Trevisan has tried hard to cross *pks3* tilling alleles with *pks2-2* but with no success (possibly because chromosome

crossing-over between the two loci may be hindered by some effect of the T-DNA present in the *PKS2* genomic sequence in *pks2-2*) (ref. from Trends in Plant Science). Martine Trevisan and Laure Allenback constructed the *pks3-4pks2-1* mutant.

2.2. Multiple mutants

pks1-1pks2-1pks4-1 and *phot1-5phot2-1gl1-1* mutants were constructed previously (Lariguet et al., 2006). *phot1-5phot2-101* was constructed by Particia Lariguet. Genotyping of the *PHOT1* locus was done as in Lariguet et al. (2004). To genotype the *phot2-101* allele, primers CF402 [5'- tac cat agt gtc att gct cac gga -3'] and JMLB2 [5'- ttg ggt gat ggt tca cgt agt ggg cca tcg-3'] were used to detect the presence of the transgene and CF401 [5'- TGG AAT CTT CTC ACA GTC ACT CCT -3'] / CF402 detected wild type genomic sequence. *aux1-22pks2-2* was generated by Martine Trevisan and Laure Allenbach by crossing.

Generation of *phot1-5pks1-1pks2-1pks4-1*

All possible *phot1/pks1/pks2/pks4* multiple mutants were obtained by crossing *phot1pks2* with *phot1pks1pks4*. *phot1pks2* and *phot1pks1pks4* were previously constructed by Isabelle Schepens. All mutants were identified in the F2 or F3 generation by genotyping. Primers and PCR conditions used for genotyping were the same as previously described (Lariguet and Fankhauser, 2004; Lariguet et al., 2006).

Generation of *nph3-6 - pks1-1/pks2-1/pks2-2/pks3-2/pks3-3/pks3-4/pks4-1* mutants

To obtain all possible *nph3/pks1/pks2-1/pks4* multiple mutants, *nph3* was previously crossed with *pks1pks2-1pks4* by Patricia Lariguet. F2 plants were genotyped by Patricia Lariguet and Martine Trevisan. All possible mutant combinations were identified in the F3 generation with the help of Martine Trevisan. *NPH3* locus was genotyped by amplifying genomic DNA with CF395([5'-GAG TGT TAA CTT GTG TAT GAT GC-3']) and CF396([5'-GAC AGC AAC GGA TAC TGA AAG-3']), digesting the 419 bp PCR product with Aci1, and separating digestion products on 3%

(w/v) agarose gel. Wild type genomic sequence yielded 309bp+110bp while mutated genomic sequence yielded 309bp+89bp+21 bp fragments. Genotyping of *pks1-1*, *pks2-1* and *pks4-1* was done as previously described (Lariguet et al., 2003; Lariguet et al., 2006).

nph3pks2-2 and *nph3pks1pks2-2* mutants were obtained by crossing *nph3-6* with *pks1pks2-2*. *pks1pks2-2* was previously constructed by Patricia Lariguet. F2 plants that showed epinastic cotyledons were genotyped at all three loci.

nph3pks3-2, *nph3pks3-3*, *nph3pks3-4* and *nph3pks3-4pks2-1* mutants obtained by crossing *nph3-6* with latest homozygous generation of backcrossed the *pks3* single or *pks3-4pks2-1* double mutants. Potential *nph3pks3* mutants were first visually selected by isolating epinastic plants in the F2 generation, and then genotyped to confirm homozygosity of mutations.

Generation of *phot2-1/pks1-1/pks2-1/pks4-1* mutants

To obtain all possible *phot2/pks1/pks2/pks4* multiple mutants *phot2* was crossed with *pks1pks2pks4*. Mutants were identified in F2 and F3 generations by genotyping. To genotype *phot2-1*, a 760 bp fragment was amplified by PCR from genomic DNA using CF347 ([5'- GAA CCT TGC AGA GTC TTC TG-3']) and CF346 ([5'-CTG CCT CAC AAT AAG GAG AG -3']), and digested with Mbo1. Wild type sequence was cut twice to yield 398bp+230bp+120bp fragments; *phot2-1* mutant sequence yielded 530bp+230 bp fragments.

Generation of *phyA-211pks1-1pks2-1pks4-1*

All possible *phyA /pks1/pks2/pks4* multiple mutants were obtained by crossing *phyApks2* (Lariguet et al., 2003) with *phyApks1pks4*. *phyApks1pks4* was previously constructed by Isabelle Schepens. All eight possible mutants were identified in the F2 generation by genotyping. Primers and PCR conditions used for genotyping were the same as previously described (Lariguet et al., 2003; Lariguet et al., 2006).

Generation of *nph3phot1* and *nph3phot2* mutants

nph3-6 plants were fertilized by *phot1-5* and *phot2-1* plants. *nph3phot1* plants were identified in the F2 generation by selection of epinastic plants followed by PCR/digestion genotyping. Only one out of eight epinastic plants in the F2 generation (*NPH3 -/-)* was heterozygous at the *PHOT2* locus (probably because *NPH3* and *PHOT2* loci are linked - 2.2megabp apart on chromosome 5). *nph3phot2* plants were obtained in the F3 generation by PCR/digestion genotyping.

Generation of *phot1phot2* mutant with trichomes

phot1-5phot2-1 mutants that bear trichomes were obtained by crossing *phot1-5phot2-1gl1-1-1* with *phot2-1*. F2 plants that were highly epinastic and that possessed trichomes were isolated and F3 seeds of *GL1* +/+ plants were used for physiological analyses.

Generation of *nph3-6aux-221* and *nph3-6aux1-2pks2-2* mutants – ongoing for future experiments

To construct *nph3aux1, aux1* plants were fertilized with *nph3* pollen. F1 plants that have lost their epinasty (*aux1* is recessive) were genotyped at *AUX1* and *NPH3* loci as follow: for *aux1-22*, primers CF484 [5' acc tga atg ttt cac acc ttc 3'] and CF485 [5' ctc cat cat cca cgg cca gc 3'] generated a 321bp PCR product, AluI digestion yielded 230bp+72bp+19bp fragments for wild type and 249bp+72bp fragments for *aux1-22* sequences. *nph3aux1* plants still need to be identified in the F2 generation. To construct *nph3aux1pks2, nph3aux1* plants were crossed with *nph3pks2* plants. F1 plants need to be analysed.

The *glabrous1* (*gl1-1*) mutant was provided by Dr. Philippe Reymond.

2.3. Transgenic lines

Crossing of *PHOT1::PHOT1-GFP* into *nph3* and *pks1pks2* mutant backgrounds

PHOT1::PHOT1-GFP nph3-6phot1-5 plants were obtained by crossing *PHOT1::PHOT1-GFP phot1-5* (Sakamoto and Briggs, 2002) with *nph3-6*. In the F2 generation *PHOT1::PHOT1-GFP* plants were identified by positive selection on 1/2MS + Basta (10µg/ml), *nph3-6* plants were identified by epinastic leaves plus genotyping, and *phot1-5* plants were identified by anti-phot1 western blot. Homozygous plants for the *PHOT1::PHOT1-GFP* transgene were identified by analyzing the segregation of F3 plants on 1/2MS + Basta (10µg/ml).

PHOT1::PHOT1-GFP phot1-5pks1-1 and *PHOT1::PHOT1-GFP phot1-5pks1-1pks2-1* plants were obtained by crossing *PHOT1::PHOT1-GFP phot1-5* (Sakamoto and Briggs, 2002) with *phot1-5pks1-1pks2-1*. In the F2 generation *PHOT1::PHOT1-GFP* plants were identified by positive selection on 1/2MS + Basta (10µg/ml), and *phot1-5pks1-1*, *phot1-5pks2-1*, *phot1-5pks1-1pks2-1* plants were screened gentoyping. Homozygous plants for the *PHOT1::PHOT1-GFP* transgene were identified by analyzing the segregation of F3 plants on 1/2MS + Basta (10µg/ml). No *PHOT1::PHOT1-GFP phot1-5pks2-1* plants are yet avaible (only *PKS2* -/+). *PHOT1::PHOT1-GFP phot1-5pks1-1pks2-1* is planned to be used in anti-GFP immunoprecipitation experiments to check if NPH3 still associates with phot1-GFP in absence of PKS1/PKS2, but experiment not yet performed.

Crossing of *PHOT2::PHOT2-GFP* into *nph3* and *pks1pks2* mutant background

PHOT2::PHOT2-GFP nph3-6 plants were obtained by crossing *PHOT2::PHOT2-GFP phot1-5phot2-2* (Kong et al., 2006) with *nph3-6*. In the F2 generation *PHOT2::PHOT2-GFP* plants were identified by positive selection on 1/2MS + Kanamycin (25µg/ml), *nph3-6* plants were identified by epinastic leaves plus genotyping (many seeds screened because *NPH3* and *PHOT2* loci are linked -

2.2megabp apart on chromosome 5), and *phot1-5* and *phot2-1* plants were identified by western blot using polyclonal antibodies that recognize both phot1 and phot2 (Kong et al., 2006). No *PHOT2::PHOT2-GFP nph3-6phot2-2* plants could be found in the F2 generation probably because *NPH3* and *PHOT2* loci are linked. However, judging from the amounts of phot2 in the western blot (*phot2-2* has a point mutation in essential phosphorylation site that leads to phot2 inactivation and also reduction in phot2 levels (Kasahara et al., 2002; Kong et al., 2006)), some lines appear to be *nph3-6 PHOT2* -/+. These lines were used in immunoprecipitation assays. However, analysis of F3 generation is still required to obtain *PHOT2::PHOT2-GFP PHOT2-/-nph3-6* plants.

PHOT2::PHOT2-GFP phot1-5phot2-2 were crossed with *phot2-1pks1-1*, *phot2-1pks2-1* and *phot2-1pks1-1pks2-1* and F2 seeds were harvested. F2 plants have not been genotyped yet.

<u>*PKS2* over-expressing lines – Lariguet et al. (2003)</u>

35S::PKS2 lines were constructed by Patricia Lariguet by transforming wild type Col-O Arabidopsis plants using vector pCF208 (Lariguet et al., 2003). *PKS2* cDNA in pCF208 is derived from pCF204 into which an EST corresponding to *PKS2* coding sequence was cloned (Christian Fankhauser). When using this *PKS2* cDNA for further sub-cloning (e.g. construction of *PKS2-GFP* fusion) I noticed by sequencing that it had the two following mismatches with TAIR's Columbia-O accession genomic sequence: A459C and C906G causing Lys153Asn and Asn302Lys substitutions, respectively. This polymorphism may originate from EST generation from another Arabidopsis ecotype or unfaithful reverse-transcription of the *PKS2* mRNA from Col-O. The two substitutions occur outside highly conserved PKS motifs (data not shown).

Crossing of *AUX1::YFP-AUX1 aux1-22* into *pks2-2* mutant background – ongoing for future experiments

AUX1::YFP-AUX1 aux1 plants (Swarup et al., 2004) were crossed with *aux1pks2* plants. F1 seeds have been collected and still need to be analyzed. *AUX1::YFP-AUX1 aux1pks2* plants will allow us to analyze PKS2 roles in intercellular AUX1 localization and in AUX1-associated protein complexes.

Crossing of *NPH3::NPH3-Cherry nph3-6* into *pks2-2* mutant background – ongoing for future experiments

NPH3::NPH3-Cherry nph3 (genomic *NPH3* sequence – mCherry – flag) seeds were provided by Ullas Pedmale and Mannie Liscum (University of Missouri, Columbia, USA). *NPH3::NPH3-Cherry nph3* plants were crossed with *nph3pks2* plants. F1 seeds have been collected and still need to be analyzed. *NPH3::NPH3-Cherry nph3pks2* plants will allow us to analyze PKS2 roles in intercellular NPH3 localization and in NPH3-associated protein complexes.

3. Construction of PKS2-GFP expressing plants

PKS2pro::PKS2-GFP and *35S::PKS2-GFP* transgenes were constructed and transformed into wild type, *pks2-2, nph3pks2-2* and *nph3pks1-1pks2-2* plants. In this study, only *PKS2pro::PKS2-GFP pks2-2* and *PKS2pro::PKS2-GFP nph3pks2-2* lines were used for experiments.

Previously, Christian Fankhauser and Patricia Lariguet generated the following vectors: *PKS2pro::PKS2-GFP* in plant binary vector pPZP312 (to generate pCF335) and *35S::PKS2-GFP* in plant binary vector pCHF5 (to generate pPL9). Since *PKS2* coding sequences in these vectors contained Lys153Asn and Asn302Lys substitutions (compared to Col-O genomic sequence), new *PKS2* promoter and coding sequence

fragments were amplified from genomic DNA (*PKS* genes do not have introns) and subcloned into intermediate vectors (pMC15, pMC16 and pMC17) to finally replace pCF335 with pMC23 and pPL9 with pMC21.

For *PKS2* 5'end, a 538 bp promoter region upstream the start codon (same as for *PKS2pro::GUS* construct – Lariguet et al., 2003) plus 396 bp coding sequence was obtained using primers MC007 [5' CTA GTC TAG ACT CCT TGA ATC GGA TAA ACA TAG 3'] (inserts Xba1 linker) plus CF142 [5' ctc tgg cta ttc caa ctg ga 3'] (inserts EcoR1 linker). The remaining 3'end *PKS2* sequence was obtained using similar strategy (several fragment – complicated strategy – see my cloning strategy in notebook – complete if I have enough time). The whole *PKS2pro::PKS2* sequence was fused in C-terminus to *GFP* coding sequence in pPZP312 containing a RBCS terminator. The *PKS2* coding sequence was cloned into the plant binary vector pCHF5 (to generate pMC21) also containing a RBCS terminator downstream *GFP* sequence. In both vectors, the Basta resistance gene expression was driven by NOS (nopaline synthase gene) promoter and terminator. In the Syngenta (GARLIC) T-DNA in *PKS2* coding sequence, the Sulfadiazine resistance marker expression was driven by the 35S promoter. Although no sequence overlap in regulator sequences were thus present in the T-DNA, strong silencing still occurred unfortunately (Daxinger et al., 2008).

PKS2pro::PKS2-GFP and *35S::PKS2-GFP* constructs were transformed into Arabidopsis Columbia-O plants via the *Agrobacterium tumefaciens* floral dip method (Clough and Bent, 1998).

4. Leaf petiole positioning

Measurement of petiole positioning was based on the protocol of Inoue et al. (2007) with many modifications. Soil was placed in 90mm × 15mm bacteria culture Petri dishes with 5 punched holes at their bottom. Dishes were placed in trays and soil surface was evened and imbibed by adding water to bottom of trays. Approximately 300 seeds were sown on each dish and stratified for 3 days to induce uniform germination. At 8:30am, seeds were placed under 150 µmol m^{-2} s^{-1}, 16hrs/8hrs

light/dark cycle (light: 7am-11pm; dark: 11pm-7am), 20°C; 55-75% RH and germinated under transparent plastic domes for 36 hours. Domes were removed thereafter to ensure that hypocotyl growth occurred at 20°C; 55-75% RH because it was noticed that high temperature and/or humidity could cause dramatic curly growth of hypocotyls under our conditions (see appendix). Unhealthy or undeveloped seedlings were progressively weeded out and healthy seedlings with similar development were grown under 100-150 µmol m^{-2} s^{-1} WL until the first pair of true leaves were 1-1.5mm long (i.e. 9 days of growth; growth stage 1.01 (Boyes et al., 2001)). At 8:30am on the 9th day, seedlings were transferred to RL 50µmol m^{-2} s^{-1} + BL 0.4µmol m^{-2} s^{-1} or RL 50µmol m^{-2} s^{-1} + BL 5.0µmol m^{-2} s^{-1} and the first pair of true leaves were let to develop for 5 days at 8 hours. Between 5:30pm and 8:00pm on the 5th day of treatment, whole Petri dishes were photographed from above using a camera stage, then individual plants were photographed from the side using a PowerShot A640 digital camera, and this always from the same angle. To measure leaf petiole positioning, the angle between the hypocotyls and the petiole was measured using ImageJ software, 90° was substracted from these values to obtain the degree angle of petioles relative to horizontal. For each genotype, both petioles of plants were analysed.

5. Leaf blade area of light capture

To measure leaf blade area of light capture, only plants of similar developmental stage were analysed because it was noticed that blade size varied with developmental stage. Only plants that had a 4th leaf of approximately 1mm in length were analysed. Using the photoshop elemement 4.0 program, the petiole-blade junction was painted in black, then the magic-wand tool with a tolerance of 60 was used to select the bright green blades from the dark-brown soil background and the blade was pasted on a black background. Then, the area of each blade was measured using the ImageJ software.

6. Gravitropism and phototropism experiment

Eexperiment was performed as previously described (Lariguet and Fankhauser, 2004). Between 36 and 46 seeds were plated on square plastic boxes containing 40 ml medium and then stratified for 3 days. Germination was induced by placing the plastic boxes side by side and vertically under 80 µmol·m^{-2}·s^{-1} white light for 6 hours. The boxes were then fixed vertically in small hand-made chamber of black cardboard that let light through only one side. The plates were then placed directly in an incubator at 25 centimeters from a blue light LED panel (Adlos AG (Schaan, Lichtenstein) with Vishay LEDs (TLCB5800 lambda max 462 nm, half band width 24 nm; Malvern, PA, USA). The irradiance of blue light at the outer limit of the square plastic boxes was set to 0.7 ± 0.002 µmol/m^2/sec using one neutral filter placed between the boxes and the LED panel. Experimental duplication was done by using a large incubator that had two levels with one vertical LED panel on each level. In this long-term experiment, plants were let to grow for 67 hours. After, 67 hours, the boxes were scanned and angles formed by the hypocotyls relative to the blue light direction (horizontal) were measured with the ImageJ image software. The horizontal blue light source was set as zero reference, and the angle formed by the triangle (horizontal line) - (base of the hypocotyl) - (top of the hypocotyl - the region between the sites of cotyledon attachment) was measured. Angles were then computed in 20° categories (i.e. 0° - 20°, 20°-40°, 40°-60°, …, 320°-340°, 340°-0°), and the frequency (expressed here as percentage) of angles within each category was calculated.

7. Photographs and image analysis

Photographs were taken with a Canon Powershot A640. Images were managed (size reduction, cropping, painting for leaf light interception measurements) on Adobe Photoshop Elements 4.0. The magic wand tool of Adobe Photoshop Elements 4.0 (tolerance level 60) was used to automatically select green leaf surfaces on bright white whatman background (leaf flattening measurements), and green leaf surfaces on dark brown soil background (area of light capture of lamina in leaf positioning assays). Area and angle measurements were performed using ImageJ software.

8. Data analysis and statistics

Measurements data were collected and graphs were constructed on Microsoft Office Excel. Statistics were done using Microsoft Office Excel.

Standard deviation (SD) error bars were used to describe the variance in biological measurements. 95% confidence intervals error bars were used as visual representation of statistical significance (inferential). If n>10 (which corresponds to all cases of leaf flattening index and leaf positioning fata), then p-value<0.05 when the error bars do not overlap (Cumming et al., 2007)

9. Generation of polyclonal antibodies specific to PKS2

Previously, antibodies against PKS1 were successfully obtained using as antigen a soluble peptide corresponding to the first 273 amino acids of the protein (Fankhauser et al., 1999). Thus, a similar fragment of PKS2 was produced but was found to be insoluble even when produced at low temperature and low IPTG concentrations (Figure 1A). Smaller PKS2 fragments were produced and PKS2(aa1-205) was soluble but highly unstable while PKS2(aa1-155) was soluble and stable (Panel A). The latter PKS2 fragment was thus purified in sufficient quantities for rabbit immunization.

Pre-immune sera of 10 rabbits were tested and two rabbits (no.1 and no.10) were selected for immunization (Panel B). Analysis of immune sera showed that rabbit no.1 developed a better immune reaction (data not shown). Subsequent boosts successfully increased the titration of anti-PKS2 antibodies in the sera of that rabbit (Panel C). anti-PKS2 antibodies from the last bleed were negatively purified by using *pks2*-null protein extracts and subsequently purified by affinity using PKS2 antigen blotted onto nitrocellulose membranes (Panel D).

9.1. Expression vector construction

PKS2 5'end sequence coding for the first 277 amino acids (until ---EED) was cloned in to pGEX-4T-1 vector to generate pMC29. Primers CF143 [5' cgg gat cca aaa tgg tga cct taa ctt cat c 3'] and MC009 [5' CAT CGG ATC CTT ATT CAT ACT TCA CAG AGA ATC CA 3'] inserted BamH1 adaptors and replaced PKS2 codon 277 (Asp) with a stop codon. *PKS2* 5'end sequence coding for the first 155 amino acids (until ---NNS) was cloned in to pGEX-4T-1 vector to generate pMC30. Primers CF143 [5' cgg gat cca aaa tgg tga cct taa ctt cat c 3'] and MC012 [5' GTA ACT GGA TCC TTA AGA GTT TTT CTT GAT GTT C 3'] inserted BamH1 adaptors and replaced PKS2 codon 155 (Ser) with a stop codon. *PKS2* 5'end sequence coding for the first 205 amino acids (until ---SSG) was cloned in to pGEX-4T-1 vector to generate pMC31. Primers CF143 [5' cgg gat cca aaa tgg tga cct taa ctt cat c 3'] and MC013 [5' GAT GGA TCC TTA ACC AGA GCT TCT TCT CTT G C 3'] inserted BamH1 adaptors and replaced PKS2 codon 205 (glutamine) with a stop codon. Proof-read *PKS2* fragments were obtained from vector pMC23 (containing *PKS2* genomic sequence from Col-O accession). PCR product was BamH1 digested and inserted into the BamHI site of the pGEX-4T-1 multiple cloning site.

9.2. Recombinant protein purification

GST-PKS2 recombinant proteins were produced for three hours in *E.coli* at 20°C using 0.1mM IPTG final concentration. Bacteria were harvested by centrifugation and resuspended in extraction buffer (EB) [1× PBS; 2.5mM EDTA; 1mM DTT; 1mM AEBSF; 1% (v/v) T X-100]. Suspension was sonicated on ice (lowest amplitude (22%); 2 secs on, 20 secs off; 6 cycles) to break open cells. Lysate was then centrifuged 16'000g for 15mins. Supernatant (SOL fraction) was added to Glutathione-Sepharose beads equilibrated with EB. Pellet (INS) was resuspended in 8.0M Urea, 0.5M NaCl, 0.5M Tris pH8.0 at room temperature and sonicated to solubilise proteins. Soluble proteins + Glutathione-Sepharose beads mixture was gently mixed for 2 hours at 4°C. Unbound (UNB) proteins were collected by gravity flow and beads were washed with 20 column-volumes of EB (W1-W3). Beads were

then equilibrated with 10 column-volumes of TMK buffer [50mM KCl; 20mM $MgCl_2$; 50mM Tris pH 7.5] (TMK1-2) and heat-shock proteins attached to affinity-purified GST-PKS2 were detached by 5mM ATP added to TMK buffer (ATP). Beads were then equilibrated again with 100mM NaCl plus 50mM Tris pH 7.5 and GST-PKS2 proteins bound to Glutathione-Sepharose beads were recovered in several elution fractions (F1-F3) by passing the column with reduced glutathione [100mM NaCl; 10mM glutathione; 50mM Tris pH 8.0]. At least 4ml of 0.5-1.0mg.ml^{-1} purified GST-PKS2 was recovered and used for rabbit immunization.

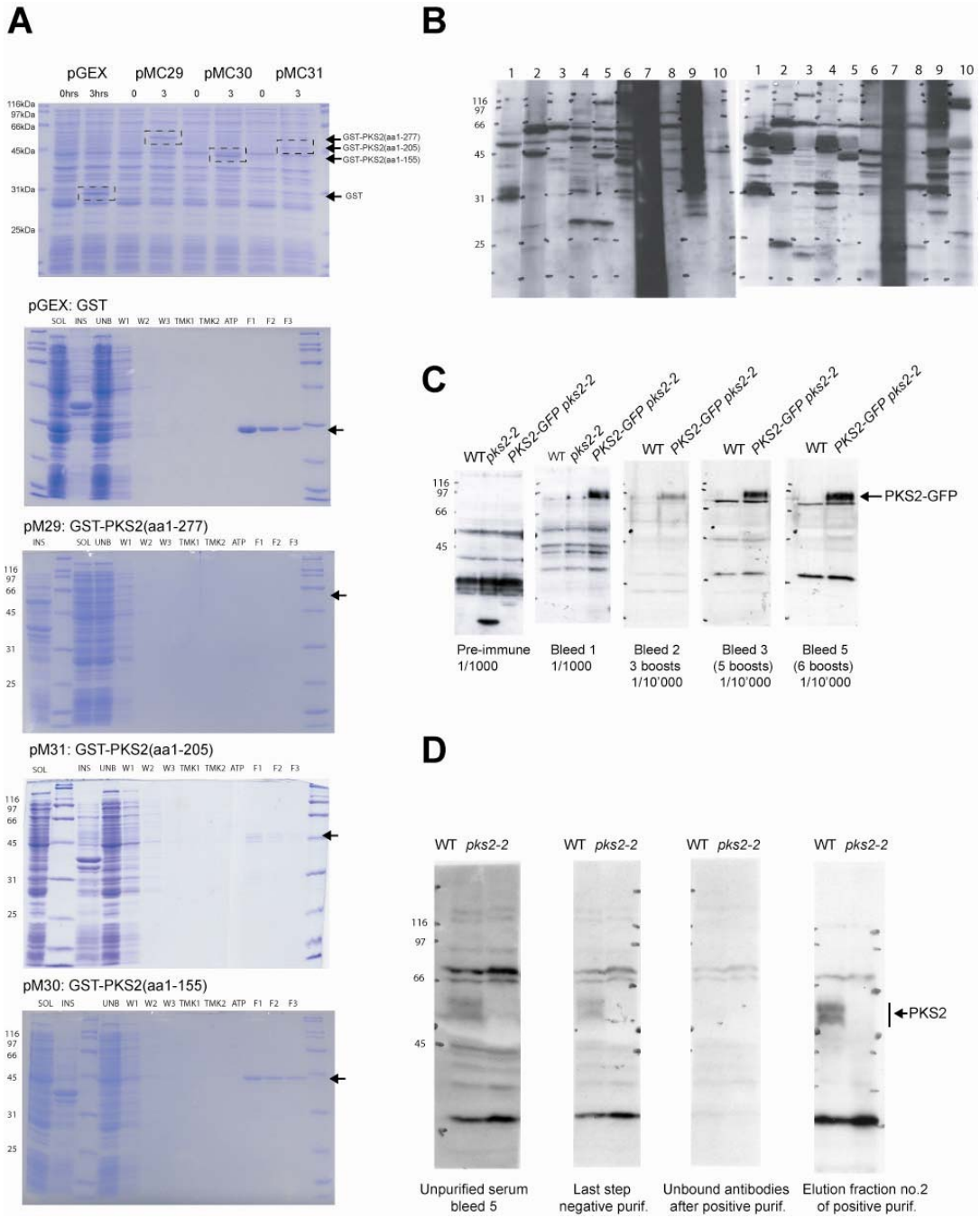


Figure 1: Preparation of polyclonal antibodies specific to PKS2.

(A) Production and purification of PKS2 antigen peptides.
First gel: Three different Glutathione-S-transferase(GST)-PKS2 recombinant proteins were produced in *E. col*i using the IPTG-inducible expression system. pGEX corresponds to the empty GST-expressing vector. pMC29-31 correspond to three GST-PKS2 peptides. Dashed boxes indicate the band corresponding to the different peptides produced after 3 hours of IPTG induction (3hrs).
Second to fourth gels: Purification of GST-PKS recombinant peptides. INS, insoluble protein fraction of bacteria lysate; SOL, soluble protein fraction; UNB, unbound proteins after incubation of soluble fraction with glutathione-couple agarose beads; W1-W3, flow-through of first three column washes; TMK1-2, flow-through of equilibrating buffer for heat-shock protein (HSP) dissociation; ATP, fraction containing dissociated HSP from glutathione-bound proteins; F1-F3, elution fractions obtained with addition of reduced glutathione.
(B) Reactivity of antibodies from pre-immune sera of 10 rabbits (1-10) against protein obtained from 3-day-old dark-grown seedlings (left) and 2-week-old light-grown plant (right) extracts.
(C) Tests of anti-PKS2 antibodies titration in sera of rabbit no.1 that was immunized by successive boosts. Protein extracts from wild type (WT) and high PKS2-GFP expressing 3-day-old etiolated seedlings (*PKS2::PKS2-GSP nph3pks2* line3) were used.
(D) Negative and positive purification of polyclonal anti-PKS2 antibodies.

9.3. Rabbit immunization and antibody purification

We used the services of the BioGenes GmbH company (Berlin, Germany). Reactivity of pre-immune sera of 10 rabbits was analysed using protein extracts from 3-day-old dark-grown seedlings and 2-week-old light grown seedlings. Two rabbits showing best patterns were chosen. 300µg proteins were injected into these rabbits for first immunization. 150µg proteins were injected at each boost. Sera were obtained at one or two-week intervals and the titration of PKS2-specific antibodies was regularly tested.

For negative purification, 500µl of the last immune serum was diluted 50× in PBS pH7.4 + Tween-20 0.1% (v/v) + 0.5% /w/v) milk. Protein extracts from *pks2-2* null dark- and light-grown plants as well as and GST proteins were blotted on nylon membranes. Strips of these membranes were mixed with the serum dilution for 3 days at 4°C and serum dilution containing unbound proteins was collected. For

positive purification, 200µg of GST-PKS2(aa1-155) antigen was blotted on ten nitrocellulose membranes and strips were prepared. Negatively purified serum dilution was mixed with these strips for 3 days at 4°C. After several washed with PBS pH7.4 + Tween-20 0.1% (v/v) + 0.5% /w/v) milk and PBS pH7.4 + Tween-20 0.1% (v/v), antibodies were recovered from strips by vortexing in 0.1M Glycine pH3.0. Solution was quickly neutralized using 1M Tris pH8 and globuline-free added to 1 $mg.ml^{-1}$ final concentration.

10. Protein extraction, western blotting and antibodies

Protein extracts (except for solubilised microsome prepration) were obtained by grinding plants in 60-100µl of 2× Laemmli sample buffer [0.125M Tris pH6.8; 4% (w/v) SDS; 20% (v/v) glycerol; 0.02% (w/v) bromophenol blue; 10% (v/v) β-mercaptoethanol] in microfuge tubes using micropestles. Protein extract was boiled 3-5min at 95°C and briefly centrifuged before gel loading. Proteins were separated on 8-12% SDS-PAGE and blotted onto nitrocellulose membranes using 1×CAPS pH11 + 10% (v/v) Methanol. Membranes were blocked in PBS pH7.4 + Tween-20 0.1% (v/v) + 0.5% /w/v) milk at 4°C overnight. Antibody dilutions used were as follow: polyclonal affinity-purified αPKS1, 1/1000 (Fankhauser et al., 1999); polyclonal affinity-purified αPKS2, 1/300 (this work); polyclonal αNPH3, 1/3000 (Motchoulski and Liscum, 1999); polyclonal αphot2,1 which recognizes both phot1 and phot2 (Kong et al., 2006), polyclonal 1/5000; polyclonal αDET3 (Schumacher et al., 1999), 1/20’000; monoclonal αGFP (JML-8, living colours), 1/6000.

11. Microsome preparation (protein fractionation) and anti-GFP immunoprecipitation

Plants were grown on 1/2MS agar in Petri dishes under 120µmol m^{-2} s^{-1} for 14-17 days. All manipulations were done at 4°C (cold room). 300-350mg of aerial parts (green tissues) were harvested and grinded in 3-volumes (~1ml) of extraction buffer (EB) [50 mM Hepes pH 7.9; 300 mM sucrose; 150 mM NaCl; 10 mM K-Acetate; 5

mM EDTA; 1mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF-prefabloc)/1% of protease inhibitor mixture for plant extracts (Sigma P9599)]. Cell debris were pelletted at 1000g for 3-5mins and 700µl of supernatant (S1) was ultracentrifuged at 75'000g for 75mins. Supernatant (S2) was collected and microsomal pellet (P2) containing insoluble membrane-associated proteins was gently resuspended in 700µl of EB + 0.5% (v/v) Triton X-100. Suspension was centrifuged at 16'000g for 5mins and supernatant containing solubilised microsomal proteins was collected (S3). Pellet (P3) was boiled in 100µl 2× Laemmli sample buffer. Monoclonal anti-GFP antibodies covalently coupled to magnetic microbeads were used to immunoprecipitate GFP-tagged proteins (Miltebyi Biotec; Order no. 130-091-125) and protein isolation was performed according to the manufacturer's instructions with minor changes. 50-60µl magnetic beads were added to 650µl S3 in a microfuge tube and gently mixed on rotating wheel for 2 hours at 4°C. Microbeads were then collected by passing the solution mixture through a metal column (pre-equlibrated with 1ml EB + Triton X-100 0.5% (v/v)) in a magnetic field. Column was extensively washes with several column-volumes of EB + Triton X-100 0.5% (v/v). After a final wash with 20mM Tris HCl pH7.5, immunoprecipitated proteins were recovered by applying 50µl of 95°C 2× Laemmli buffer to the column (elution fraction 1 – E1). This was repeated to recover elution fraction 2 (E2).

12. Confocal microscopy

Dark-grown or light grown seedlings were grown on agar in Petri dishes as previously described. Samples were mounted in water or 80% (v/v) glycerol between glass slides separated with one or two layers of transparent tape and sealed using nail polish. Confocal microscopy was performed on an inverted confocal LSM510 Axiovert 200M Zeiss microscope with the help of Arnaud Paradis and Chitose Kami (Cellular Imaging Facility; CIG; University of Lausanne). Laser monochromatic excitation light λ_{exc}=488nm was obtained from Argon / Krypton gas mixture. Emission light was collected using a short-pass 505-530nm filter for GFP signal (converted into green) and long-pass 650nm filter for plastid signal (converted into red). Image preparations (image merge, Z-stacks, scale bars) were done using the

Zeiss LSM software or the NIH image software ImageJ (http://rsbweb.nih.gov/ij/). Objectives used are described in figure legends.

13. GUS staining and tissue sections

GUS staining was done based on the protocol of Lagarde et al. (1996) (Lagarde et al., 1996). Briefly, plant tissues were prefixed for 45mins at room temperature in prefixing solution [0.5% (v/v) formaldehyde; 0.05% Triton X-100; 50mM NaPO4 pH7], rinsed in 50mM NaPO4 ph7 and incubated at 37°C in solution containing coloration substrate [0.5mM K-ferricyanide; 0.5mM K-ferrocyanide; 0.05% (v/v) Triton X-100; 1mM X-Gluc; 50mM NaPO4 pH7]. Duration of coloration varied between 1 and 24 hours. Tissues were then fixed in 2% (v/v) formaldehyde + 0.5% (v/v) glutaraldehyde + 100mM NaPO4 pH7 for 3 hours at 4°C and rinsed with 100mM NaPO4 pH7. Green tissues were clarified using a series of ethanol concentration (10-70% (v/v)). Micrographs of samples were obtained using a Nikon SMZ1500 stereomicroscope.

14. Experimental procedures of experiments for which data was not shown

Protocols for the following experiments were not described in this report because the data was not shown. Please see Appendix of results (report given to Christian Fankhauser) for a full account of *Materials and Methods* of the following experiments:

- Bi-molecular fluorescence complementation (Bi-FC) assays: *PKS1, PKS2, PKS4, NPH3, phot1, phot2* and *phyA* cDNAs were all cloned in Bi-FC vectors (Walter et al., 2004) and onion bulb epidermis was used for transient co-expression assay with the reporter control DsRED.
- Sections of GUS-stained hypocotyl, cotyledons and of non-stained leaf number 5 (to analyse possible tissue / cell phenotype in epinastic mutants).

- Photobleaching experiments to test for *PKS* role in phot2-mediated avoidance response (Kasahara et al., 2002). Leaf blade hyponasty in the *phot2* mutant under extremely high light.
- Infloresence stem gravitropism (Fukaki et al., 1996).
- Tropism experiments of on horizontal agar plates (percent of hypocotyls detached from agar surface under different light treatments).
- Effect of PKS2 loss-of-function in *pgp19-101* background.
- Root gravitropism of *aux1-22* and *aux1-22pks2-2.*
- Leaf positioning complementation assay (quantification of petiole angles) in *PKS2pro::PKS2-GFP pks2-2* lines.

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