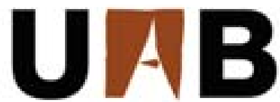

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Unraveling New Roles and Substrates for Protein Kinase CK2 in *Arabidopsis thaliana*

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Summary

This thesis is part of a research project that aims to study the role of the serine/threonine protein kinase CK2 in plant development, using Arabidopsis thaliana as a model. Despite being one of the first kinases identified, the signaling pathways in which CK2 is involved are not yet fully characterized.

The first part of this thesis describes the involvement of CK2 in the signaling pathway of salicylic acid (SA), and the control exercised by this hormone in the expression of genes coding for auxin membrane transporters (the PIN proteins) and their regulatory kinase PINOID (PID). Former members of the group where this thesis was carried out had obtained a dominant negative mutant of CK2 (CK2mut plants). These plants showed altered root phenotypes (decrease of the main root length and absence of lateral root formation) and changes in the transcription levels of genes encoding several of the PIN proteins (PIN1-PIN4 and PIN7) and of the kinase PINOID (PID) (Marques-Bueno et al., 2011a). Here, we show that CK2mut plants contain high levels of salicylic acid, which are responsible for the root phenotype of CK2mut plants. We also demonstrate that treatment of Arabidopsis wild-type plants with exogenous SA inhibits the transcription of genes coding for proteins PIN1-PIN4 and PIN7, while it stimulates the transcription of the PID encoding gene. This effect is similar to that observed in roots of CK2mut plants, except for PIN4 and PIN7 genes, which are overexpressed, suggesting that the repressive effect of SA on PIN4 and PIN7 expression requires a functional CK2. Moreover, SA stimulates the expression of CK2 subunits, whereas the loss of CK2 activity in CK2mut plants produces an increase in the transcript levels of genes related to SA biosynthesis. We propose the existence of a negative feedback loop between CK2 and SA, needed to maintain the homeostasis of SA. This chapter also shows that overexpression of a catalytically active α subunit of CK2 improves the root system of Arabidopsis plants.

The second part of this thesis focuses on the searching and characterization of plant CK2 substrates. For this purpose, we performed a large scale yeast two-hybrid screen that resulted in the identification of 28 potential CK2 substrates. Among them, we found four members of the same protein family, called NPH3/RPT2 (NRL), including NPH3, the founder member of the family. NPH3 is an essential element of the phototropic signaling pathway, and its activity in this pathway depends on its phosphorylation state and on its role as a substrate adapter within the Cullin3-Ring E3 ligase ($CRL3^{NPH3}$) ubiquitination complex. $CRL3^{NPH3}$ ubiquitinates the membrane-associated blue light photoreceptor phototropin 1. In the dark, NPH3 is phosphorylated and inactive, while in light conditions it is defosforilated and active and directs ubiquitination of phot1. Recently, it has been proposed that ubiquitination of phot1 promotes its internalization from the plasma membrane into the cytoplasm. Here we show that CK2 phosphorylates NPH3 in vitro, and that CK2 activity is required for the in vivo NPH3 phosphorylation in darkness. In addition, phosphorylation of NPH3 by CK2 is important to keep the protein inactive. Moreover, we observe that the lack of CK2 activity causes internalization of phot1 even in darkness, which could be responsible for the aphototropic phenotype of plants without CK2 activity. This internalization is, however, independent of the presence of NPH3 and therefore independent of ubiquitination. Surprisingly, internalization of phot1 observed in light conditions is also independent of the presence of NPH3.

Introduction

Cells must constantly react and adapt to changes in their environment and/or intracellular homeostasis. External and internal stimuli are detected by membrane-bound and/or cytoplasmic receptors, which transfer the signals to intracellular regulatory proteins. Posttranslational modifications of proteins are often responsible for the transmission and modulation of these signals. One of the most important post-translational modifications is phosphorylation. It constitutes one of the basic mechanisms of molecular signalling (Hunter, 2000), involved in almost every aspect of cell physiology. In particular, phosphorylation-dependent protein interactions are vital for transducing signals intracellularly. Phosphorylation can also produce changes in the subcellular location of a protein, modulate protein stability and turnover (i.e. create a phosphodegron, leading to ubiquitin-dependent protein degradation) or modulate (activate or reduce) the activity of a substrate protein (Hunter, 2012).

The phosphorylation status of a protein at any given time is the result of the antagonistic action of two types of enzymes: protein kinases and protein phosphatases. Protein kinases catalyse the transfer of the γ-phosphoryl group from ATP (or GTP) to specific residues within a protein substrate, mostly to serine, threonine, tyrosine or histidine residues. By means of phosphopeptide analysis of human cells, it has been shown that phosphorylation on Ser, Thr and Tyr residues occurs at a ratio of 88:11:1, respectively (Olsen et al., 2006). The phosphate groups are hydrolised from the protein substrate by Ser/Thr phosphatases, Tyr phosphatases or dual-specificity phosphatases. In general, phosphatases have been considered as promiscuous enzymes, showing little specificity for their target substrates, however, complex protein interaction networks have been shown to modulate the subcellular compartmentalization and substrate docking of eukaryotic protein phosphatases (DeLong, 2006; Sacco et al., 2012).

Comparison of the sequences and estructures of the catalytic domains of eukariotic protein kinases lead to their initial classification in two groups: serine/threonine kinases and tyrosine kinases (Hanks et al., 1988). The actual accepted classification of eukaryotic protein kinases (Manning et al., 2002), divides this superfamily into two groups: the ''conventional'' protein kinases, wich includes the previosly classified S/T kinases and Tyr kinases (Hanks and Hunter, 1995), and the ''atypical'' protein kinases, a small group of kinases that do not share clear sequence similarity with the conventional kinases, but have experimentally tested protein kinase activity. Histidine kinases are not included in any of these classifications.

Plant protein kinases are involved in a wide range of signalling pathways such as perception of biotic agents, light quality and quantity, plant hormones, and a variety of abiotic growth-limiting conditions. In addition, they also function in circadian rhythms, cell cycle regulation, modulation of vesicle transport and of channel activities, and regulation of cellular metabolism (see (Lehti-Shiu and Shiu, 2012)). Genome analysis of the model plant Arabidopsis thaliana (Arabidopsis) revealed that the number of genes encoding protein kinases amounts to 4% of its whole genome (Champion et al., 2004). A recent phylogeny of Arabidopsis kinases (excluding histidine kinases) showed a clear division into two major clades. Around 60% of all protein kinases belong to the large superfamily of receptor kinases (RLK), while the remaining 40% fall into the clade of soluble kinases, which contain the most prominent eukaryotic kinase families involved in intracellular signal transmission cascades (Zulawski et al., 2014). The large number of receptor-like kinases is not a unique feature of Arabidopsis, but it seems to be a characteristic of angiosperm plants that may have contributed to their adaptative evolution (Lehti-Shiu and Shiu, 2012). Moreover, plant kinomes are, in general, significantly larger than those of other eukaryotes (i.e: protein kinase encoding genes of Arabidopsis correspond to about 4% of the genome, while in yeast is ~1.8% and in Homo sapiens is ~1.7%), likely as a result of successive rounds of gene and genome duplications. This functional redundancy plays an important role in plant genetic robustness (Champion et al., 2004; Lehti-Shiu and Shiu, 2012). Due to the sessile nature of plants, a plausible hypothesis to explain the large amount of existing plant kinases is that plants must constantly integrate a huge number of environmental and intracellular signals necessary to adapt their morphogenesis and physiology to new or changing environmental constraints.

Additionally, it is estimated that aproximatly 30% of the nuclear encoded proteins of Arabidopsis thaliana require phosphorylation (Champion et al., 2004). The authors concluded that each one of the thousand predicted kinases present in Arabidopsis should

phosphorylate an average of seven proteins. They also suggested that this figures might be an underestimation of the real situation because (1) many proteins are phosphorylated at multiple sites; (2) some kinases have overlapping activities; and (3) some kinases phosphorylate only one or few substrates (Champion et al., 2004). Thus, in order to phosphorylate so many proteins it is necessary the existence of kinases capable of phosphorylating multiple substrates. One example of such kinase is protein kinase CK2.

I. Protein kinase CK2

General features

Protein kinase CK2 (formerly misnamed casein kinase 2 or II) is a Ser/Thr kinase present in all eukaryotes with a high degree of structural and functional conservation. Protein CK2 is considered one of the most pleiotropic members of the eukaryotic protein kinase superfamily. It is estimated that human CK2 is responsible for the generation of ~24% of the phosphoproteome and ~10% of the phosphosecretome (Venerando et al., 2014). But, although CK2 was one of the first protein kinases discovered in animals (Burnett and Kennedy, 1954), the biological role of CK2 is still far from being fully characterized. Most of the known CK2 substrates are proteins involved in signal transduction, gene expression and other nuclear functions (Meggio and Pinna, 2003). Concordantly, CK2 is involved in many cellular processes, such as proliferation, neoplasia, transcriptional control and apoptosis (reviewed in (Ahmed et al., 2002; Litchfield, 2003; Meggio and Pinna, 2003; Filhol and Cochet, 2009; St-Denis and Litchfield, 2009)). In plants, CK2 has been studied in several species such as maize (Riera, Peracchia, de Nadal, et al., 2001), tobacco (Espunya et al., 1999; Salinas et al., 2001), Arabidopsis (Espunya and Martínez, 1997; Sugano et al., 1999; Salinas et al., 2006), wheat (Kato et al., 2002), mustard (Ogrzewalla et al., 2002) and broccoli (Klimczak and Cashmore, 1994) and, although the number of identified substrates of plant CK2 is smaller than that of animals, CK2 has been involved in the regulation of several important pathways. Among them, housekeeping processes such as protein translation, chromatin structure, and cell cycle (Dennis and Browning, 2009; Moreno-Romero et al., 2011; Moreno-Romero et al., 2012). Additionally, CK2 activity modulates responses to various hormones and stresses (Marques-Bueno et al., 2011a; Mulekar et al., 2012), as well

as light signalling and circadian rhythms (Sugano et al., 1999; Bu, Zhu, Dennis, et al., 2011), the latter being an evolutionary conserved role of CK2 (Mizoguchi et al., 2006).

The amino acid consensus sequence required for CK2 phosphorylation is characterized by the presence of multiple negatively charged residues downstream from the serine or threonine (with preference for Ser residues). The minimal consensus sequence is X-S/T-X-X-E/D/pS, where X represents any non-basic amino acid residue (Meggio and Pinna, 2003). The presence of a negative charge (which can be borne by a glutamic, aspartic, or a phosphorylated amino acid residue) at position n+3, and to a lesser extent at positions n+1 and n+2, is crucial for CK2 phosphorylation. An additional requirement is the absence of a proline residue at position n+1. A basic region located within the catalytic domain of CK2α is responsible for the substrate recognition, and this explains the acidic nature of the CK2 phosphorylation sequences. Although less studied, the ability of CK2 to phosphorylate tyrosine residues was initially reported in *Saccharomices cerevisiae* (Wilson et al., 1997) and later on in mammals (Vilk et al., 2008). Moreover, autophosphorylation of CK2 in Tyr residues has also been reported (Donella-Deana et al., 2001). For this reason, CK2 must be classified as a protein kinase with dual-specifity. Tyr phosphorylation by CK2 can occur within a typical CK2 consensus sequence, but using peptide arrays it has been demonstrated that the minimal Tyr phosphorylation consensus sequence is X-Y-D, where X represents any hydrophobic residue (Vilk et al., 2008). The in silico substrate prediction analyses available are based on the information for mammals and yeast consensus sequences; the comparatively small set of CK2 substrates and phosphopeptides known in plants difficults the establishment of plant CK2 consensus sequences and the study of specific requirements (if any) of plant CK2 phosphorylation.

Protein kinase CK2 has some characteristic features that make it distinguishable from the rest of the kinases. One of them is the ability to use both ATP and GTP as phosphate donor, with similar Km (Dobrowolska et al., 1999). Another distinguishing feature (somehow responsible of CK2 high pleiotropy) is its constitutive activity, a phenomenon that results from its particular structure (see below). Moreover, CK2 activity is independent of classical secondary messengers such as cyclic nucleotides, Ca2+ or phosphatidylinositol. Nonetheless, CK2 activity is not deregulated, but several mechanisms act to modulate it and to confere differential specificity against its substrates. For instance, CK2 activity is stimulated by polycations such as polylysine or polyamines, and it is reduced by polyanions notably by heparin (Tuazon and Traugh, 1991).

CK2 structure

The typical molecular structure of CK2 is an heterotetramer of 130-150 kDa (Figure 1), composed of two catalytic subunits (CK2α, Mr = 36-44 kDa) and two regulatory subunits (CK2β, Mr = 24-26 kDa) (Litchfield, 2003). The structure of human CK2 heterotetramer was inferred from crystallographic analysis (Niefind et al., 2001) and showed that the four subunits form a butterfly-shaped structure, composed by a central pre-stablished β-β dimer, which acts as a stable core for the binding of the two CK2α subunits. The two α subunits interact with the C-terminal domain of each one of the β monomers. These domains are the most distally located in the dimer, therefore, the two alpha subunits do not interact with each other (Figure 1a) (Niefind et al., 2009).

It has been proposed that the CK2 holoenzyme is a transiently formed and dinamic heterocomplex (Niefind et al., 2001; Filhol et al., 2004) and thus, that several subpopulations of CK2 monomers and tetramers coexist within the cells (Filhol et al., 2004; Olsten and Litchfield, 2004). Additionally, both the catalytic and the regulatory subunits have been found associated with polypeptides other than their CK2 counterparts and, in some cases, the functional significance of these associations has been demonstrated (Bibby and Litchfield, 2005). The presence of the CK2α monomeric form is more common in plants than in animals. It has been isolated from several plant species such as Arabidopsis, wheat, maize and broccoli (Yan and Tao, 1982; Dobrowolska et al., 1992; Klimczak and Cashmore, 1994; Espunya and Martínez, 1997).

The catalytic subunit

The catalytic CK2α subunit is highly evolutionary conserved in eukaryotes, suggesting its involvement in crucial processes for cell viability. This fact was demonstrated in *Saccharomices cerevisiae*, where deletion of the two genes encoding the CK2α subunit was lethal, and the phenotype could be rescued by transformation with the CK2α subunit from human or from *Caenorhabditis elegans* (Padmanabha et al., 1990). Based on sequence similarities, the CK2α protein has been grouped within the CMGC (for cyclin-dependent kinases (CDK), mitogen-activated kinases (MAPK), glycogen synthase kinase-3 (GSK-3) and CK2) family of the eukaryotic protein kinase superfamily (Miranda-Saavedra and Barton, 2007). The members of this family are characterized by the presence of an small helixα insertion within their C-terminal domain (Kannan and Neuwald, 2004). In a recent classification of the Arabidopsis kinome, CK2α falls into the cluster of soluble kinases

(Zulawski et al., 2014). The amino acid conservation in CK2α from different plant species is often >90%, while when plant CK2α is compared to that from animals and yeast the sequence similarity decreases until values of 50% (Espunya et al., 2005).

The three-dimensional structure of CK2α was first obtained from the protein purified from *Zea mays* (Niefind et al., 1998). The structure was resolved by X-ray crystallography in the presence of ATP and Mg2+ (Figure 1b), and it allowed the interpretation of some unique features of CK2. The general structure of CK2α presents the basic characteristics of other protein kinases: an amino-terminal domain rich in β sheets, a carboxy-terminal domain rich in α helix and, the catalytic active site between both domains. However, important structural differences with other kinases explain the particular enzymatic characteristics of CK2α. Some of these differences are found in the activation segment and in the αC helix of the N-terminal domain. In most eukaryotic protein kinases, conformational changes in the activation segment determine the ON/OFF state of their catalytic activity. In CK2α, these two key regulatory elements adopt always the conformation of the active state (Niefind et al., 2009), which is mostly maintained by the intramolecular interaction between the N-terminal segment, the activation segment and the αC helix (Niefind et al., 1998). Moreover, this interaction seems important for the full activation of CK2α (Sarno et al., 2002). The three-dimensional structure in Figure 1b shows the stabilizing contacts between the N-terminal segment (blue), the activation segment (yellow) and the αC helix (red). Another distinct feature of CK2α is the absence of phosphorylation in its activation segment, in contrast to what occurs in its closest-related kinases (CDKs, MAPK and GSK-3). In these kinases, phosphorylation of a conserved Thr and Tyr residue in the activation segment produces a conformational change that stabilizes the proteins and switch them to their active form (Niefind et al., 2009).

Another peculiarity of CK2α is the presence of a cluster of basic residues (a lysine-rich region) located at the beginning of the αC helix of the N-terminal domain, which is involved in the recognition of substrates and regulatory proteins (Roher et al., 2001), and determines the acidophilic character of this enzyme (Sarno et al., 1996). This Lys cluster is also involved in the inhibition of CK2 by heparin (Vaglio et al., 1996). Furthermore, this region may contain a potential nuclear localization sequence (NLS). Close in space, face to face with this cluster, is located the Lys68 residue, which is essential for ATP binding. Deletion of Lys68 produces total inhibition of CK2α activity. Additionally, Phe176, wich is a conserved in most of the kinases, has been substituted to Trp 176 in the CK2α polipeptide chain. This substitution has been associated with the ability of the enzyme to use both ATP and GTP as

phosphate donors (Niefind et al., 1998), and in the maintenance of a rigid "open" active state of the catalytic domain (Battistutta, 2009).

Contrary to the tightly uphold open conformational state of Zm-CK2α, human CK2α subunit contain a partially flexible interdomain region (or hinge region) that can result in an intermediate "inactive" form, due to its movement into the ATP binding site (Niefind et al., 2001; Niefind et al., 2009).

The knowledge acquired from the elucidation of the CK2α structure has allowed to design specific inhibitors of this protein (Niefind and Issinger, 2010), which are very good tools in the research of CK2 functions and became of special importance for human health due to the anti-apoptotic nature of CK2 and the high levels of expression found in many different cancer types.

The regulatory subunit

The regulatory subunit of CK2 (CK2β) also presents a high degree of conservation through evolution, but to a lesser extent than CK2α subunit (Maridor et al., 1991; Boldyreff et al., 1993). Additionally, CK2β has no sequence similarity with any other known protein, except with its functional homolog protein Stellate found in *Drosophila melanogaster* (Bozzetti et al., 1995; Egorova et al., 2009). The cristallographyc resolution of the CK2β structure (as found in the human CK2 holoenzyme) showed that this subunit is composed by two distinguishable domains, one rich in α helix and the other in β sheets, and by two unstructured tails, one at the N-terminal and the other at the C-terminal site of the protein (Figure 1c) (Niefind et al., 2001). A Zn2+ atom is coordinated to four cysteine residues forming a Zinc finger domain, which is responsible for the formation of the dimer CK2β-CK2β stabilized by hydrophobic interactions. The N-terminal tail contains an acidic domain which become distantly exposed in the tetrameric structure, and that can interact with polyamines and/or regulatory proteins of CK2 (Chantalat et al., 1999). Close to it, a sequence with homology to the cyclins destruction box is located, which could regulate the stability of the protein (Bibby and Litchfield, 2005). In the C-terminal side, a loop rich in basic residues is involved in the interaction with the N-terminal domain of the catalytic subunit CK2α (Niefind et al., 2009).

Although evolutionary conserved, sequence similarities between plant β subunits is around 70%, but it is only less than 40% when plant sequences are compared to those from yeast or human (Espunya et al., 2005). These differences can be explained by several

distinctive features found in plant β subunits: i) an extra N-terminal extension of about 90 aminoacids, not found in the β subunits of other species; ii) a shorter C-terminus (about 20 amino acids less); and iii) an acidic loop at the N-terminal tail that is poorly conserved at the aminoacid level (Riera, Peracchia and Pagès, 2001; Velez-Bermudez et al., 2011). The extra N-terminal extension shares no homology with any previously characterized functional protein domain. It contains several conserved motifs, such as two clusters of glycines of variable length and several autophosphorylation consensus sequences (Espunya et al., 2005). To date, no tridimensional structures of plant β subunits, neither of CK2 holoenzymes are available, which could allow us to infer the functional specialization of the distinctive features of plant CK2 subunits.

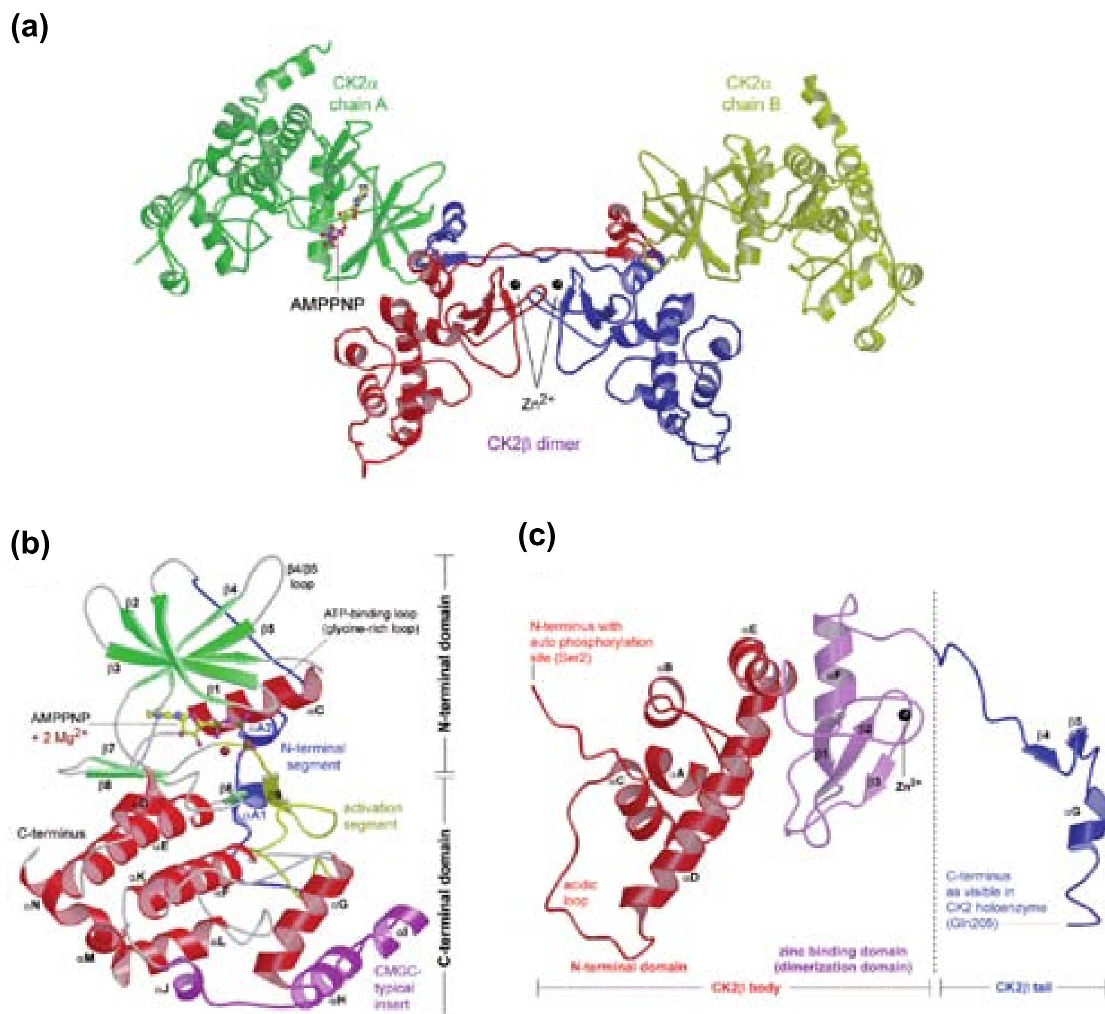

Figure 1. Tridimensional structure of protein kinase CK2.
(a) Structure of the CK2 holoenzyme complex. **(b)** Structure of the CK2α catalytic subunit from *Zea mays* in complex with AMPPNP and magnesium ions. **(c)** Architecture of CK2β subunit, extracted from the structure of the human CK2 holoenzyme. Modified from Niefind et al., (2009).

Regulation of protein kinase CK2 activity

As previously mentioned, the catalytic subunit of CK2 is constitutively active but not deregulated (Litchfield, 2003). A model has been proposed, in which the equilibrium between different conformational states of CK2α would modulate its catalytic activity (Olsen et al., 2010). According to this model, three different CK2α forms can exist inside the cell: an active state (inferred from structure analysis), a partially inactive state (also inferred from structure analysis) and an inactive state. The inactive state would be stabilized by small ligand molecules (still unidentified), while the active state is stabilized by interaction with the CK2β dimer. Recently, new insights about the so-called “regulation by aggregation” of CK2 were obtained, proposing that high-order interactions between CK2 tetramers might play a regulatory role in modulating CK2 activity (Pagano et al., 2005; Schnitzler et al., 2014).

In addition to stabilizing the CK2α subunit, the presence of the CK2β subunit affects two other functional characteristics: the activity and the specificity of CK2 (Allende and Allende, 1995). In most cases, formation of the tetrameric holoenzyme increases the CK2α enzymatic activity; one exception is the substrate calmodulin, wich is most efficiently phosphorylated by the CK2α monomer (Meggio et al., 1994). Moreover, the dimeric β complex interacts with several partners other than CK2α (Litchfield, 2003; Arrigoni et al., 2008), thus, it has been suggested that CK2β acts as a docking platform for substrates and effectors (Bibby and Litchfield, 2005).

Beside the regulation of CK2 catalytic activity per se, another intriguing question is the regulation of substrate specificity. Regarding this, the interaction with CK2β constitutes one step in stablishing specificity; however, additional steps must exist. One to be considered is the existence of different isoforms of CK2, due to the existence of multiple genes encoding each one of the two subunits. In the human genome, two genes encoding the α subunit, which give rise to three protein isoforms (α,α’ and α’’), and one gene encoding the regulatory subunit, have been identified (Lozeman et al., 1990; Allende and Allende, 1995; Shi et al., 2001). In plants, both CK2 subunits are often encoded by multigene families, thus, increasing the number of possible tetrameric combinations. In the Arabidopsis genome, four genes coding for the alpha subunit (αA, αB, αC and αcp) and four genes coding for the beta subunit (β1, β2, β3 and β4) have been identified (Sugano et al., 1998; Lee, 1999; Riera, Peracchia and Pagès, 2001; Espunya et al., 2005).

Another mechanism to achieve substrate specificity is the regulation of intracellular localization. In animals, CK2 activity has been detected in the nucleus, cytoplasm, plasma membrane, lipid rafts, clathrin-coated vesicles, mitochondria, endoplasmic reticulum, cytoskeleton and centrosomes (Faust and Montenarh, 2000; Korolchuk and Banting, 2002; Gil et al., 2011). In Arabidopsis, the catalytic subunits αA, αB and αC are located in the nucleus, predominantly in the nucleolus, and the αcp subunit is only located in the chloroplast. On the other hand, the regulatory subunits β1 and β3 are located in the nucleus and in the cytosol, the β2 subunit is only located in the nucleus and the β4 only in the cytosol (Salinas et al., 2006). The existence of multiple CK2α/β isoforms suggests that the differential expression of these subunits, together with their variable localization in the cell compartments, can contribute to the regulation of CK2 functions (Faust and Montenarh, 2000). Indeed, specific functions have been associated to each plant isoform (Riera, Peracchia and Pagès, 2001). In Arabidopsis, only the β3 and β4 regulatory subunits appear to be involved in circadian-clock regulation (Sugano et al., 1999; Perales et al., 2006) and specific CK2 holoenzymes present differential substrate specificity (Dennis and Browning, 2009). In tobacco, it has been reported a differential expression of genes encoding protein kinase CK2 subunits during the cell cycle (Espunya et al., 2005). In maize, a differential expression of CK2β subunits during embryogenesis has been reported, whereas all the CK2α subunits show a similar pattern of expression (Riera, Peracchia, de Nadal, et al., 2001).

The existence of several subpopulations of CK2 within a cell has been suggested (Bibby and Litchfield, 2005). There are evidences of free monomeric catalytic and regulatory subunits of CK2 in animals and plants, as well as of their involvement in functions independent from those associated to the CK2 heterotetramer. The dinamical interconvertion between the CK2 holoenzyme and the CK2 monomers might constitute another level of regulation of CK2 activity and specificity. Additional regulatory mechanisms, such as phosphorylation or protein-protein interactions, are necessary to modulate the balance of CK2 subpopulations and CK2 substrate specificity (Filhol et al., 2004). Some of these mechanisms are briefly described below and summarized in Figure 2.

Regulation by phosphorylation. It is not yet clear whether phosphorylation/dephosphorylation of CK2 subunits is one of the mechanisms for the regulation of its activity and/or specifity. In mammals, CK2α and CK2β subunits can be both autophosphorylated or phosphorylated by other kinases in a cell-cycle dependent manner,

but the functional significance of these phosphorylation events remains elusive. Moreover, CK2α inactivation could be mediated by trans-autophosphorylation of Tyr residues present in the activation segment of this subunit (Donella-Deana et al., 2001). When compared to animal CK2β, Arabidopsis and maize β subunits contain additional autophosphorylation sites, most of them located in the N-terminal region (Espunya et al., 2005). In Arabidopsis, autophosphylation of the CKβ4 subunit produces its degradation by the 26S proteasome pathway (Perales et al., 2006). Additionally, phosphorylation of CK2 subunits can modulate the interaction with other proteins, which modulate its activity or subcellular localization (Messenger et al., 2002). More experimental data are still necessary to take conclusions about whether or not CK2 activity is regulated by phosphorylation. What it became clear is that the action of CK2 does not appear to be "vertical" in the phosphorylation cascade, contrary to what occurs for most of kinases. However, CK2 is susceptible to hierarchical, or 'primed', phosphorylation and, thus, to regulation by phosphorylation, at least, at the substrate level (Meggio and Pinna, 2003).

Regulatory interactions. The activity of CK2 is independent of known secondary messengers. However, it has been recently reported that CK2 activity can be regulated by phosphatidylinositol phosphates in clathrin-coated vesicules (Korolchuk et al., 2005). Moreover, interaction with compounds with negative charges, such as heparin, inhibit CK2 activity in vitro, while compounds with positive charges, such as polyamines, act as activators of CK2 activity (Tuazon and Traugh, 1991). Studies with purified CK2 from Arabidopsis demonstrated that the presence of polycations (polylysine, polyarginina and protamine) caused a shift in the incorporation of phosphate from the CK2β subunit to the CK2α, whereas the presence of polyamines (spermine and spermidine), produced an increase in the incorporation of phosphate to the CK2β subunit (Espunya and Martínez, 1997).

Protein-protein interactions. Interactions of CK2 with other proteins may contribute to the regulation of CK2 activity. These proteins are not necessarily CK2 substrates, but the interaction with them may stabilize or alter CK2 activity. It is also plausible that binding to certain proteins can modulate the subcellular localization of CK2 subunits. Some studies performed in animals have shown that CK2 interacts with proteins such as tubulin, which may target CK2 to specific locations or intracellular structures (Litchfield, 2003). In plants, a possible example of this type of regulation can be found in the interaction of CK2 with nuclear matrix proteins (Meier et al., 1996; Samaniego et al., 2006). These proteins, in addition to having a structural role, could provide a platform for intranuclear

protein-protein or protein-nucleic acid interactions, and thus participate in the organization of replication and transcription (Ahmed et al., 2000).

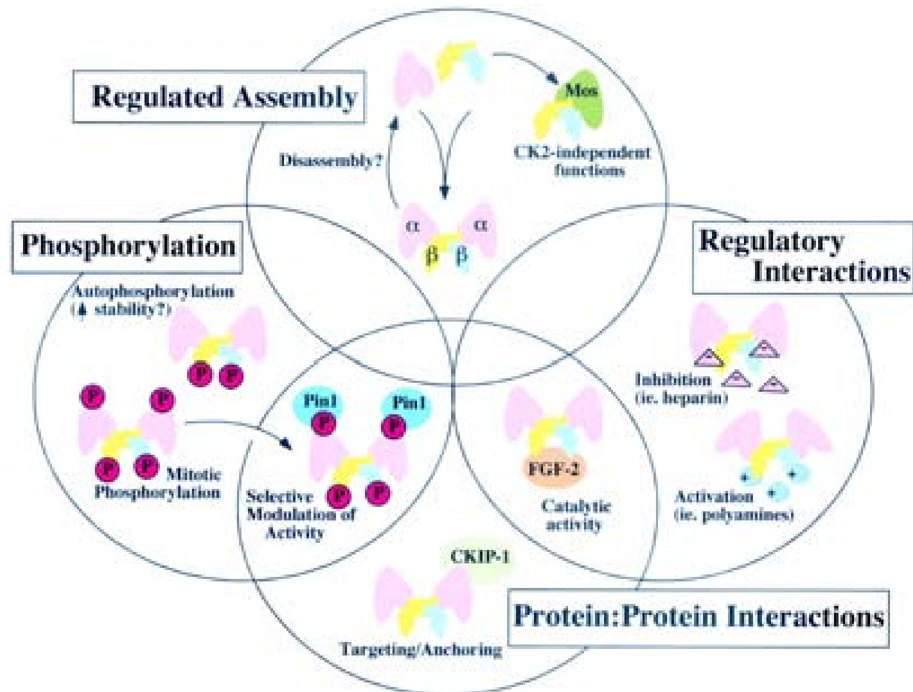

Figure 2. Possible mechanisms of regulation of CK2 activity and CK2 substrate specificity.
Extracted from Litchfield (2003).

Strategies to study protein kinase CK2, Substrates and functions

Inhibition of CK2 as a strategy to study CK2 functions

I. Chemical inhibition of CK2

To date, two types of CK2 inhibitors have been described: the classical ATP-competitive inhibitors, and the non-ATP-competitive inhibitors. The latter ones bind to the "remote cavity" formed in the flexible hinge region of the human CK2α subunit. However, the most used and studied inhibitors belong to the ATP-competitive type.

One of the earliest described CK2 inhibitors of the ATP-competitive type was DRB, (5,6-dichloro-1-(β-D-ribofuranosil)-benzamidazol) (Zandomeni et al., 1986) but it was later shown that this compound inhibits with the same efficiency both CK2 and CK1, and also other kinases (Meggio et al., 1990). A long list of this type of CK2 inhibitors have been described (reviewed in (Battistutta, 2009; Cozza et al., 2012)), which fall into a few classes: anthraquinones, xanthenones, indoloquinazolines, coumarins, flavonoids, tyrphostins and pyrazolotriazines derivatives. However, some of these inhibitors have a low inhibitory capacity or are not specific of CK2 (Sarno et al., 2003; Pagano et al., 2008). With the exception of the anthraquinone quinalizarin which is one of the most specific inhibitor of CK2 (Cozza et al., 2009; Pagano et al., 2008).

Currently, the most used ATP-competitive CK2 inhibitors belong to the class of tetralogenobenzo-imidazoles and are derived from DRB. They were obtained by substituting the DRB radicals with bromur atoms (see Figure 3a), giving rise to TBBz (tetrabrombo-benzimidazoles) and TBB (4,5,6,7-tetrabromobenzatriazol). Both TBBz and TBB are stronger and more specific than DRB. Later on, the diethylamino derivative of TBBz, the DMAT, was obtained, which is more powerful than TBBz and TBB. These three compounds are highly selective because they were designed considering that the hydrophobic pocket adjacent to the ATP binding site is much smaller in CK2 than in other kinases (Battistutta et al., 2001). Studies with these three inhibitors revealed that the most effective and selective on CK2 activity was TBB (Sarno et al., 2001; Pagano et al., 2008).

Part of the results presented in this work have been obtained using TBB as a specific inhibitor of the catalytic subunits of Arabidopsis CK2. Previous studies performed in the lab, showed that inhibition with TBB produced similar effects than genetic inhibition of CK2.

II. Genetic inhibition Substrates and functions

Several genetic strategies have been used to study the in vivo function of protein kinase CK2. Reduction of the intracellular levels of CK2α has been performed by means of antisense RNA and siRNAs, and by generation of knockout mutants and of catalytically inactive mutants. However, interpretation of the results obtained with these strategies should be made with caution, due to the following aspects: i) CK2 has a relative high level of expression in the cell, and its activity is “constitutive” (Ruzzene and Pinna, 2010), thus, a remaining basal level of endogenous CK2 expression and activity may exist; ii) the partial functional redundancy between the different CK2 isoforms, implying that loss of activity in

one isoform can be compensated by the others; for instance, a mouse knockout in one of the CK2α subunits gave rise to an sterile but viable animal (Xu et al., 1999), and disruption of both catalytic subunits in *S. cerevisiae* is necessary to have a lethal phenotype (Padmanabha et al., 1990).

Taking into account all this information, former members of the lab obtained a dominant negative mutant of CK2 in Arabidopsis thaliana, which has been used to gain new insights into the role of CK2 in plant systems, particularly Arabidopsis development. This strategy, previously used in mammalian cells, is based on the elimination of CK2 activity by overexpression of a catalytic inactive CK2α subunit. This subunit can still interact with β subunits and with CK2 substrates, thus competing with the endogenous active α subunits and producing and overall reduction of CK2 activity (Vilk et al., 1999; Lebrin et al., 2001). The catalytic inactive α subunit was obtained by site-directed mutagenesis of the CKA3 gene of *Nicotiana tabacum* (GeneBank / EMBL accession no. AJ438263), substituting lysine K63 by methionine (Espunya et al., 2005; Moreno-Romero et al., 2008). This substitution abrogates the phosphotransferase activity of the kinase. Moreover, to prevent the lethal effects of constitutive expression of the transgene, the mutated open reading frame (CK2mut) was cloned under a dexamethasone (Dex) inducible promoter. Dex, an analogue of glucocorticodes, binds to the chimeric GVG transcription factor (Aoyama and Chua, 1997), which DNA coding sequence is in the same construct. The GVG is constitutively expressed in plant cells and accumulates in the cytoplasm, but, as other steroid receptors, it migrates to the nucleus after binding to the inductor (Dex). In the nucleus, it recognizes the Gal4 6xUAS promoter and activates the expression of CK2mut trangene. The above construction was introduced in the genome of Arabidopsis by stable transformation with Agrobacterium tumefaciens and, posteriorly, homozygous plants were selected. A scheme of the dominant negative mutant line obtention (thereafter, CK2mut line) is depicted in Figure 3b.

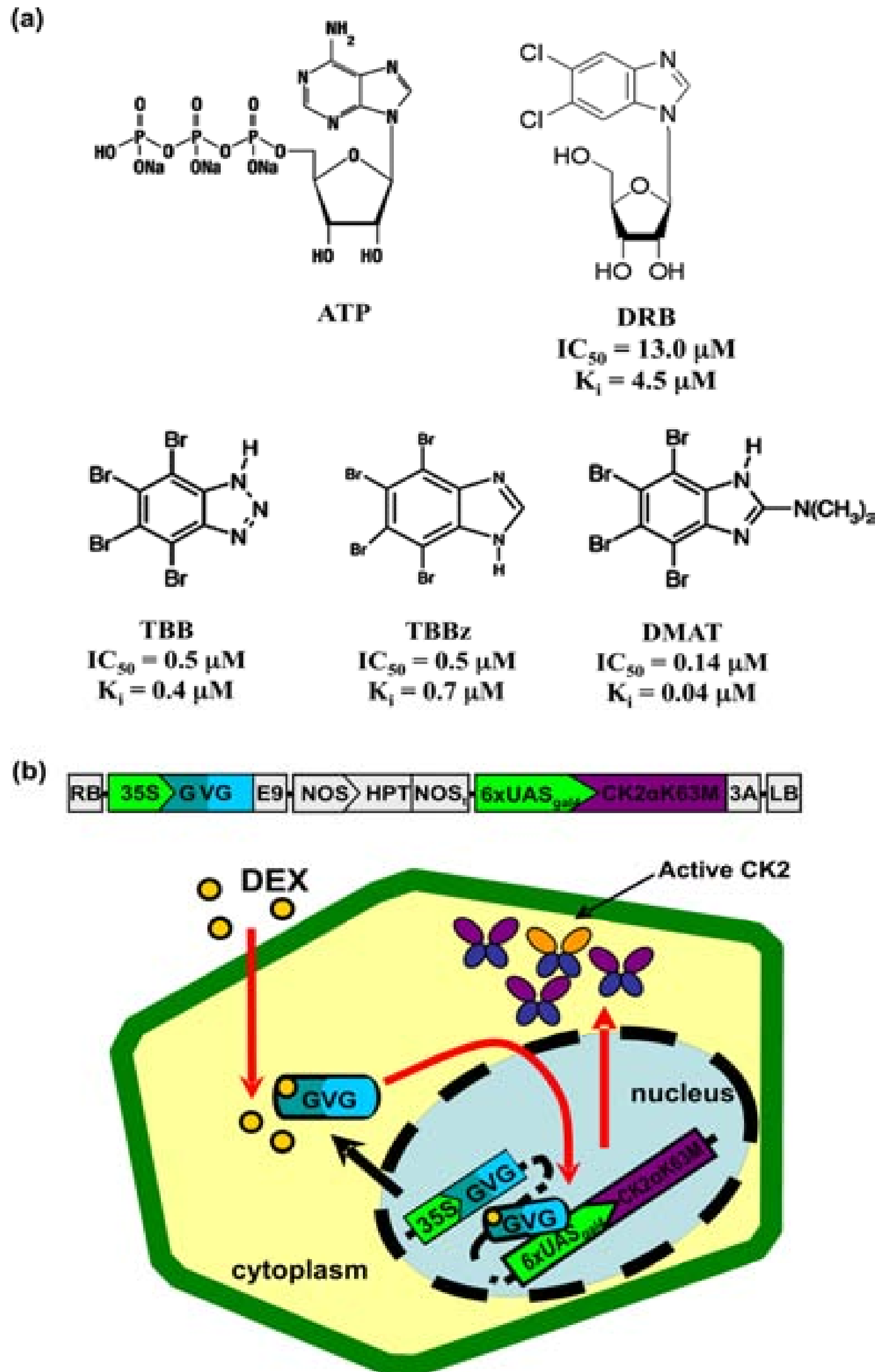

Figure 3. Strategies used to inhibit CK2 activity.
(a) Chemical structure of ATP and some of the commercially available ATP-competitive CK2 inhibitors (DRB and its derivates). Extracted from (Duncan et al., 2008). **(b)** Scheme of the construct used to transform Arabidopsis plants, and working mechanism of the dominant negative mutant (CK2mut).Abbreviations: R, right end of the T-DNA; 35S, 35S promoter; GVG, chimeric transcription factor inducible by dexamethasone (DEX); E9, polyadenylation sequence of pea rbcS-E9; NOS, nopaline synthase promoter; HPT, hygromicine phosphotransferase; NOSt, polyadenylation sequence of the nopaline synthetase; 6XUASgal4, promoter regulated by GVG; CK2αK63M, tobacco CK2α subunit with the inactivating K63M mutation; 3A, polyadenylation sequence of Pea RBC-3 A; L, left end of the T-DNA.

CK2 Substrates and functions

In animals, more than 300 substrates of protein kinase CK2 have been described. However, this is likely a reduced list of the real number of possible CK2 substrates, because, as mentioned above, CK2 is one of the major kinases in the generation of mammalian phosphoproteome. For some of the substrates, the physiological effect of CK2 phosphorylation is known, but in most cases the only known information is the existence of the phosphorylation. In general, CK2 substrates can be classified as: (1) proteins that control DNA and RNA synthesis; (2) transcription and translation factors; (3) proteins involved in growth, proliferation and cellular differentiation; and (4) proteins involved in signal transduction pathways.

In plants, the number of identified CK2 substrates is much lower than in animals. Among them, we can find transcription factors involved in the transduction of photomorphogenic light signals and of circadian rhythms, transcription factors and storage proteins of the seeds, proteins of the basic machinery for DNA transcription and RNA translation, transcription factors and other proteins related to stress responses, proteins involved in the synthesis of ATP and lipids, structural proteins of the proteasome, nuclear matrix proteins and nucleoid proteins, enzymes involved in lysine catabolism, and viral proteins. Table 1 shows a list of all the CK2 substrates known to date in different plant species.

Table 1. Summary of the CK2 substrates identified from plants.
Modified from (Mulekar and Huq, 2013).

Name	Type of protein	Source	Role	Pathway	Reference
C2	Proteasome protein	Rice	Protein degradation	Proteasome machinery	(Umeda et al., 1997)
eIF2α	Translation initiation factor	Wheat, Arabidopsis	Enhance multifactor complex formation	Translation	(Dennis et al., 2009)
eIF2β	Translation initiation factor	Wheat, Arabidopsis	Enhance multifactor complex formation	Translation	(Dennis et al., 2009)
eIF3c	Translation initiation factor	Wheat, Arabidopsis	Enhance multifactor complex formation	Translation	(Dennis et al., 2009)
eIF4B	Translation initiation factor	Wheat, Arabidopsis	Enhance multifactor complex formation	Translation	(Dennis et al., 2009)
eIF5	Translation initiation factor	Wheat, Arabidopsis	Enhance multifactor complex formation	Translation	(Dennis et al., 2009)

eIF5A	Translation initiation factor	Maize	Nucleocytoplasmic shuttling regulation	Translation	(Łebska et al., 2010)
HD2B	Translation initiation factor	Wheat, Arabidopsis	Enhance multifactor complex formation	Translation	(Dennis and Browning, 2009)
p34	Ribonucleo-protein	Spinach chloroplast	mRNA 3'-end processing	Translation	(Kanekatsu et al., 1993)
p36	eIF-2 subunit	Wheat germ	Guanine nucleotide Exchange	Translation	(Janaki et al., 1995)
P-proteins	Ribosomal proteins	Maize	Complexes with 60S ribosomal subunits	Translation	(Hasler et al., 1991)
PDH65	DNA helicase	Pea	Opens duplex DNA during nucleic acid transactions	Transcription	(Tuteja et al., 2001)
SIG1	Plastid sigma factor	Mustard	Directs plastid RNA polymerase to promoter	Transcription	(Ogrzewalla et al., 2002)
SIG6	Plastid sigma factor	Arabidopsis	Directs plastid RNA polymerase to promoter	Transcription	(Schweer et al., 2010)
HMGd	High Mobility Group proteins	Broccoli	Chromatin-associated proteins	Transcription, recombination	(Klimczak et al., 1994)
HMGB1	High Mobility Group proteins	Maize	Chromatin-associated proteins	Transcription, recombination	(Stemmer et al., 2002)
HMGB2/3	High Mobility Group proteins	Maize	Chromatin-associated proteins	Transcription, recombination	(Stemmer et al., 2002)
SSRP1	Structure-specific recognition protein	Maize	Chromatin-associated proteins	Transcription, replication	(Krohn et al., 2003)
DNA topoisomerase I	DNA topoisomerase	Pea	Relaxation of superhelical DNA tension	DNA metabolism	(Tuteja et al., 2003)
Lamina-like	Lamina matrix protein	Pea	Lamina matrix protein	Nuclear matrix proteins	(Li and Roux, 1992)
MFP1	Lamina matrix protein	Tomato	Lamina matrix protein	Nuclear matrix proteins	(Meier et al., 1996)
90Kda MFP1	Lamina matrix protein	Onion	Lamina matrix protein	Nuclear matrix proteins	(Samaniego et al., 2006)
MFP1	MAR-binding filament-like protein 1	Tomato	DNA-binding coiled-coil protein	Component of thylakoid membrane and nucleoids	(Jeong et al., 2004)
TOC159	GTPases	Arabidopsis	Contact of preproteins with the chloroplast surface	Chloroplast protein import	(Agne and Kessler, 2010)
gp96	Lipoxygenase	Soybean	Oxygenation of unsatu- rated fatty acids	Lipid synthesis	(Ohtsuki et al., 1995)
CFOCF1-ATPase	ATPase synthase b subunit	Spinach chloroplasts	ATP synthesis	ATP synthesis	(Kanekatsu et al., 1998)
Apyrase	ATP hydrolysing enzyme	Pea	ATP hydrolysis	ATP synthesis	(Hsieh et al., 2000)
LKR/SDH	Metabolic enzymes	Maize	Enzimes involved in lysine catabolism	Lysine catabolism	(Stepansky et al., 2006)
Calreticulin	Ca^{2+}-binding protein	Spinach	Ca^{2+} metabolism	Seed storage	(Baldan et al., 1996)
Opaque2	bZIP transcription factor	Maize	Binds to promoter of zein seed storage protein gene family	Seed storage	(Ciceri et al., 1997)
β-conglycinin	Storage protein	Soybean	Storage protein	Seed storage	(Ralet et al., 1999)

aEPA	BBI-type proteinase inhibitor CII	Soybean	Inhibitor of Bowman-Birk-type Serine proteinase	Seed storage	(Katano et al., 2005)
EmBP-2	bZIP transcription factor	Maize	Bind to ABRE	ABA/stress-induced pathway	(Nieva et al., 2005)
Rab 17	LEA protein	Maize	Stress-induced protein	ABA/stress-induced pathway	(Plana et al., 1991)
TAS-14	LEA protein	Tomato	Stress-induced protein	ABA/stress-induced pathway	(Godoy et al., 1994)
ZmBZ-1	bZIP transcription factors	Maize	Bind to ABRE	ABA/stress-induced pathway	(Nieva et al., 2005)
TGA-2	bZIP transcription factor	Arabidopsis	Binds to promoters	Salicylic acid response	(Kang and Klessig, 2005)
p23	Co-chaperone protein	Arabidopsis	Chaperone ass ociates with Hsp90	Hormone signalling?	(Tosoni et al., 2011)
AT-1	Transacting factor	Pea	Binds to AT-rich promoter	Light signalling	(Datta and Cashmore, 1989)
ATBP-1	Transacting factor	Pea	Binds to AT-rich promoter	Light signalling	(Tjaden and Coruzzi, 1994)
GBF1	bZIP transcription factor	Broccoli	Binds to G-box promoter	Light signalling	(Klimczak et al., 1992)
HFR1	bHLH transcription factor	Arabidopsis	Promotes photomorphogenesis	Light signalling	(Park et al., 2008)
HY5	bZIP transcription factor	Arabidopsis	Promotes photomorphogenesis	Light signalling	(Hardtke et al., 2000)
PIF1	bHLH transcription factor	Arabidopsis	Represses photomorphogenesis	Light signalling	(Bu, Zhu, Dennis, et al., 2011)
CCA1	Myb-related transcription factor	Arabidopsis	Circadian clock regulator	Circadian clock	(Sugano et al., 1998)
LHY	Myb-related transcription factor	Arabidopsis	Circadian clock regulator	Circadian clock	(Sugano et al., 1998)
BaMV CP	Coat protein	*N.benthamiana*	Coat protein of Bamboo mosaic virus	Cell-to-cell movement of BaMV	(Hung et al., 2014)
ToMVMP	Coat protein	tobacco	Movement protein of tomato mosaic virus	Cell-to-cell movement of ToMV	(Matsushita et al., 2003)

Source species: Arabidopsis (*Arabidopsis thaliana*); Broccoli (*Brassica oleracea*); Maize *(Zea mays)*; Mustard *(Sinapis alba)*; *N. benthamiana* (*Nicotiana benthamiana*); Onion *(Allium cepa)*; Pea (*Pisum sativum)*; Rice *(Oryza sativa)*; Spinach *(Spinacia oleracea)*; Soybean *(Glycine max)*; Tobacco *(Nicotiana tabacum)*; Tomato *(Lycopersicon esculentum)*; Wheat *(Triticum aestivum)*.

Plant protein kinase CK2 had been extensively involved in light signalling and circadian rhythms, as well as in translation and cell cycle regulation (for a revision of CK2 functions, see (Mulekar and Huq, 2013)). A further involvement of CK2 in the cell cycle was reported in the CK2mutant line. It was found that inhibition of CK2 produced an arrest of the cell cycle at early G2 and/or G1/S phases (Moreno-Romero et al., 2008). These results were in concordance with previous observation in BY-2 tobbaco cells that CK2 subunits expression fluctuates during the course of the cell cycle, being higher in G1/S and M phases (Espunya et al., 1999). Additionally, tissues with high mitotic activity such as pollen and

meristem show a higher level of CK2 transcripts (Moreno-Romero et al., 2011; Espunya and Martínez, 2003).

In addition to cell-cycle analysis, the dominant negative mutant line of CK2 has allowed to study the involvement of CK2 in DNA repair (Moreno-Romero et al., 2012). It was shown that CK2mut plants are hypersensitive to a wide range of genotoxic agents, among them ionizing (γ) radiation, but, surprisingly these plants had and enhanced ability to repair double-strand DNA breaks produced by this type of radiation. Moreover, CK2mut plants showed deffects in the maintenance of genomic stability, which can account for their high sensitivity to DNA damaging agents.

Germination of CK2mut plants in Dex-containig medium produced severe developmental deffects (Moreno-Romero et al., 2008): depigmented and non-expanded cotyledons, broader and depigmented hypocotyl, and shorter roots. In dark grown seedlings, CK2mut plants showed a partially de-etiolated phenotype. These developmental defects could be explained, in part, by alterations in cell expansion and in cell-cycle progression. Moreover, prolonged induction of the CK2mut transgen was lethal confirming the vital nature of CK2 function in plants. Furthermore, carrying out transient inductions of the CK2mut transgene, plants showed defects in both lateral root formation and growth of the main root (Moreno-Romero et al., 2008). Some of the altered phenotypes obtained by CK2 inhibition are typically regulated by the phytohormone auxin (Moreno-Romero and Martínez, 2008). Indeed, CK2 was found to be involved in auxin signalling, and specifically in auxin transport (Marques-Bueno et al., 2011a; Marques-Bueno et al., 2011b). CK2 defective plants showed reduced basipetal auxin transport as well as misregulation of gene expression, of protein abundance and of subcellular localization of some members of the PIN-formed gene family encoding auxin exporters (Marques-Bueno et al., 2011a), and of the regulatory kinase PINOID (PID).

CK2mut plants contain high levels of the salycilic acid (SA) hormone, but no changes in the total auxin content. The first part (Chapter I) of the work presented here deals with the cross-talk between SA and auxin and its consequences in modulating the expression of the PIN and PID genes. The second part of this study (Chapter II) aimed to find new substrates of protein kinase CK2. We performed a high throughput yeast-two-hybrid screening in order to identify new CK2 interacting proteins. We then chose for a further study some members of the NPH3/RPT2 protein family, in particular, the founding member NPH3. A general overview of each topic will be presented in the next sections of the introduction, and they will be specifically introduced in each chapter.

II. Overview of the crosstalk between salycilic acid signalling and auxin signalling networks

Hormones are intrinsic modulators of plant growth and development and also central players of the signalling pathways necessary to respond to external cues. Several types of phytohormones are known to date: abscisic acid, auxin, brassinosteroids, salicylic acid, cytokinins, ethylene, gibberellins, jasmonates and strigolactones. The different plant hormones have independent and specific roles in plant biology, but they are also interdependent and function by stablishing complex webs of hormonal signalling pathways (Depuydt and Hardtke, 2011).

Salicylic acid (SA) is a simple phenolic compound that has been largely studied due to its central role in plant innate immunity. Moreover, it has also been identified as a regulator of abiotic stress responses and of growth development. Crosstalk between SA and ethylene and SA and jasmonic acid is important in defense responses, and more recently, a crosstalk between SA and auxin has been reported to regulate those processes as well. On the other hand, it has been suggested that SA together with gibberellins and abscisic acid regulates several developmental features.

In the next sections I present a general overview on SA biosynthesis and functions, as well as on the crosstalk between SA and auxin, with particular focus on the different SA-related mutants used in the first chapter of this thesis work.

SA biosynthesis

In plants, SA (2-hydroxybenzoic acid) can be synthesized by two distinct enzymatic pathways that require the primary metabolite chorismate as the initial compound (Figure 4). On one hand, the chorismate-derived L-phenylalanine can be transformed into SA via the cinnamic acid pathway. The first step of this pathway is the conversion of L-phenylalanine into cinnamic acid, in a reaction catalyzed by the phenylalanine ammonia lyase (PAL) enzyme. Then, cinnamic acid is converted to SA via either coumaric acid or benzoate intermediates, through two independent series of enzymatic reactions (Figure 4). On the other hand, chorismate can also be directly converted into SA by a two-step process

involving the enzymes isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL), with isochorismate as an intermediate compound ((Vlot et al., 2009) and references therein).

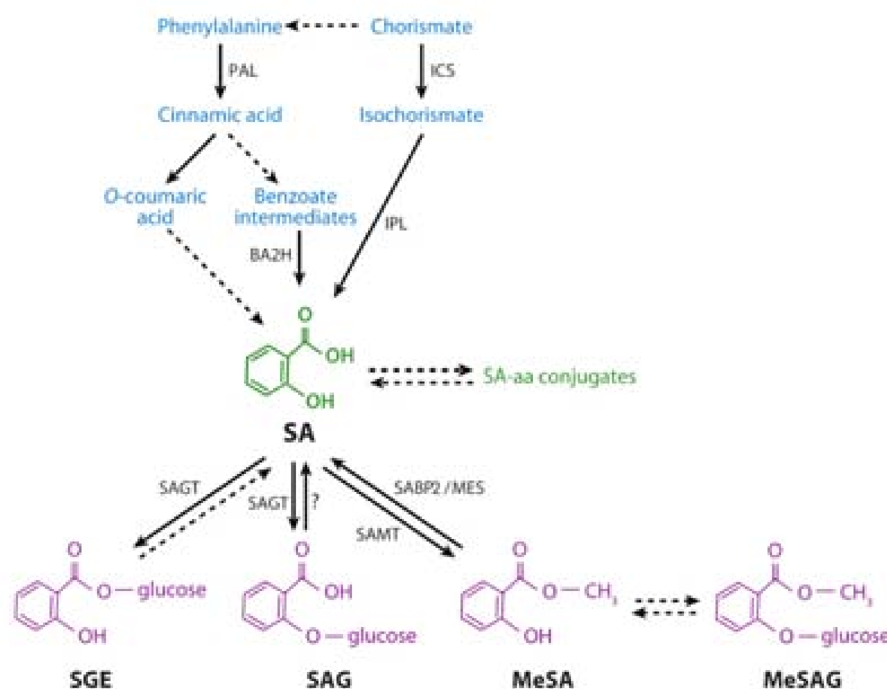

Figure 4. Simplified scheme of pathways involved in SA biosynthesis and metabolism. Abbreviations: PAL, phenylalanine ammonia lyase; ICS, isochorismate synthase; IPL, isochorismate pyruvate lyase; BA2H, benzoic acid-2-hydroxylase; SA, salicylic acid; SAGT, SA glucosyltransferase; aa, amino acid; SAMT, SA methyltransferase; SABP2, SA-binding protein 2; MES, methyl esterase; SGE, salicyloyl glucose ester; SAG, SA *O-β*-glucoside; MeSA, methyl salicylate; MeSAG, methyl salicylate *O-β*-glucoside. From (Vlot et al., 2009).

In Arabidopsis, the majority of the pathogen-induced SA biosynthesis is by the isochorismate pathway. Plants carrying the sid2 (for SA induction deficient 2) mutation only produce 5%-10% of the SA quantities observed in pathogen-challenged wild-type plants (Wildermuth et al., 2001). *sid2* plants contain a mutation in the isochorismate synthase 1 (ICS1) gene encoding a chloroplastic ICS responsible for the conversion of chorismate to isochorismate (Figure 4). In Arabidopsis there are two ICS encoding genes, ICS1 and ICS2, but the contribution of ICS2 to the total SA production is very low (Garcion et al., 2008). Moreover, ics1 ics2 double mutants exhibit residual pathogen-induced SA production, wich is in agreement with the presence of additional SA biosynthesis pathways, such as that iniciated by the activity of the PAL enzyme (Garcion et al., 2008). SA produced via ICS1 is necessary for the establishment of both local-acquired resistance (LAR) and systemic acquired resistance (SAR) (Wildermuth et al., 2001).

Although free SA can be found within plant tissues, several SA conjugated forms are also present. Formation of conjugates occurs via methylation, glucosylation and amino acid conjugation (Boatwright and Pajerowska-Mukhtar, 2013). Most of the SA produced in planta is converted into the glycosylated conjugates in a reaction catalyzed by pathogen-inducible SA glucosyltransferases (SAGT) (Figure 4). Two SAGT enzymes exist in Arabidopsis; one of them produces O-β-glucoside (SAG) and the other salicyloyl glucose ester (SGE) ((Vlot et al., 2009) and references therein). Althought both glycosylates can coexit, SAG is preferentially formed and thus, it constitutes the most abundant SA form in plant tissues. SAG is a signalling inactive form, which is storaged into the vacuol until it becomes activated (converted back to SA) by hydrolisis (Dean et al., 2005). It has also been suggested that conversion of SA to SAG could constitute a mechanism exploited by pathogens to increase plant susceptibility by decreasing the free-active SA content (Boatwright and Pajerowska-Mukhtar, 2013). Additionally, conversion of SA to its methylated form has also been suggested as a pathogen-induced mechanism to increase plant susceptibility, due to the volatile nature of methyl salicylate (MeSA) (Loake and Grant, 2007). Thus, formation of this volatile compound causes depletion of intracellular SA. Moreover, MeSA might act as a mobile signalling molecule, inducing defences in systemic tissues or even in nearby plants (Spoel and Dong, 2012). However, the exact role of MeSA in the systemic acquired resistance (SAR) is still a matter of discussion, since Arabidopsis mutants defective in SA carboxyl methyltransferase (SAMT), which catalyses the conversion of SA to MeSA, can still establish SAR (Attaran et al., 2009). MeSA can also be found in its glucosylated form (Figure 4).

SA can also be conjugated with amino acids (aa) and these compounds can activate plant defense responses (Loake and Grant, 2007) (Figure 4). In Arabidopsis, the altered expression of acyl-adenylate/thioester-forming enzyme (GH3.5), which is involved in the conjugation of amino acids to SA and to indole acetic acid, increases disease susceptibility (Staswick et al., 2005; Zhang et al., 2007). Additional members of the GH3 protein family have also been related to plant pathogenesis. For example, GH3.12 conjugates aa to 4-substituted benzoates (such as 4-aminobenzoate and 4-hydroxybenzoate), but not to SA, a 2-substituted benzoate. GH3.12 mutant plants (*pbs3*/*gdg1*/*win3*) contain reduced levels of SA and increased pathogen susceptibility (Nobuta et al., 2007), but this phenotype is rescued by exogenous SA treatment, suggesting that GH3.12 products (such as 4-hydroxybenzoate-glutamic acid) act upstream and stimulate the SA biosynthesis. Moreover, SA inhibits GH3.12 conjugating activity and thus, SA modulates its own synthesis (Okrent et al., 2009). Additionally, the amino acid conjugates of SA might act as mobile molecules to

transport SA to neighbour cells, where they would be hydrolysed to free active SA (Nobuta et al., 2007).

SA functions

Defense

Plants activate distinct defense mechanisms depending on the feeding requirements and the degree of virulence of the phatogens encountered. SA induces defense against biotrophic pathogens (that feed and reproduce on live host cells), whereas jasmonic acid (JA) activates defense against necrotrophic pathogens (that kill host cells for nutrition and reproduction). Crosstalk between these defense signaling pathways is important to optimize the response against the pathogens attack.

Plants (and animals) possess membrane-localized pattern recognition receptors (PRRs) that recognize molecular signatures called pathogen-associated molecular patterns (PAMPs), which identify whole classes of pathogens (such as chitin for fungi or peptidoglycan for bacteria) (Boller and Felix, 2009). This recognition triggers the so-called PAMP-triggered immunity (PTI) as a first step to activate defense upon pathogen attack. To overcome this barrier, plant pathogens have evolved to suppress PTI by injecting effectors into the host cells (Jones and Dangl, 2006); plants, on their turn have evolved to counteract this virulence strategy by expressing specific resistance (R) proteins. Plant R proteins mostly contain nucleotide-binding leucine-rich repeat (NB-LRR) domains, and localize to the cytoplasm where they either directly interact with pathogen effectors or indirectly detect their activities. Detection of the pathogens effectors triggers immunity (ETI), rendering the pathogen avirulent. ETI activation by biotrophic pathogens induces several defenses responses, such as a rapid burst of reactive oxygen species (ROS), biosynthesis and accumulation of SA, rapid programmed cell death (PCD) (called hypersensitive response, HR) at the infection (local) site, and increased expression of antimicrobial PR (pathogenesis-related) genes (reviewed in Wu et al., (2014)). In addition to the local induced defenses, avirulent pathogens activate the production of mobile signal molecules (i.e. MeSA) that trigger systemic defense responses, which protect the plant from a second infection. This latter process is called systemic acquired resistance (SAR) and can be induced by exogenous application of SA even in the absence of the pathogen. SAR is a long-lasting

broad-spectrum defense that, contrary to ETI, promotes cell survival (reviewed in Fu and Dong, (2013)).

SA promotes large-scale transcriptional changes that are mediated by the trascriptional cofactor NPR1 in combination with transcription factors of the TGA family and of the WRKY family (see SA signalling section). Additionally, a NPR1-independent pathway is also active in the SA-mediated transcriptional response. Mutant plants with altered endogenous SA levels have been invaluable tools to study the involvement of SA in plant defense responses. It was demonstrated that reduced amounts of SA produced enhanced susceptibitily to pathogen infection, whereas increased amount of SA produced enhanced resistance. Table 2 shows a summary of defense-related phenotypes encountered in SA mutants used in this work. Additional examples are reviewed in (Vlot et al., 2009).

Table 2. Arabidopsis genotypes used in this work, showing altered SA levels and abnormal defense responses.

Transgene or mutation	Gene function	Effect on SA levels	Defense phenotype	References
NahG	Bacterial salicylate hydroxylase	**Upon pathogen attack, SA accumulates up to 20-fold less than in wt plants**	Inactive SAR ; No expression of PR genes in systemic leaves; Increased susceptibility to virulent and avirulent pathogens.	(Delaney et al., 1994)
			Disease resistance and PR expression restored by treatment with the SA synthetic analog, 2,6-dichloro-isonicotinic acid (INA)	(Nawrath and Métraux, 1999)
sid2	Isochorismate synthase 1		Enhanced pathogen susceptibility ; No SAR induction Reduced PR1 expression.	(Nawrath and Métraux, 1999)
			Resistance and PR expression restored by treatment with SA or INA.	
cpr1	F-Box protein	**Up to 5-fold increase of total SA**	Constitutive expression of *PR* genes; Enhanced pathogen resistance resistance.	(Bowling et al., 1994)
			Constitutive disease resistance is suppressed by the SA-deficient *eds5* mutant	(Clarke et al., 2000)

crp5	unknown	Constitutive expression of *PR* genes; Enhanced pathogen resistance Spontaneous HR-like lesions	(Bowling et al., 1997)
		Constitutive disease resistance is suppressed by the SA-deficient *eds5* mutant	(Clarke et al., 2000)
cpr6	unknown	Constitutive expression of *PR* genes; Enhanced pathogen resistance	(Clarke et al., 1998)
		Constitutive disease resistance is suppressed by the SA-deficient *eds5* mutant	(Clarke et al., 2000)

Non-defense functions

SA also functions as a hormonal signal that regulates plant responses to several abiotic stresses, such as drought, chilling, heavy metal tolerance, heat and osmotic stress (Vlot et al., 2009; Miura and Tada, 2014). Morever, althought less studied, SA has also been associated to the modulation of plant growth and development, in processes such as seed germination, vegetative growth, photosynthesis, respiration, thermogenesis, flower formation, seed production, senescence and autophagy. The positive or negative effects exerted by SA in these processes are often concentration-dependent and influenced by environmental factors such as salinity (revised in Rivas-San Vicente and Plasencia, (2011)). Some of the SA-related developmental processes might be mediated by regulation of gene expression, as in the case of seed germination, flowering, and/or senescence. In addition, SA role in modulating intracellular levels of reactive oxygen species (ROS) are important for disease resistance as well as for photosynthesis, respiration and senescence (Vlot et al., 2009; Rivas-San Vicente and Plasencia, 2011).

Exogenous SA treatments affect vegetative growth, and the consequences depend on the plant species, developmental stage, and SA concentration tested (Rivas-San Vicente and Plasencia, 2011). In Arabidopsis, exogenous SA (100 µM and 1 mM) reduces the number and density of trichomes on leaves (Traw and Bergelson, 2003). The function of SA during Arabidopsis growth has been further characterized by the obtention of Arabidopsis mutants containing altered levels of endogenous SA. For instance, Arabidopsis mutants with constitutively high levels of SA, such as *cpr1*, *cpr5* or *cpr6* (for constitutive expressor of PR genes; (Bowling et al., 1994; Bowling et al., 1997; Clarke et al., 1998)) show a dwarf

phenotype, whereas Arabidopsis NahG transgenic plants, which contain low levels of endogenous SA by expression of a bacterial salicylate hydroxylase, have a high growth rate (Abreu and Munné-Bosch, 2009). A summary of the developmental deffects of the SA-related mutants used in this work can be found in Table 3.

Table 3. **Arabidopsis genotypes used in this work with altered SA levels and abnormal growth.** Adapted from Rivas-San Vicente and Plasencia, (2011).

Transgene or mutation	Gene function	Effect on SA levels	Growth phenotype	References
NahG	Bacterial salicylate hydroxylase	**2- to 4-fold reduction of SA levels in leaves**	Increased growth (leaf rosette biomass at early stages of reproduction 1.7-fold more than wild type).	(Abreu and Munné-Bosch, 2009)
			Faster growth rate at low temperature (4ºC) associated with enlarged cell size, extensive endoreduplication, and increased expression of CycD3.	(Scott et al., 2004)(Xia et al., 2008)
sid2	Isochorismate synthase		Increased growth (leaf rosette biomass at early stages of reproduction 1.7-fold more than wild type).	(Abreu and Munné-Bosch, 2009)
cpr1	F-Box protein	**Up to 5-fold increase of total SA**	Small, narrow, dark green leaves densely covered with trichomes on the adaxial surface and relatively long siliques compared with the wild type.	(Bowling et al., 1994)
			Growth much more inhibited at 5ºC.	(Scott et al., 2004)
			The dwarf phenotype reverts when grown under high light (HL) conditions.	(Mateo et al., 2006)
crp5	unknown		Significantly smaller than the wild type, and reduction in both trichome number and development.	(Bowling et al., 1997)
			The dwarf phenotype partially reverts under HL conditions.	(Mateo et al., 2006)
cpr6	unknown		Loss of apical dominance and a reduction in overall plant size.	(Clarke et al., 1998)
			The dwarf phenotype partially reverts under HL conditions.	(Mateo et al., 2006)

SA signalling

Most of the knowledge about SA signaling comes from the study of its role in plant immunity. In order to activate downstream signaling events, SA must bind to intracellular targets or receptors. Using biochemical approaches, several SA-binding proteins (SABP) have been identified, most of them being metabolic enzymes such as catalase (wich modulates the levels of ROS species, i.e: H_2O_2), iron-containing enzyme such as aconitase, lipoxidase and peroxidase (Rüffer et al., 1995) and an enzyme with methyl salicylate (MeSA) esterase activity (SABP2). SABP2, which activity is inhibited by SA binding, converts the biologically inactive MeSA to active SA in the systemic tissues and is required for SAR (Forouhar et al., 2005). On the other hand, SABP3 is a chloroplastic localized carbonic anhydrase (CA) that also binds SA (Slaymaker et al., 2002) although it is unlikely a SA receptor because SA signaling is localized in the cytoplasm. SA is synthesized in the chloroplast and then exported to the cytoplasm, mediated by the EDS5 (ENHANCED Disease Susceptibility 5) transporter. eds5 mutant has reduced amounts of SA and increased susceptibility to pathogens (Serrano et al., 2013).

NPR1 (Nonexpresser of PR genes 1, also known as NIM1, SAI1) has been reported as a master regulator of SA-dependent signaling, as *npr1* mutants are defective in SA responses (no induction of SAR neither expression of PR genes) and insensitive to SA treatment (Cao et al., 1994; Delaney et al., 1995; Ryals et al., 1997; Shah et al., 1997). NPR1 contains two conserved protein–protein interaction domains, BTB (Bric-a-brac, Tramtrack, Broad-complex) and ankyrin repeat (Cao et al., 1997). Althought the genetic data suggests that NPR1 could be a bona fide SA receptor, no conclusive data about its ability to bind SA have been obtained yet, and opposite results have been recently reported (Fu et al., 2012; Wu et al., 2012). While Fu and co-workers (2012) could not detect SA binding to NPR1 in a ligand-binding assay, Wu and colleagues (2012) reported that NPR1 binds SA in an equilibrium dialysis assay using copper as a cofactor. In constrast, NPR1 has been shown to interact with several members of the TGA subfamily of the bZIP (basic leucine zipper) family of transcription factors, and it has been proposed to act as a co-activator necessary for the transcriptional reprogramming at the onset of plant immunity responses (Zhang et al., 1999; Zhou et al., 2000; Wang et al., 2006). For instance, the promoter regions of WRKY-encoding genes are targets for NPR1/TGA complexes, and the cis-elements recognized by WRKYs proteins (W boxes) are abundant in the promoters of SAR-related genes, such as ICS1, NPR1 and PR1 (Fu and Dong, 2013). Moreover, some WRKYs are involved in a feedback negative regulation of SA synthesis. WRKYs are thought to be required in the activation

phase of the defense response, as well as in repressing the response when the infection disappears (Fu and Dong, 2013).

Under non-inductive conditions, NPR1 is mostly found as an oligomer in the cytosol, the structure of which is maintained by cross-linking of NPR1 monomers through disulfur bonds. Pathogen infection or SA treatment produces redox changes that favours NPR1 reduction from the oligomeric to the monomeric form (Mou et al., 2003), followed by translocation to the nucleus where NPR1 acts as a transcription cofactor (Kinkema et al., 2000). In addition to this mode of regulation, it has been reported that proteasome-mediated NPR1 degradation is also important for its role in plant immunity (Spoel et al., 2009). NPR1 degradation plays a dual antagonistic role. In the absence of pathogen infection (or of increased SA levels), nuclear NPR1 monomers are degraded to avoid constitutive activation of the defense responses. Upon infection, NPR1 degradation is necessary for maximum activation of defense gene expression, due to constant removal and incorporation of NPR1 to gene promoters (Spoel et al., 2009). NPR1 degradation is mediated by a Cullin 3-based E3 ligase which uses BTB-containing proteins as substrate adaptors (Pintard et al., 2004). Interestingly, NPR3 and NPR4, two NPR1 homologs that are also BTB-containing proteins, were found to interact with both NPR1 and Cullin 3, and were required for NPR1 degradation (Fu et al., 2012). Moreover, the interaction between NPR3/4 and NPR1 is modulated by SA in a concentration-dependent manner. NPR3 and NPR4 bind SA with different affinities and outcomes. SA binding disrupts NPR4 interaction with NPR1, while in NPR3 has the opposite effect. It has been proposed that NPR3 and NPR4 are sensors of the SA gradient produced during pathogen infection, and that this gradient determine cell death or survival (Enyedi et al., 1992; Fu et al., 2012; Yan and Dong, 2014) (Figure 5). The increased SA levels produced after pathogen infection facilitates the interaction between NPR3 and NPR1 (NPR3 having lower affinity for SA than NPR4), (Fu et al., 2012) and thus degradation of NPR1. As NPR1 is known to supress programmed cell death during ETI (Rate and Greenberg, 2001), the SA-induced NPR1 degradation allows progression of cell death in infected tissues. On the other hand, in the surrounding tissues SA accumulates at lower levels, hampering NPR4–NPR1 interaction and allowing accumulation of NPR1, which promotes cell survival and SA-mediated resistance (Yan and Dong, 2014).

In addition to NPR1-mediated SA signaling, several genetic screenings for npr1 supressors have supported the idea of the existence of SA-dependent NPR1-independent signalling pathways, and thus of the existance of additional (still unknown) SA receptors (revised in An and Mou, (2011)). For example, one of this screenings lead to the

identification of SNI (Suppressor of npr1-1, inducible 1) as a negative regulator of defense responses (Li et al., 1999), demonstrated because in the *sni npr1* doble mutant, SA-mediated gene expression and pathogen resistance was restablished. SNI has been found to be a subunit of a complex involved in DNA damage responses and genome stability, named SMC 5/6 (structural maintenance of chromosome protein complex 5/6) (Yan et al., 2013). Moreover, the authors suggested that activation of DNA damage responses by the SA-dependent NPR1-independent pathway is an important step in the activation of plant immune responses.

Another example of the existance of NPR1-independent pathways in plant immunity was obtained from the SA-accumulating mutants *cpr1*, *cpr5* and *cpr6*. The constitutive disease resistance exhibited by cpr mutants was only partially rescued when crossed with the SA-insensitive *npr1* mutant (Clarke et al., 2000).

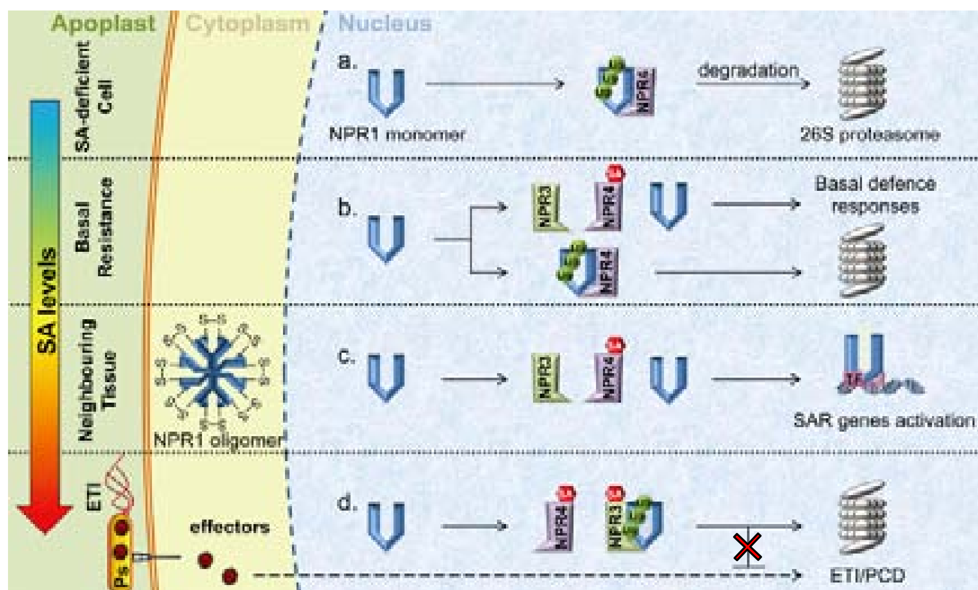

Figure 5. Model for salicylic acid (SA) gradient perception *in planta*.
(a) Binding of NPR1 by NPR4 in the absence of SA leads to NPR1 degradation via the 26S proteasome. **(b)** Basal SA levels allow binding of SA to NPR4, thereby limiting the ability of NPR4-mediated NPR1 degradation and activating basal resistance responses **(c)** Moderate SA levels accumulated in systemic tissues (ETI - effector-triggered immunity- in neighbouring cells) allow SA binding to NPR4, limit NPR4–NPR1 interaction and, induces NPR1-dependent expression of systemic acquired resistance (SAR) genes. A pool of NPR1 undergoes degradation via NPR3 interaction. **(d)** Cells subjected to direct avirulent pathogen attack experience high SA accumulation, leading to subsequent NPR3 dependent NPR1 degradation and induction of ETI/programmed cell death (PCD). Adapted from Boatwright and Pajerowska-Mukhtar, (2013).

Crosstalk between salicylic acid and auxin

SA, JA and ET act cooperatively to regulate plant defense responses. More recently, the phytohormone auxin, althought mostly known by its role in plant growth and development, has been also involved in plant defense. Some plant pathogens have evolved the ability to produce auxin or stimulate auxin byosinthesis in plant host cells to disrupt the normal growth of infected plants (Chen et al., 2007; Robert-Seilaniantz et al., 2007). On the other side, plants have evolved some mechanisms to reduce auxin signalling upon pathogen infection. These findings suggest the existence of an antagonistic crosstalk between SA and auxin that modulates the optimal response to pathogens attack.

SA-accumulating mutants (such as *cpr6*) exhibit lower levels of free IAA and reduced sensitivity to auxin, as well as morphological phenotypes similar to auxin-deficient and auxin-insentive mutants (Wang et al., 2007). Treatment of Arabidopsis plants with exogenous SA causes transcriptional repression of genes invoved in auxin polar transport, such as AUX1 (encoding an auxin impoter) and PIN7 (encoding a member of the PIN family of auxin exporters) (Wang et al., 2007). Moreover, exogenous SA also affects and genes involved in auxin signaling. Auxin perception is due to members of a small family of F-box proteins, named TIR1 and its paralogs AFB1-3 (Dharmasiri et al., 2005). Auxin binding to SCFTIR1/AFBs results in degradation of transcriptional repressors (the Aux/IAA proteins) via the SCF E3-ubiquitin ligase and the proteasome. Then, Aux/IAA degradation allows activation of gene expression by the ARF family of transcription factors (Gray et al., 2001). Elevated levels of SA cause transcriptional repression of TIR1, AFB1, and of some members of the Aux/IAA family (Wang et al., 2007), and produces TIR1 protein downregulation (Iglesias et al., 2011) and AUX/IAA protein stabilization (Wang et al., 2007). By contrast, SA-treated tir1 afb2 mutant seedlings show higher induction of PR-1 expression than SA-treated wild-type plants, thus indicating that disruption of auxin signaling interferes with SA-regulated PR-1 induction and supporting the idea of an antagonistic crosstalk between these two hormones (Iglesias et al., 2011). In addition, it has recently been reported that SA interferes with clathrin-mediated endocytic protein trafficking of the auxin transporters PIN1 and PIN2, adding another level within the SA-auxin crosstalk. This effect is independent of transcription and of NPR1-mediated SA signalling (Du et al., 2013).

III. Overview of phototropism

In contrast to animals, shape and organogenesis in plants are mostly determined post-embryonically. As sessile organisms, plants show an enormous plasticity, i.e.: the ability to modulate their growth rate and development according to variations in the environmental factors, light among them. Phototropism, the reorientation of plant growth toward (positive phototropism) or away (negative phototropism) light, is an example of such an adaptative process (Holland et al., 2009). Stems generally exhibit positive phototropism as a mechanism to maximize light capture for photosynthesis or to ensure survival of germinating seedlings. On the other hand, roots show negative phototropism, which has been shown to increase root growth efficiency under drought conditions (Galen et al., 2007a; Galen et al., 2007b).

Ultraviolet A (UV-A) and blue light (BL) (320–500 nm) are the most effective wavelengths to stimulate phototropism. Three distinct classes of UV-A/BL photoreceptors have been identified in plants (Banerjee and Batschauer, 2005): cryptochromes (cry), which play a major role in plant photomorphogenesis (together with the red/far-red light receptors, phytocromes (phy)); the Zeitlupe/Adagio protein family (ZTL/ADO), which includes F-box proteins that mediate targeted proteolysis of proteins involved in circadian rhythms and flowering; and phototropins (phot), which regulate light-dependent processes, such as phototropism, and serve to optimize the photosynthetic efficiency of plants and to promote growth.

The phototropic curvature follows the Cholodny–Went theory, which states that in response to tropic stimulus, the asymmetric distribution of the phytohormone auxin causes differential growth of the two sides of the plant organ, and, as a consequence, organ bending. In etiolated plants, unilateral BL produces auxin accumulation at the shaded side of the hypocotyls, where it stimulates cell expansion (Sakai and Haga, 2012). Figure 6 shows the asymetric auxin distribution in Arabidopsis hypocotyls as visualized by the auxin repoter DR5rev::GFP.

In monocots, there is a spacial separation between the site of perception of the phototropic light and the site where the curvature is produced. Perception of light occurs at the tip of the coleoptile of dark grown seedlings, while the auxin-induced asymetric growth occurs above in the stem. By contrast, in dicots, it has recently been reported that light perception and bending occurs at the same region of the hypocotyl of etiolated seedlings. In

Arabidopsis, the region in and above the elongation zone is sufficient to trigger the phototropic response (Preuten et al., 2013). Moreover, an independent study on dark acclimated de-etiolated Arabidopsis seedlings, showed that the region in and above the hypocotyl apex is necessary to iniciate lateral auxin fluxes and phototropic bending (Christie et al., 2011). Thus, the morphological and/or physiological changes occurring upon de-etiolation can influence the spatial patterns of light sensing and bending outcome (Preuten et al., 2013). Both studies, however, concluded that cotyledons are not involved in the phototropic response.

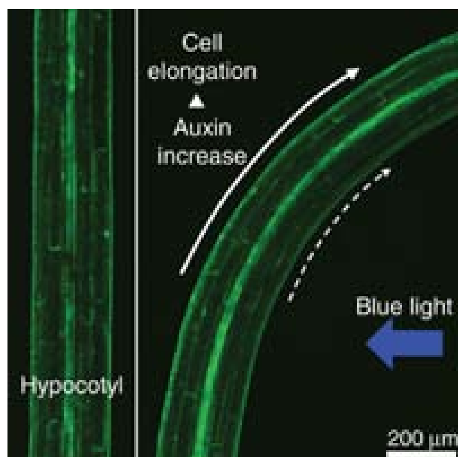

Figure 6. Distribution of auxin during hypocotyl phototropism in Arabidopsis. Two-day-old etiolated seedlings harboring the auxin reporter gene *DR5rev:GFP* were used. The hypocotyl was stimulated with unilateral irradiation of blue light for 3 h at 0.2 μmol m−2 s−1. Extracted from (Sakai and Haga, 2012).

In the next sections I present and overview on the blue-light receptors phototropins (phot1 and phot2) and their downstream signaling components involved in the positive phototropic response. Most of the actual knowledge on this tropic response is based on the study of hypocotyls from dark-grown (etiolated) Arabidopsis seedlings, as a working model.

Blue-light phototropic receptors: phototropins

Analysis of Arabidopsis seedlings with altered phototropic responses lead to the indentification of two blue-light (BL) receptors for phototropism, named phototropin 1 (phot1) and phototropin 2 (phot2). Phot1 is involved in the phototropic response at low BL intensities, while both phot1 and phot2 are necessary for the phototropic response at high BL intensities. *phot1* (also called *nph1*) Arabidopsis mutant shows non-phototropic hypocotyl and root phenotypes at BL intensities <10 $\mu mol \cdot m^{-2} \cdot s^{-1}$, whereas at higher fluence rates (10 and 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) the hypocotyls show a clear positive phototropic response, mediated by phot2

activity (Sakai et al., 2000). By contrast, the *phot2* (or *npl1*) single mutant shows normal phototropic responses at all the BL intensities, due to phot1 activity. Only the *phot1phot2* (or *nph1npl1*) double mutant shows a strong aphototropic phenotype at both low and high BL (Sakai et al., 2001). These differences in fluence rate requirements might result from differences in protein levels; phot1 is abundant in etiolated seedlings and decreases in response to prolonged blue light exposure, whereas phot2 protein levels are almost undetectable in the dark, and they increase upon irradiation (Christie and Murphy, 2013).

Phototropin structure and activation

Analysis of the protein sequence of phototropins allowed to group them into the VIIIb subfamily of the AGC (for cAMP-dependent protein kinase, cGMP-dependent protein kinase G and phospholipid-dependent protein kinase C) family of eukaryotic protein kinases (Bögre et al., 2003). The polypeptide chain of phototropins contains two functional domains: a N-terminal photosensor domain and a C-terminal serine/threonine kinase domain (Figure 7). Moreover, the N-terminal photosensor domain contains two LOV (light, oxygen, or voltage) domains, LOV1 and LOV2, which bind flavin mononucleotid (FMN) as a cofactor, and function as a blue-light sensor (Christie et al., 1999). The FMN and a conserved cysteine residue within the LOV domain form a covalent adduct upon illumination, that is reversed in darkness (Christie, 2007).

The LOV2 domain acts as a repressor of the phototropin kinase activity. This activity is exerted in coordination with a conserved helix-α (named Jα-helix) located next to the C-terminal end of the LOV2 domain. In the dark, the Jα-helix interacts with LOV2; upon BL irradiation formation of the cysteinyl-adduct disrupts this interaction (Harper et al., 2003) resulting in the unfolding of the Jα-helix and the activation of the C-terminal kinase domain (Figure 7). This structural change leads to phototropin autophosphorylation and phosphorylation of substrates. Phot1 autophosphorylation is mostly mediated by intermolecular interactions (Kaiserli et al., 2009). The LOV1 domain has been suggested to act in photoreceptor dimerization, although in the case of phot1 LOV1 is not necessary for intermolecular phosphorylation (Kaiserli et al., 2009).

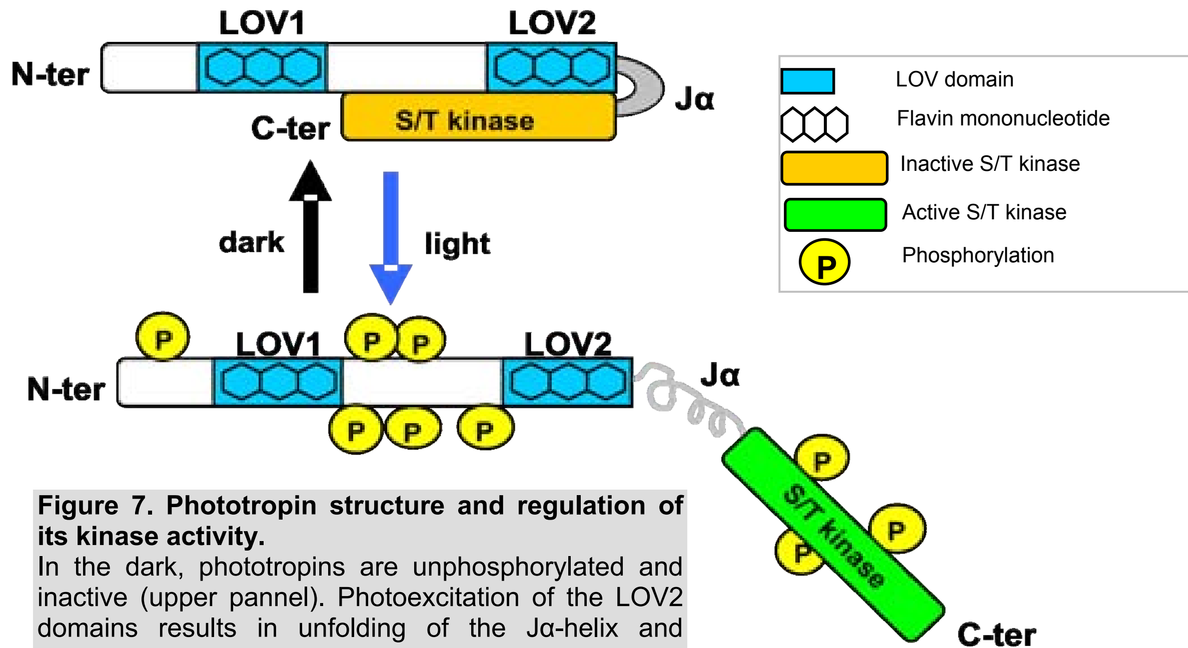

Figure 7. Phototropin structure and regulation of its kinase activity.
In the dark, phototropins are unphosphorylated and inactive (upper pannel). Photoexcitation of the LOV2 domains results in unfolding of the Jα-helix and activation of the C-terminal kinase domain (bottom panel), which consequently leads to autophosphorylation of the photoreceptor. Adapted from Christie, (2007).

Phot1 is found phosphorylated at multiple serine and treonine residues upon BL treatment (Salomon et al., 2003; Inoue, Kinoshita, Matsumoto, et al., 2008; Sullivan et al., 2008; Deng et al., 2014). The biological significance of this phosphorylation is still not clear, but autophosphorylation at serine 851 (located within the kinase activation loop) in Arabidopsis phot1 and the equivalent position in phot2 is necessary to activate the phototropic response, as demonstrated by the loss of phototropism produced by mutations of these residues (Inoue, Kinoshita, Matsumoto, et al., 2008; Inoue et al., 2011). One of the functions of phototropin autophosphorylation may be regulation of its subcellular localization, at least for phot1. Both phot1 and phot2 are hydrophilic in nature, but they localize at the plasma membrane in the dark. Upon BL irradiation, one fraction of phot1 dissociates from the plasma membrane and moves into the citosol (Sakamoto and Briggs, 2002; Wan et al., 2008; Kaiserli et al., 2009; Sullivan et al., 2010), whereas one fraction of phot2 localizes at the Golgi apparatus (Kong et al., 2006; Aggarwal et al., 2014). In the case of phot2, it has been shown that the kinase domain but not autophosphorylation is necessary for its localization at the Golgi apparatus (Aggarwal et al., 2014), whereas phot1 kinase domain and phosphorylation at S851 are required for phot1 internalization (Kaiserli et al., 2009). By contrast, phot1 autophosphorylation is not necessary for the phot1 turnover observed after prolonged BL treatment (Sullivan et al., 2010). Moreover, phot1 intracellular movement has

been suggested to be dependent on clathrin mediated endocitosis (Kaiserli et al., 2009; Roberts et al., 2011).

Phototropic signaling downstream phot1

Several proteins have been identified to interact with Arabidopsis phot1 *in vivo*. Briefly, two members of the plant specific NPH3/RPT2 protein family (see below), three members of the phytochrome kinase susbtrate family (PKS), and the ABCB19 auxin transporter (see next section). Among the PKS family, PKS1 and PKS2 interact with phot1 (and also with NPH3), and additionally, PKS4 has been shown to be a phot1 substrate (Lariguet et al., 2006; de Carbonnel et al., 2010; Demarsy et al., 2012). Characterization of the phototropic impaired triple mutant *pks1pks2pks4* has suggested that the role of PKS proteins in phototropism might be the modulation of auxin-regulated gene expression or the modulation of lateral auxin transport (Kami et al., 2014). Moreover, a yeast-two-hybrid screen of an Arabidopsis cDNA library using phot1 as a bait lead to the identification of 1) a NPH3-like protein of unknown function (At1g30440), 2) a 14-3-3λ protein, and 3) two ADP-ribosylation factors (ARF2 and ARF7) that are members of the Ras superfamily of GTP-binding proteins, which are important modulators of vesicle trafficking (Sullivan et al., 2009). The 14-3-3λ interaction with phot1 was further confirmed *in vivo*, and shown to be dependent on BL-induced phot1 autophosphorylation (Sullivan et al., 2009).

The Signal transducer NPH3 and the NRL protein family

NPH3 (for Non-phototropic hypocotyl 3) is a key component of the phototropic signaling pathway. *nph3* loss-of-function Arabidopsis mutants show defects in hypocotyl and root phototropism under a broad range of light intensities (Liscum and Briggs, 1995; Sakai et al., 2000; Inada et al., 2004), and thus, NPH3 has been associated with the phot1-dependent phototropic signalling under low BL conditions, and with the phot1/phot2-mediated signaling under high BL conditions. Moreover, NPH3 seems indispensable for the formation of the lateral auxin gradient upon directional light treatment. Loss-of-function mutant plants of the rice NPH3 ortholog CPT1 are aphototropic due to the lack of lateral auxin redistribution (Haga et al., 2005).

NPH3 is the founding member of the plant specific NPH3/RPT2 (or NRL) protein family, which is composed of 33 members in Arabidopsis. Three distinct domains can be identified within the NPH3 protein sequence and, by extension, in the NRL proteins: a Broad-

complex, Tramtrack, and Bric-a-brac (BTB) domain located at the N-terminus, a centrally located NPH3 domain, and a Coiled-coil (CC) domain at the C-terminus of the protein (Motchoulski and Liscum, 1999; Pedmale et al., 2010) (Figure 8). While the BTB and CC domains are involved in protein-protein interactions, the function of the NPH3 domain remains unknown.

Figure 8. Scheme of the NPH3 structure.
BTB, Broad-complex, Tramtrack, and Bric-a-brac; NPH3, Non-phototropic hypocotyl 3; CC, coiled-coil domain.

As the phototropins, the NPH3 protein is hydrophylic in nature but is found associated to the plasma membrane, where it interacts with phot1 (Motchoulski and Liscum, 1999; Lariguet et al., 2006). The interaction takes place between the C-terminal CC domain of NPH3 and the N-terminal photosensory domain of phot1 (Motchoulski and Liscum, 1999). However, in contrast to phot1, NPH3 is found phosphorylated in dark conditions and dephosphorylated after BL exposure. The exact mechanism by which NPH3 is dephosphorylated is not known, but it depends on phot1 activity (Pedmale and Liscum, 2007). In addition, NPH3 also interacts with Cullin3 (CUL3), forming a Cullin3 Ring E3 ubiquitin ligase that uses NPH3 as a substrate adaptor ($CRL3^{NPH3}$) (Roberts et al., 2011). It has been reported that phot1 is ubiquitinated upon irradiation with either low or high BL intensities, and that this ubiquitination depends on the formation of the $CRL3^{NPH3}$ complex. Low BL induces mono- and multiubiquitination (i.e: addition of one or multiple single ubiquitin moieties) of phot1, and high BL induces mono-/multi- and polyubiquitination (i.e: addition of an ubiquitin chain)(Roberts et al., 2011). The biological significance of phot1 ubiquitination is not clearly understood, but it has been suggested that mono-/multiubiquitination induces phot1 internalization from the plasma membrane, promoting intracellular phot1 signaling by interaction/phosphorylation of cytoplasmic proteins. On the other hand, polyubiquitination has been proposed to target phot1 for degradation via the 26S proteasome as a means to bring about receptor desensitization at high BL intensities (Roberts et al., 2011). Figure 9 shows a schematic model of the phot1-NPH3 signaling modul.

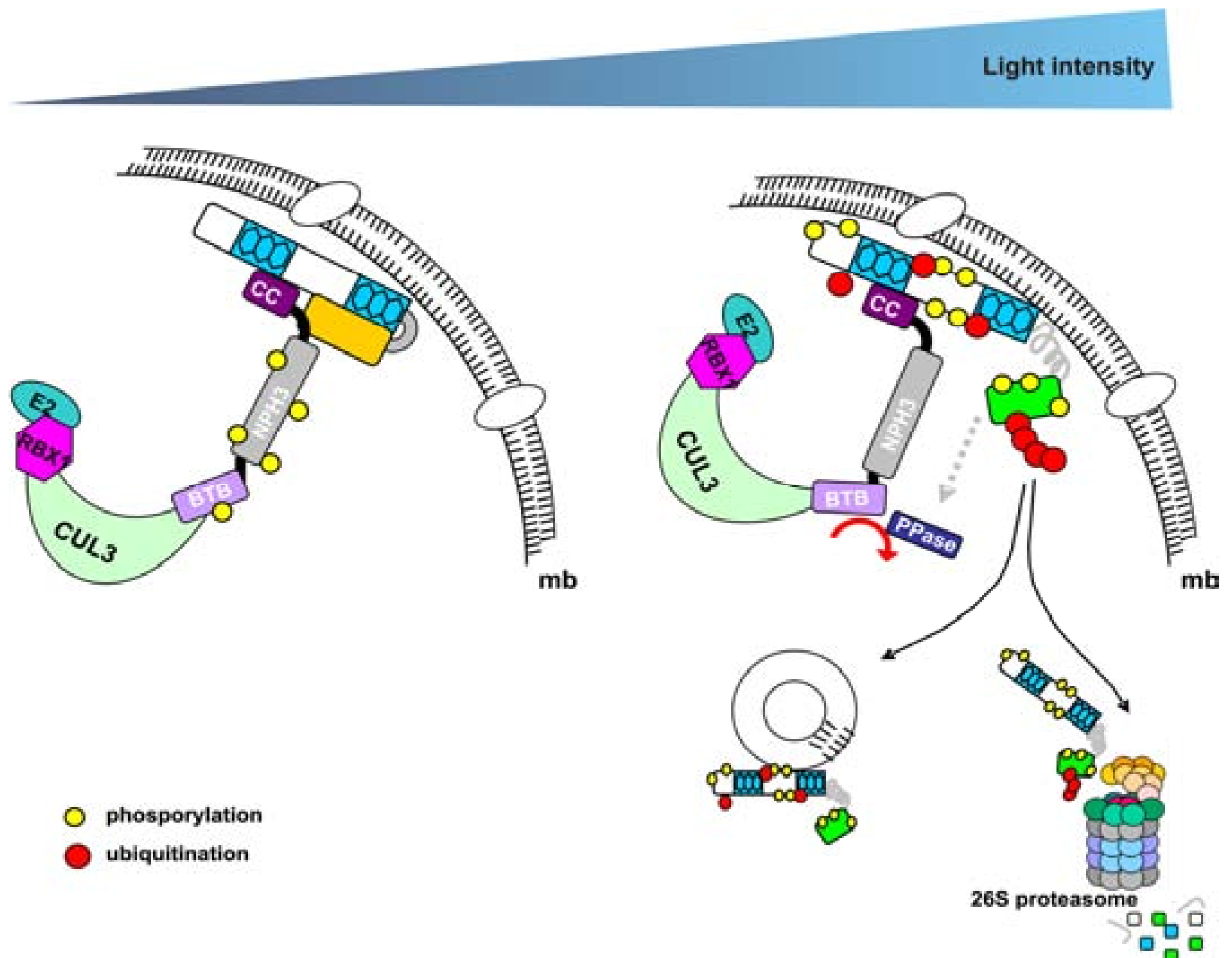

Figure 9. Schematic model of the phot1-NPH3 signaling modul.
In the dark (left pannel), phot1 is found at the plasma membrane in its dephosphorylated inactive state, and NPH3 is in its phosphorylated signaling-incompetent state. Upon BL irradiation (right pannel), phot1 becomes activated and autophosphorylated. Morevorer, phot1 would activate a protein phosphatase (by an unknown mechanism), which dephosphorylates NPH3. The dephosphorylated NPH3 state is capable of transducing phot1-mediated signals, probably through its activity as a substrate adaptor of a CRL3 complex. Phot1 is ubiquitinated by the $CRL3^{NPH3}$ which leads to phot1 internalization (when mono-/multiubiquitinated) or degradation by the 26S proteasome.
CUL3 (cullin3), RBX1 and the E2 ligase constitute the cullin3 ring E3 ubiquitin ligase complex.
Abbreviations: mb, membrane, PPase, phosphatase.

The co-founder member of the NRL family, named RPT2 (Root Phototropism 2) has also been directly involved in phototropism. *rpt2* mutants show aphototropic root phenotypes, and defects in hypocotyl phototropism under high-intensity BL conditions (when both phot1

and phot2 are active) (Sakai et al., 2000). RPT2 locates at the plasma membrane and interacts with both phot1 and NPH3. The RPT2-phot1 interaction takes place between the N-terminal domain of phot1 and the N-terminal BTB domain of RPT2 while, NPH3-RPT2 interaction occurs by dimerization through their respective BTB domains (Inada et al., 2004). It has been hypothesized that a $CRL3^{NPH3/RPT2}$ complex could be the responsible of phot1 polyubiquitination under high BL conditions (Hohm et al., 2013), as supported by the fact that BTB-containing proteins usually interact with the CRL3 complexes as homo-/heterodimers and oligomers (Perez-Torrado et al., 2006).

As previously mentioned, the outcome of the phototropic signaling is the formation of an auxin gradient between the lit and the shaded sides of the hypocotyl, which produces elongation of the cells located in the shaded side and thus, asymetric growth and phototropic curvature towards the light source. It is worth noting that several members of the NRL family act redundantly in auxin-mediated organogenesis and root gravitropism (which involves asymetric auxin distribution), by modulating the subcellular localization of two members of the PIN-formed protein family, PIN1 and PIN2, which are polar-localized auxin-exporters (Furutani et al., 2007; Furutani et al., 2011). These NRL proteins share high degree of sequence similarity, and grouped within the NPY/MAB4 subclass, composed by Naked pins in yuc 1 (NPY1)/macchi-bou4 (MAB4)/ At4g31820, NPY2/At2g14820, NPY3/At5g67440, NPY4/ At2g23050 and NPY5/At4g37590. NPY1 (also known as ENP, for Enhancer of Pinoid) functions in concert with the AGC3 kinase PINOID (Furutani et al., 2007), which regulate the subcellular localization of the PIN proteins by phosphorylation (Dhonukshe et al., 2010). Whether or not the NRL homologs NPH3 and RPT2 (together with the AGC kinase phot1) can modulate auxin signaling and transport to mediate the phototropic curvature is not known.

Additional evidences about the function of the NRL family members in the auxin-regulated cell elongation and differentiation came from the characterization of several Arabidopsis mutants in NPH3-like encoding genes. It was concluded that SETH6/At2g47860 is required for pollen germination and pollen tube growth (Lalanne et al., 2004), an, mutation in defectively organized tributaries 3 (DOT3)/At5g10250 causes several developmental deffects, such as dwarf phenotype, abnormal vein patterning, reduced primary root, fused rosette leaves and low fertility (Petricka et al., 2008). To date, only 9 of the 33 NRL proteins have been described.

Auxin transport and signaling

Polar auxin transport (i.e: the active, cell-to-cell auxin transport) is the result of the coordinated action of the membrane localized auxin importers of the AUX/LAX protein family and the auxin exporters of the Pin-formed (PIN) and of the MDR/PGP protein families (Peer et al., 2011). Arabidopsis contains eight PIN members: *PIN1–PIN4* and *PIN7* genes encode plasma-membrane localized auxin exporters, while *PIN5*, *PIN6*, and *PIN8* genes encode PIN proteins localized to endomembrane structures, such as endoplasmic reticulum (ER)(Mravec et al., 2009), which are likely involved in intracellular auxin movement (Zazímalová et al., 2010). The polar localization of the PIN proteins at the plasma membrane determines the directionality of auxin fluxes. For instance, in the central vasculature of the shoots PIN1 is localized at the basal side of the cells, driving auxin from the shoot apex to the root (Gälweiler, 1998; Noh et al., 2003; Grunewald and Friml, 2010).

The involvement of the PIN proteins in formation of lateral auxin fluxes in response of unilateral light stimulus is still an open question. The lack of *pin* Arabidopsis mutants showing strong aphototropic phenotypes, and the functional redundancy of these proteins together with the strong developmental deffects shown by the high order *pin* mutants, have difficulted this study. However, some PIN proteins, such as PIN3, have been proposed to function as modulators of lateral auxin transport in phototropism (Ding et al., 2011). PIN3 is expressed in the hypocotyl's endodermis, central vasculature, and epidermis. In etiolated Arabidopsis seedlings kept in darkness PIN3 is apolarly localized in the endodermis, and upon BL irradiation it becomes more abundant at the inner side of the endodermic cells from the illuminated side of the hypocotyl (Ding et al., 2011). Thus, PIN3 re-localization could drive auxin back to the vasculature, and reduce the auxin content in the epidermis cells of the illuminated side (Figure 10). However, *pin3* mutant plants only show a moderate reduced phototropic response. On the other hand, using dark acclimated de-etiolated seedlings, Christie et al., (2011) concluded that neither PIN3 nor ABCB19 (see below) are reponsible for lateral auxin redistribution, as both *pin3* and *abcb19* mutants have the same pattern of auxin distribution as wild-type plants. However, these authors reported a reduction of PIN3 protein below the region of the hypocotyl curvature upon BL treatment, thus suggesting that PIN3 could promote auxin accumulation at the bending zone and indirectly contribute to lateral auxin distribution (Christie et al., 2011). Additionally, PIN7 has been suggested to play a role in mobilizing auxin in the hypocotyl elongation zone (Christie and Murphy, 2013), since PIN7 protein is abundant in the epidermis and cortex of this zone (Christie et al., 2011). In

support to this, it has been found that *pin7* mutants are slightly aphototropic (Ding et al., 2011; Christie et al., 2011).

Polar localization of the PIN proteins is, in part, regulated by phosphorylation. The AGC3 S/T kinase PINOID and its close homologs WAG1 and WAG2 phosphorylate the membrane-localized PIN proteins (Dhonukshe et al., 2010), and promote PIN localization at the apical (top) membrane of the cells. By contrast, dephosphorylation by the PP2A phosphatase produces their localization at the basal (bottom) membrane. The exact role of PIN phosphorylation in phototropism is unclear. It has been reported that light-dependent activation of phot1 reduces PINOID transcript levels at the illuminated side of the hypocotyl, and that this reduction stimulates polar localization of PIN3 at the inner cell wall of the endodermis cells (Ding et al., 2011). Whether phot1 could modulate auxin fluxes by phosphorylation of PINs or their regulatory proteins, such as AGC3 kinases or NPH3-like proteins, remains unknown. By contrast, it has been demonstrated that phot1 binds *in vivo* and phosphorylates *in vitro* the auxin exporter ABCB19, a member of the MDR/PGP family (Christie et al., 2011). ABCB19 is a negative regulator of the phototropic curvature, as demonstrated by the enhanced phototropic curvature of *abcb19* mutants (Noh et al., 2003). Moreover, ABCB19 has been shown to stabilize PIN1 in specific membrane locations and thus to promote the vertical auxin flux from shoots to roots (Noh et al., 2003). Disruption of ABCB19 activity by phot1 phosphorylation or abcb19 mutation reduces the vertical auxin flux, and increases the auxin content in the region above the elongation zone in dark aclimated de-etiolated seedlings (Christie et al., 2011) (Figure 10). Auxin accumulation in this zone can lead to lateral auxin distribution and phototropic bending.

In summary, upon BL irradiation, the auxin efflux transporters PIN3 and ABCB19 might restrict the vertical flow of auxin from the shoot apex to the root, while lateral redistribution of auxin may involve the activity of additional PIN proteins (Christie and Murphy, 2013; Hohm et al., 2013)(Figure 10).

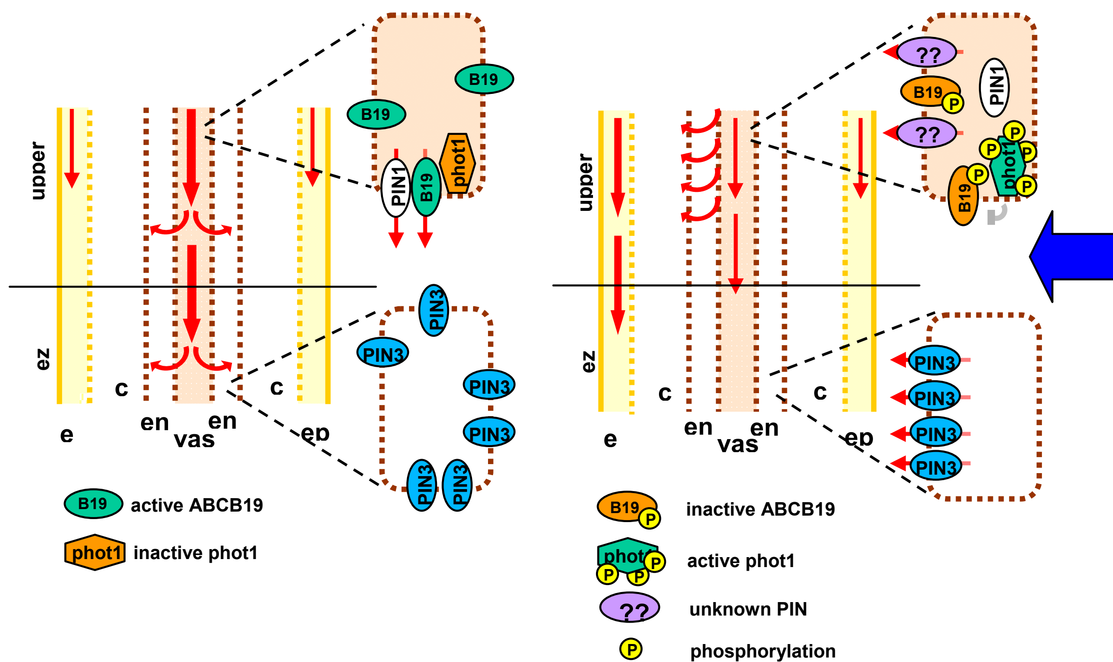

Figure 10. Scheme of auxin transport in the upper hypocotyl (upper) and the elongation zone (ez).
The scheme has been performed by using data from Arabidopsis etiolated seedlings and from dark aclimated de-etiolated seedlings. Details of the model are explained in the text. The left pannel corresponds to dark grown seedlings, while the right pannel correspond to seedlings treated with unilateral BL. Red lines depict auxin fluxes.
Abbreviations: e, epidermis; c, cortex; en, endodermis.

The increase of auxin concentration at the shaded side of the hypocotyl promotes the transcription of auxin-regulated genes, and thus differential gene expression between the lit l and shaded sides of the phototropically stimulated hypocotyl (Figure 11). Esmon et al. Esmon et al., (2006) identified eight TROPIC STIMULUS-INDUCED (TSI) genes, differentially expressed at the shaded side of the hypocotyls of *Brassica oleracea*. Among them, two genes, EXP1 and EXP8, encoded α-EXPANSIN proteins that mediate cell wall extension at low pH (Liscum et al., 2014). The involvement of auxin signaling in phototropism was first discovered by the isolation of the Arabidopsis mutant *nph4* (Liscum and Briggs, 1995; Liscum and Briggs, 1996). The non-phototropic hypocotyl phenotype of nph4 was due to a mutation in the gene encoding auxin response factor 7 (ARF7) (Harper et al., 2000), a transcriptional activator of auxin regulated genes. Later on, loss-of-function mutations of genes encoding auxin receptors of the TIR/AFB family and gain-of-function mutation of the auxin repressor MASSUGU2 (MSG2)/IAA19 confirmed the involvement of auxin signaling in

positive phototropism, as all these mutants have aphototropic hypocotyls (Tatematsu et al., 2004; Whippo and Hangarter, 2005).

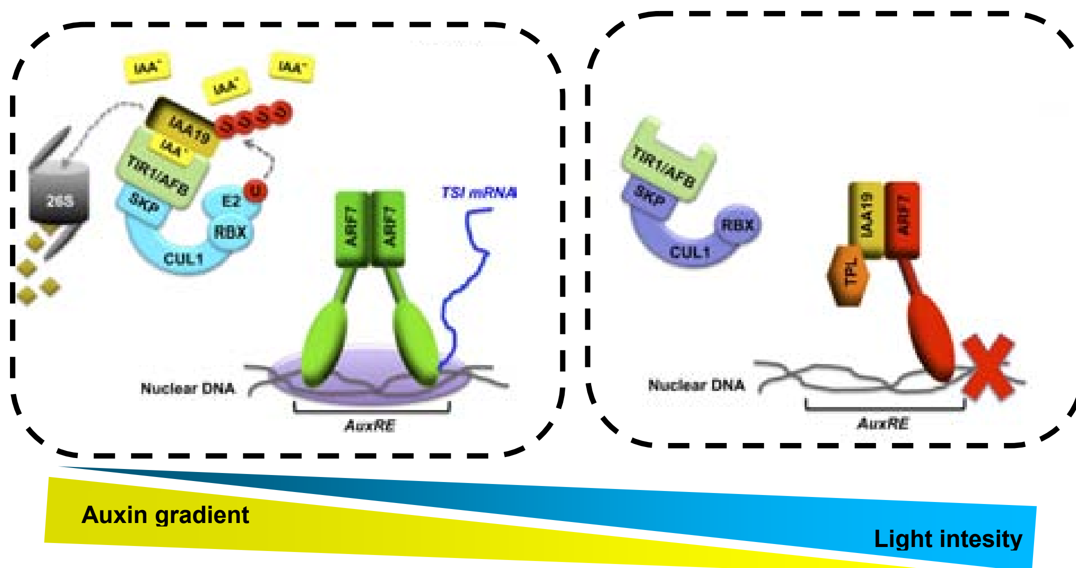

Figure 11. Model for regulation of transcription by auxin.
Cells at the illuminated side of the hypocotyl contain basal levels of auxin (right panel). In the nucleus, the ARF transcription factors, such as NPH4/ARF7 (red), are bound to their DNA target sequences (AuxRE) forming heteromeric complexes with a dominant transcriptional repressor protein, such as AUX/IAA19 (gold) and a corepressor, such as TPL (Topless) (orange). This complex is transcriptionally inactive, and, thus, transcription of auxin-regulated genes is repressed. Also present in the nucleus is the $SCF^{TIR1/AFB}$ auxin receptor complex (blue-violet and light green) in its inactive state. By contrast, BL-stimulated lateral auxin fluxes increase the auxin concentration of the cells at the shaded side of the hypocotyl (left pannels). In the nucleus of these cells, auxin stimulates the binding of AUX/IAA proteins, such as IAA19, to the $SCF^{TIR1/AFB}$ complex (green and light blue), which in turn promotes polyubiquitination of AUX/IAA proteins and its subsequent degradation by the 26S proteasome (gray). Removal of AUX/IAA proteins releases the corepressor TPL and allows homodimerization of ARF proteins, which stimulates transcription of target genes, such as TSI genes. Modified from (Liscum et al., 2014).

Objectives

This thesis work had the following objectives:

1. Generation of Arabidopsis transgenic lines overexpressing a catalytically active subunit of protein kinase CK2 ($CK2^{OE}$) and characterization of the phenotypic traits of these plants, with particular emphasis on roots.

2. Analysis of the role of CK2 in the cross-talk between salicylic acid (SA) and auxin signalling pathways. Within this general objective we stablished two specific objectives:

   2.1. Characterization of the effects of SA accumulation on root growth in Arabidopsis thaliana.

   2.2. Study of gene expression of the basic machinery for polar auxin transport in SA-treated plants, SA mutants and CK2 mutants.

3. Identification and characterization of new substrates of the plant protein kinase CK2 in Arabidopsis thaliana, with special emphasis on putative proteins involved in auxin-signalling pathways.

Chapter 1: Functional interplay between protein kinase CK2 and salycilic acid sustains PIN transcriptional expression and root development

Summary

We have previously reported that CK2-defective *Arabidopsis thaliana* plants (CK2mut plants) were severely impaired in root development and auxin polar transport, and exhibited transcriptional misregulation of auxin-efflux transporters (Marques-Bueno et al., 2011a). In this work we show that CK2mut roots accumulate high levels of salicylic acid (SA) and that the gene encoding isochorismate synthase (*SID2*) is overexpressed, strongly suggesting that CK2 activity is required for SA biosynthesis via the shikimate pathway. Moreover, SA activates transcription of CK2-encoding genes, and thus, SA and CK2 appear to be part of an autoregulatory feedback loop to fine-tune each other's activities. We also show that exogenous SA and constitutive high SA levels in *cpr* mutants reproduce the CK2mut root phenotypes (decrease of root length and of number of lateral roots), whereas inhibition of CK2 activity in SA-defective and SA-signalling mutants lead to less severe phenotypes, suggesting that the CK2mut root phenotypes are SA-mediated effects. Moreover, exogenous SA mediates transcriptional repression of most of *PIN-FORMED (PIN)* genes, which is the opposite effect observed in CK2mut roots. These results prompted us to propose a model in which CK2 acts as a link between SA homeostasis and transcriptional regulation of auxin-efflux transporters. We also show that CK2 overexpression in Arabidopsis has neither impact on SA biosynthesis nor on auxin transport, but it improves the Arabidopsis root system. Thus, unlike in mammals, an excess of CK2 in plant cells does not produce neoplasia, but it might be advantageous for plant fitness.

Introduction

The protein kinase CK2 is an ubiquitous Ser/Thr kinase, with a tetrameric structure composed of two catalytic (α) and two regulatory (β) subunits (Niefind et al., 2001; Litchfield, 2003). Pharmacological and genetic tools have demonstrated that CK2 activity is essential for cell survival in yeast, mammals and plants (Padmanabha et al., 1990; Moreno-Romero et al., 2008). Loss-of-function mutants of CK2 are in most cases not viable; however, development of cell culture techniques and use of conditional mutants made it possible to get important information about the biological functions in which CK2 is involved. Pioneering studies revealed a phenotype of cell cycle arrest by inhibition of CK2 activity (Pepperkok et al., 1994; Hanna et al., 1995; Espunya et al., 1999; Moreno-Romero et al., 2011), and extension of these studies led to formulate the hypothesis that CK2 might function as a cell survival factor by acting on chromatin remodelling and other epigenetic mechanisms (Ahmed et al., 2002; Moreno-Romero et al., 2012).

CK2 subunits are encoded by multigene families, which in plants include more members than in mammals and yeast. For instance, the *Arabidopsis thaliana* genome contains four genes encoding the CK2α subunit (one of them with predicted chloroplast localization), and four genes encoding the CK2β subunit (Salinas et al., 2006). Small gene families have been also reported in other plant species (Riera, Peracchia, de Nadal, et al., 2001; Espunya et al., 2005; Salinas et al., 2006). Antisense expression of a CK2α-encoding gene revealed some negative effects on light-regulated responses (Lee, 1999), and generation of an Arabidopsis CK2α1α2α3 triple mutant resulted in late flowering, reduced hypocotyl growth, smaller cotyledon size, reduced number of lateral roots, and ABA-signalling defects (Mulekar et al., 2012). A stronger impact on plant development was obtained by construction of a CK2 dominant negative mutant. This mutant was generated by conditional overexpression of a CK2α-inactive subunit in Arabidopsis (Moreno-Romero et al., 2008). Long-term induction of the transgene was lethal, confirming that CK2 activity is essential for cell survival. Short-time induction, however, resulted in phenotypes similar to those exhibited by auxin-defective mutants, affecting cell expansion, gravitropism, phototropism, and lateral root formation. The authors demonstrated that auxin transport was partially impaired in this mutant and that most of *PIN*-formed (PIN) genes, encoding auxin-efflux transporters, were misregulated (Marques-Bueno et al., 2011a; Marques-Bueno et al., 2011b). Moreover, some of the PIN transporters showed a tendency to be found internalized in endosome-like particles.

The direction of auxin flux within the plant is largely determined by the polar localization at the plasma membrane (PM) of the auxin transporters, among them a subset of PIN proteins (PIN1, 2, 3, 4, and 7) (Petrásek et al., 2006). PIN localization and function requires clathrin-mediated endocitosis (Kitakura et al., 2011). Auxin inhibits PIN endocitosis via rapid depletion of clathrin light- and heavy-chains from PM, by a mechanism that requires AUXIN BINDING PROTEIN1 (ABP1)-mediated auxin signaling (Robert et al., 2010; Sauer and Kleine-Vehn, 2011). Clathrin light chains (CLCs) and heavy chains (CHCs) are associated to both PM and trans-golgi network/early endosome, and this localization is differentially regulated by auxin. The mechanism by which ABP1 regulates clathrin-mediated trafficking and auxin signaling has been recently proposed (Wang et al., 2013).

Salicylic acid (SA) is an important signaling molecule mainly involved in plant defense (Vlot et al., 2009). Unexpectedly, it has been recently found that SA has an effect on the endocitic traffic of auxin-efflux transporters. High levels of SA interfere with PIN1 and PIN2 internalization, therefore increasing the levels of those proteins at the PM (Du et al., 2013). These results show that SA and auxin converge in the regulation of clathrin-dependent endocitic mechanism that regulates PIN trafficking and, ultimately, both auxin flux and distribution. The components of the SA-mediated mechanism are not yet known.

Over a decade ago, the involvement of CK2 in the transcriptional regulation of SA-signalling pathways was postulated (Hidalgo et al., 2001). The authors demonstrated that CK2 inhibitors hindered the transcriptional activation of early SA-regulated genes in tobacco cell extracts. Later on, Kang and Klessig, (2005) demonstrated that CK2 phosphorylated *in vitro* several members of the TGA family of transcription factors (TFs). These TFs recognize the *as-1* cis-acting element that confers response to SA and other hormones. Moreover, CK2-mediated phosphorylation had an inhibitory effect on TGA2-binding to *as-1*, which is contradictory with the results from other authors (Stange et al., 1997; Hidalgo et al., 2001). On the other hand, TGA-binding to DNA is positively regulated by interaction with NPR1 (nonexpressor of pathogenesis-related (PR) genes), a master regulator of plant defence. NPR1 resides in the cytoplasm as an oligomer and is translocated to the nucleus upon SA increase (usually after pathogen attack), triggering the transcription of defence genes. However, a rapid turnover of NPR1 in the nucleus is essential to promote gene transcription; this is achieved by phosphorylation and ubiquitination, which targets NPR1 to the proteosome (Spoel et al., 2009). Recent results suggest that two adaptor proteins, NPR3 and NPR4, which are SA receptors with different affinities, also bind NPR1 and might be involved

in the regulation of NPR1 stability (Zhang et al., 2006; Fu and Dong, 2013). NPR1 also directly activates the expression of the plant-specific WRKY family of transcription factors, which might act as either transcriptional activators or repressors (Wang et al., 2006). NPR1 expression is itself under the regulation of the WRKY factors (Yu et al., 2001). WRKY factors bind to DNA sequences called W-boxes that have been implicated in plant defence responses to pathogens. Moreover, the gene promoter of isochorismate synthase, an enzyme involved in SA biosynthesis, is enriched with W boxes (Wildermuth et al., 2001). In a transcriptional profiling study, Maleck et al., (2000) discovered that W boxes are overrepresented in a cluster of genes sharing the induction pattern of *PR-1*, suggesting a role for WRKY factors in the systemic acquired resistance (SAR).

On the other hand, the transcriptional responses to auxin are driven by the well-characterized Auxin Response Factors (ARFs) that bind the Auxin Response Elements (AuxREs). However, promoters of the auxin-regulated genes in Arabidopsis and rice show a high occurrence of b-ZIP and MYB-responsive elements located close to AREs (Berendzen et al., 2012). This observation led to propose that these two families of TFs might act as modulators of the auxin-elicited transcriptional responses. The b-ZIP family of TFs is composed of 162 elements in Arabidopsis, which have been classified in different subfamilies according to their structural features, and the Arabidopsis MYB family is composed of 198 genes. Both families of TFs control responses to light, biotic and abiotic stress and plant development, among other biological processes (Jakoby et al., 2002; Bailey et al., 2003; Yanhui et al., 2006).

In this work, we have used gain-of-function and loss-of-function mutants of the protein kinase CK2 to further investigate the role of this kinase in auxin-signalling functions. We show that plants overexpressing CK2 exhibit improved root systems, due to faster growth of the main root and earlier emergence of lateral roots, providing evidence that CK2 might be an important target to improve plant fitness. Moreover, we show that depletion of CK2 activity increases the roots' endogenous salicylic acid levels (SA), indicating that CK2 activity is required for SA biosynthesis. Moreover, our data show a link between SA and auxin transport, which requires a functional CK2. We propose a model in which CK2 and SA are part of a regulatory feed-back loop, underpinning control of root development and auxin transport.

Experimental procedures

Plant material

Arabidopsis thaliana (Col-0 ecotype) and the transgenic lines generated in the same genetic background were grown at 21 to 22ºC under 16 h photoperiod light (140 µE m^{-2} sec^{-1}). For in vitro germination and culture, seeds were surface sterilized and grown in Murashige and Skoog (MS) plates (Duchefa Biochemie BV, http://www.duchefa.com/) supplemented with 0.5% (w/v) sucrose and 1.2% (w/v) agar. Generation of CK2mut plants has been described in Moreno-Romero et al., (2008). The SA-deficient *sid2-1* mutant was sexually crossed with the CK2mut line and homozygous plants were selected by hygromycin resistance and by PCR. *cpr1*, *cpr5* and *cpr6* mutants (Clarke et al., 2000) were a king gift of X. Dong (Duke University, Durham, NC, USA). *NahG* and *npr1-1* mutants (Delaney et al., 1994; Durrant and Dong, 2004) were obtained from P. Tornero (IBMCP-Valencia, Spain).

Plant treatments and phenotypes

Expression of *CK2mut* transgene was induced with 1 µM Dexamethasone (DEX) for the indicated times, and controls with DEX solvent (ethanol) were performed in all cases. Salicylic acid (SA) was dissolved in ethanol and treatments were performed at 0.25 mM for the indicated times. Treatments with 10 µM 4,5,6,7-tetrabromobenzotriazol (TBB) dissolved in DMSO were performed on 5-day-old WT plantlets for 16 h. Lengths of primary roots were measured in seedlings grown vertically in Petri dishes and analyzed using the IMAGEJ software (rsb.info.nih.gov/ij). The number of lateral roots was determined using a Leica DMRB optical microscope, and the results are represented as frequency distributions in histograms (frequency denotes the number of plants containing the indicated number of emerged lateral roots or of lateral root primordia) or as mean values ± SD. For the root gravitropic assay, 5-day-old seedlings grown on vertically-oriented plates were reoriented by 90º, left to grow for 24h, and reoriented again by 90º. Plants were scanned with a Bio-Rad GS-700 Imaging Densitometer (Bio-Rad Laboratories, www.biorad.com). Statistical analyses of data were performed either in EXCEL (Microsoft, www.microsoft.com) or the R program (www.R-project.org), using the Student's two-tailed *t*-test for independent samples ($p \leq 0.05$).

Generation of transgenic CK2^{OE} plants

CK2α-encoding sequence was amplified by PCR, using specific primers based on the *NtCKA3* cDNA sequence (GenBank/EMBL bank accession no. AJ438263). The product was cloned into the pE3n vector (Dubin et al., 2008), giving rise to the pE3n-CKA3 plasmid with a *c*-myc-encoding epitope at the 5'-end, and suitable for recombination by the Gateway system. The pE3n-CKA3 was then recombined with the destination vector pMDC32 (Curtis and Grossniklaus, 2003), giving rise to the expression subclone pMDC32-CKA3. The pMDC32-CKA3 was introduced into *Agrobacterium tumefaciens* GV3101 pMP90, and transgenic *Arabidopsis* plants were generated by the modified floral dip method (Logemann et al., 2006). The CK2^{OE} x *DR5::GFP* line was obtained by sexual crossing, and *DR5::GFP* detection was performed by confocal microscopy, as in (Marques-Bueno et al., 2011a).

RT-PCR analysis

Total RNA was extracted with Trizol (Life Technologies, www.lifetechnologies.com/) and first-strand cDNA synthesized with iScript cDNA synthesis kit (Bio-Rad Laboratories). Quantitative PCR was performed using a Bio-Rad CFX96 real-time PCR Detection System and SYBR Green Master Mix (Bio-Rad Laboratories). The specificity of the PCR reactions was confirmed by melting curve analysis (55–95ºC). The -ΔCt values were calculated relative to either *EF-1-α* (*at5g60390*) or *actin2* (*at3g18780*) (Livak and Schmittgen, 2001). The annealing temperature used to amplify the *actin2* by quantitative RT-PCR was 60º and the specific primers were the following: F: tgcttgcaccaagcagcatgaa; R: ccgatccagacactgtacttcctt. The specific primers and the annealing temperature for the rest of genes have been previously described (Marques-Bueno et al., 2011a; Moreno-Romero et al., 2012). Statistical analyses of data were performed with either the Student's two-tailed *t*-test for independent samples ($p \leq 0.05$) (Excel, Microsoft) or with ANOVA ($p \leq 0.05$) (R program, www.R-project.org).

Protein extracts, western blots, enzymatic activities and hormone analysis

Proteins were extracted from frozen root tissue homogenized in cold protein extraction buffer (50 mM Tris–HCl, pH 7.5, 100 mM KCl, 10% glycerol, 0,1% Triton x-100, 2mM DTT, 5mM EDTA, 0,5% (w/v) polyvinylpolypyrrolidone). For immunoblots, proteins

were electrophoresed on 10% SDS-PAGE gels, transferred to immobilon-P membranes (Millipore, www.millipore.com), and then incubated with 1:1000 anti-*c*-myc antibodies (GenScript, www.genscript.com). The immunocomplexes were revealed using the Lumi-Light Western Blotting Substrate system (Roche, www.roche.com). Loading of equal quantities of proteins was controlled by Bradford's analysis using BSA as a standard, and by Ponceau-staining of the membranes. Indole-3-acetic acid and salicylic acid (SA) were determined as described in Müller and Munné-Bosch, (2011). CK2 enzymatic assays in crude extracts were performed as described by Espunya et al., (1999) using 50 µM of the specific peptide RRRADDSDDDDD (Jena Bioscience GmbH, www.jenabioscience.com) and [γ^{32}P]-ATP (1000-2000 c.p.m. $pmol^{-1}$). Proteins were extracted with Tris-HCl pH 7.5 50 mM, NaCl 50 mM, MgCl2 10 mM, PMSF 1 mM, beta-glicerophosphate 25 mM, NaF 20 mM, sodium orthovanadate 0.2 mM, and protease Inhibitor cocktail 1/1000 (Sigma). One enzymatic unit was defined as the amount of enzyme that incorporates 1 pmol of ^{32}P into the substrate per minute at 30ºC. All the assays were made in triplicate.

In silico analysis of promoters

SA and auxin responsive *cis*-elements were searched using the Patmatch software of TAIR web site (Yan et al., 2005) (http://www.arabidopsis.org/). Sequences of TAIR10-3000bp Loci Upstream Sequences and TAIR10 5' UTRs datasets were screened for the presence of the regulatory elements. For the sake of simplicity, only 2,000 bp upstream from the transcriptional start site were analyzed. Analysis of ATH1 Affymetrix arrays data was performed as in Marques-Bueno et al., (2011a).

Results

Generation and characterization of Arabidopsis transgenic plants overexpressing a catalytic subunit of the protein kinase CK2

The CK2α-encoding sequence was amplified by PCR using *NtCKA3* cDNA as a template (GenBank/EMBL bank accession no. AJ438263). For immunodetection purposes, a *c*-myc-encoding epitope was introduced at the N-terminal end of the coding sequence. The construct was cloned into a binary vector and used to transform Arabidopsis plants by means of *Agrobacterium tumefaciens.* Several independent transgenic lines were isolated and

brought to homozygosis using hygromycin as a selection factor. Expression of the CK2α transgene was confirmed by RT-PCR using specific primers (one of them corresponding to the *c*-myc-encoding region and the other to the CKA3-encoding sequence) (Figure 12a). We also performed quantitative RT-PCR reactions to amplify separately the transcripts of *AtCK2αA* and *AtCK2αB* genes (the two CK2α-encoding genes predominantly expressed in Arabidopsis, (Moreno-Romero et al., 2011)) and of *CKA3*, in order to compare the total *CK2α* transcript levels in WT and transgenic plants. The results are shown in Figure 12b. As *CKA3* is only expressed in $CK2^{OE}$ transgenic plants and *CK2αA* and *CK2αB* are similarly expressed in WT and transgenic plants we conclude that the total amount of transcripts encoding the CK2α subunit is higher in $CK2^{OE}$ transgenic plants. Moreover, accumulation of the CKA3 transgenic protein was detected using a *c*-myc antibody (Figure 12c) and measurement of CK2 activity in whole-cell extracts incubated with radiolabelled ATP and with a CK2-specific peptide (see Experimental procedures) revealed CK2 activity increments ranging from 5 to 36 % in $CK2^{OE}$ lines, compared to wild-type plants (Figure 12d).

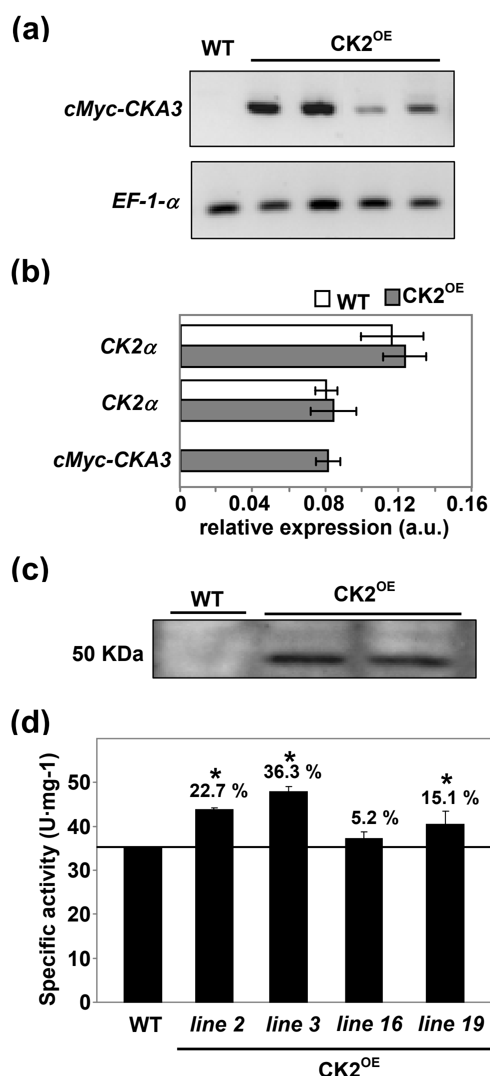

Figure 12. Molecular characterization of Arabidopsis transgenic lines overexpressing CK2α subunit.
(a) Transgenic Arabidopsis lines, previously selected by Hy^R (F3 generation), were analyzed by RT-PCR, using specific primers to amplify the CK2α transgene. Amplified *EF-1-α* transcript levels were used as loading control. **(b)** Quantification of CK2α-encoding gene expression in $CK2^{OE}$ roots. Transcript levels of endogenous Arabidopsis CK2α-encoding genes (*CK2αA* and *CK2αB*) and of CK2α transgene (cMyc-*CKA3*) were measured separately by quantitative RT-PCR. Values are the means of three biological replicates (±SD) and are shown as relative expression versus that of the constitutive *actin2* gene (*at3g18780*). **(c)** Western blot, using an anti-*c*-myc antibody. Only two of the several analyzed lines are shown. **(d)** Overall CK2 activity in wild-type and $CK2^{OE}$ transgenic lines. The data shown are the mean of three replicates (±SD), and two independent experiments were performed. The activity percentage for each $CK2^{OE}$ line (relative to wt) is shown above each bar. (*) Asterisks denote statistically significant differences using Student's *t*-test at $p \le 0.05$. *Abbreviations:* WT, wild-type Arabidopsis plants; $CK2^{OE}$, CK2α-overexpressing plants; a.u., arbitrary units.

We then investigated whether CK2α overexpression ($CK2^{OE}$) produced significant phenotypic changes in Arabidopsis roots. Figure 13a shows that the growth rate of primary root was slightly but significantly enhanced in the different $CK2^{OE}$ lines (Figure13a). We also found that those lines exhibited an increment in the number of lateral roots (LRs) (Figure 13b-c, and Figure S1a).To further investigate the effect of CK2α overexpression in LR formation we performed a detailed study in one of the lines showing the highest increment in CK2 activity (line 3). We distinguished between lateral root primordia (LRP) and emerged roots (according to the classification in Péret et al., (2009)). Figure 13b shows that $CK2^{OE}$ plants had more emerged lateral roots but less LRPs than wild-type (WT) plants. These results suggest that lateral roots arise earlier in $CK2^{OE}$ plants. To check this hypothesis, we quantified the number of LRPs in younger seedlings (5-day-old); we found an average of 4 LRP and 8 LRP in WT and $CK2^{OE}$ seedlings, respectively (Figure 13c). Moreover, lateral root density (number of lateral roots per mm) was higher in $CK2^{OE}$ seedlings (Figure S1b). Meristem size, however, was similar in $CK2^{OE}$ and WT seedlings (Figure S1c), as well as auxin distribution, as measured by the expression of *DR5::GFP* reporter in $CK2^{OE}$ x *DR5::GFP* seedlings (F3 generation) (Figure S1d). On the other hand, $CK2^{OE}$ seedlings showed normal gravitropic response (not distinguisble from that of WT plants) (Figure S1e). Additional pictures of $CK2^{OE}$ root phenotypes are shown in Figure S2.

We conclude that CK2α overexpression does not alter either the basic pattern of root morphology or the distribution of auxin and/or auxin-regulated responses such as root gravitropism. Moreover, CK2α overexpression increases the rate of root growth and development, resulting in improved root system that might have important consequences for the efficiency of nutrients uptake.

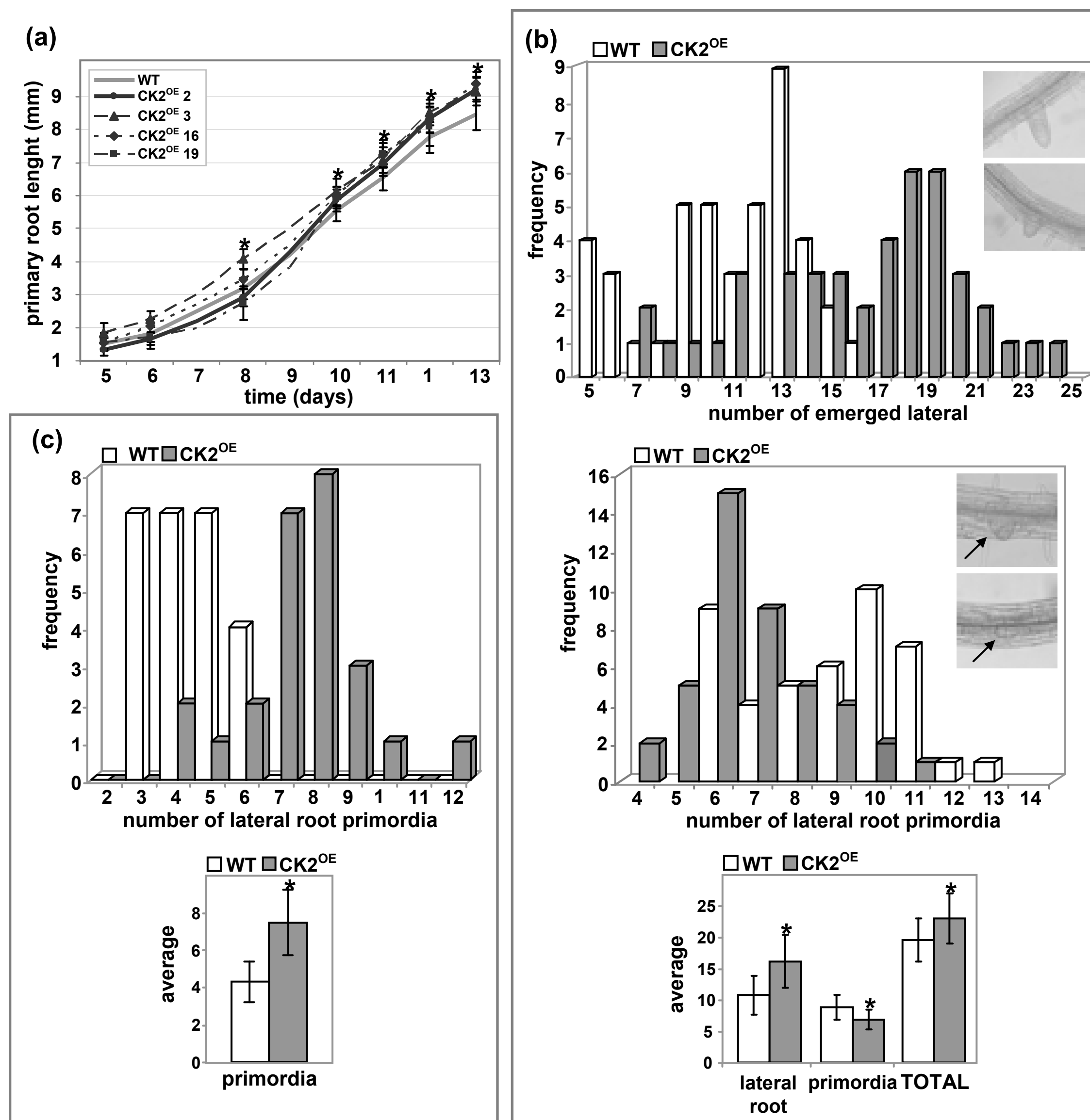

Figure 13. **Root phenotypes of CK2α-overexpressing plants.**
(a) Quantification of root lengths (primary roots) in WT and CK2OE seedlings. Results shown for WT and for four independent transgenic lines are the mean values ±SD (n=10-25); the experiment was repeated two times with similar results, and only the data from one of them is shown. (*) Asterisks denote statistically significant differences between WT and CK2OE lines at the indicated times. **(b)** Number of lateral roots in 10-day-old seedlings (CK2OE3). The histograms show frequency distributions of the number of emerged lateral roots (top) or of root primordia (middle), according to the classification in Peret *et al*. (2009). The frequency denotes the number of plants containing the indicated number of emerged lateral roots or of lateral root primordia. Mean values ±SD (n≥40) are shown at the bottom panel; three independent experiments were performed. The insets show pictures of lateral roots at the indicated stages. **(c)** Number of lateral roots in 5-day-old seedlings (CK2OE3). Data are represented as in (b), but note that only root primordia are seen at this developmental stage (top). Mean values ±SD (n≥25) are shown at the bottom panel; three independent replicates were performed. *Abbreviations:* WT, wild-type Arabidopsis plants; CK2OE, CK2α-overexpressing plants; SD, standard deviations. Statistical analyses were performed using Student's *t*-test at p≤0.05, and statistical significances are marked with asterisks (*).

Protein kinase CK2 is a component of the auxin- and SA-signalling pathways

We have previously shown that CK2-defective plants (CK2mut plants) were impaired in auxin polar transport, although the content of indole-3-acetic acid (IAA) was unchanged in CK2mut seedlings (Marques-Bueno et al., 2011a). We now determined IAA and SA content in excised roots of WT, CK2mut and $CK2^{OE}$ lines. Our results show similar IAA levels in $CK2^{OE}$, CK2mut (+/-Dex) and WT roots (Figure 14a). However, CK2mut roots exhibited a spectacular increase in salicylic acid (SA) content, as compared to WT and $CK2^{OE}$ roots. This high SA content was only detected after induction with dexamethasone, indicating that it was a consequence of *CK2mut* transgene expression (Figure 14a). It has been recently reported that auxin and SA counteract during the adaptative response to stress (Wang et al., 2007; Iglesias et al., 2011), and thus we wondered whether the previously described IAA-related phenotypes of CK2mut plants were a consequence of their elevated SA levels. To check this hypothesis, Arabidopsis WT plants were incubated with SA. Figure 14b shows that exogenous SA triggers inhibition of both root length and lateral root formation, phenocopying the morphological characteristics of CK2mut roots (Marques-Bueno et al., 2011a). We then crossed CK2mut plants with the SA-deficient *sid2* mutant. *sid2* is a loss-of function mutant of the isochorismate synthase 1 (ICS1) (Wildermuth et al., 2001), an enzyme of the shikimate pathway, involved in SA biosynthesis in plants. We isolated homozygous lines for both mutations (CK2mut x *sid2* double mutant, F3 generation), and we obtained a partial but significant recovery of the WT root phenotype (Figure 14c). The recovery of the root length was small but statistically significant. Moreover, the recovery of the number of lateral roots was clearly visible and statistically significant in CK2mut x *sid2* double mutant. Quantification of SA in the CK2mut x *sid2* double mutant revealed that DEX-mediated induction of *CK2mut* transgene still increased SA levels in the *sid2* background (Figure 14a), which agrees with the partial but not complete recovery of the WT root phenotypes.

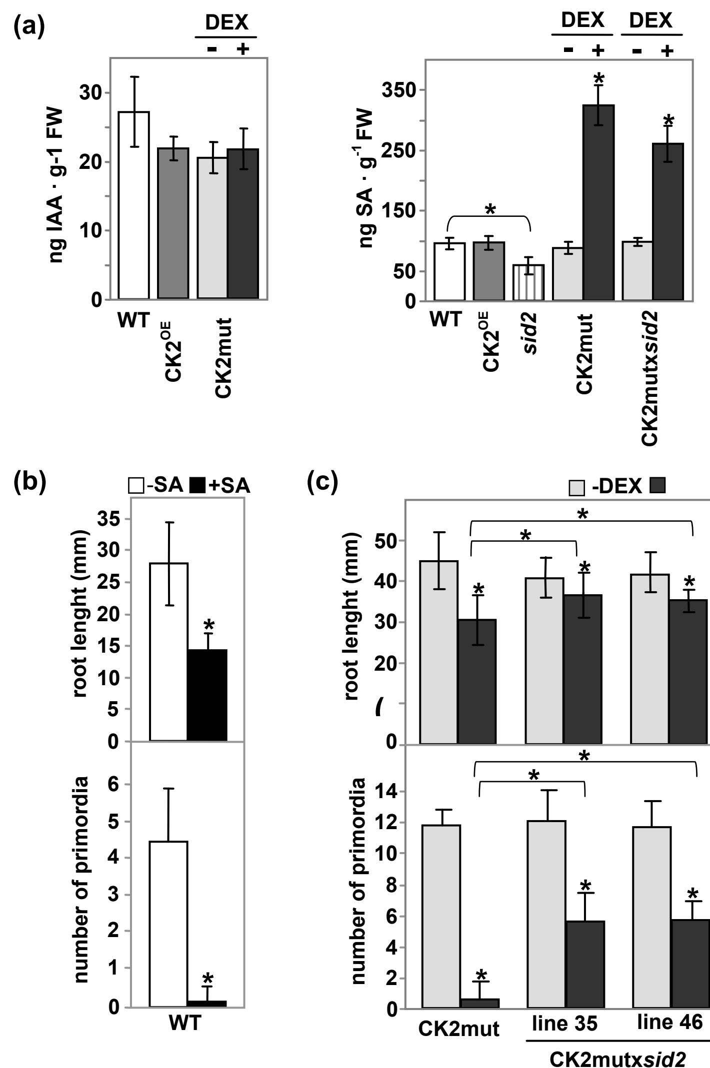

Figure 14. Influence of salicylic acid on root phenotypes.
(a) Quantification of indole-acetic acid (IAA) and salicylic acid (SA) in 10-day-old roots of different Arabidopsis lines. CK2mut and CK2mut x *sid2* lines were incubated with either dexamethasone (+DEX) or ethanol (-DEX) for the last 72 h before hormone determinations. Values shown are the mean (±SE) of 10 biological replicates. **(b)** Root phenotypes of Arabidopsis wild-type seedlings incubated with 0.25 mM SA for 48 h. Mean values (±SD) are shown (n≥20). **(c)** Root phenotypes of CK2mut x *sid2* double mutant (± DEX, as in a). The CK2mut line (± DEX) was used as a control. Mean values (±SD) are shown (n≥20). Statistical analyses were performed using Student's *t*-test at p≤0.05, and significant differences were marked with asterik (*). *Abbreviations:* FW, fresh weight; CK2OE, CK2-overexpressing line; SE, standard errors; SD, standard deviations.

To corroborate the idea that the CK2mut root phenotypes are a direct consequence of the elevated SA levels in the mutant, we used additional mutants affected in either SA content or SA-signalling. The *CONSTITUTIVE EXPRESSER OF PR1* mutants *(cpr1, cpr5 and cpr6* mutants*)* show high levels of SA (Clarke et al., 2000). We measured the primary root length and the number of lateral roots in *cpr1, cpr5 and cpr6* and we found that they exhibited shorter roots and fewer lateral roots, with the only exception of the number of lateral roots in *cpr1*, which was similar to that in WT plants (Figure 15a). We also used two Arabidopsis mutants that are impaired in SA-mediated effects, the SA-defective *NahG* mutant (expressing bacterial SA hydroxylase, (Delaney et al., 1994), and the *npr1-1* mutant *(NONEXPRESSER OF PATHOGENESIS-RELATED PROTEIN1)*, which is impaired in SA-signalling (Durrant and Dong, 2004)**.** *NahG* and *npr1-1* mutants were incubated with 4,5,6,7-tetrabromobenzotriazole (TBB), a strong inhibitor of CK2 (Shugar, 1994) that has been previously used by us in combination with the CK2 mutant (Moreno-Romero et al., 2008; Marques-Bueno et al., 2011a; Moreno-Romero et al., 2012). We first measured IAA and SA levels in WT plants incubated with TBB. Figure 15b (left panel) shows that IAA levels were unchanged, whereas SA levels increased significantly, as it happened in Dex-treated CK2mut plants. Moreover, incubation of the SA-defective *NahG* plants with TBB produced a slight increase of SA, but to a much less extent than in WT plants (Figure 15b, right panel). The effect of TBB on the number of lateral roots is shown in Figure 15c. TBB-treated WT plants show a significant decrease of lateral roots (number of LRs in TBB-treated plants versus that in control plants: 0.48), whereas TBB-treated *NahG* or *npr1-1* mutants show a higher ratio of LRs in TBB-treated versus control plants (0.74 and 0.71, respectively). The experiments were performed with a high number of individuals (≥40) and the statistical analyses show significant differences between genotypes.

The experiments with TBB did not allow us to assess the differences in the root growth rate. TBB produced complete growth arrest in all genotypes, at the different concentrations tested. Indeed, we had previously observed and reported that the effect of TBB on plant phenotypes was qualitatively similar but quantitatively much stronger than the effect of *CK2mut* gene expression (Moreno-Romero et al., 2008; Marques-Bueno et al., 2011a).

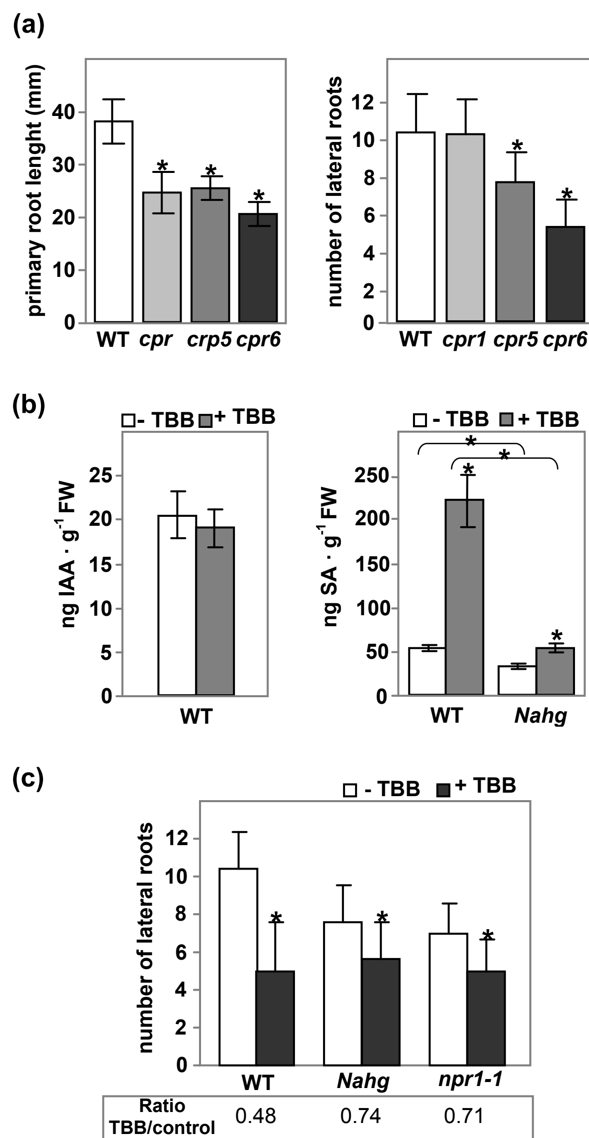

Figure 15. Salicylic acid mutants and inhibition of CK2 activity with 4,5,6,7-tetrabromobenzotriazol (TBB).
(a) Primary root length and number of lateral roots in *CONSTITUTIVE EXPRESSER OF PR1 (cpr)* mutants. Experiments were performed with 10-day-old seedlings of *cpr1*, *cpr5* and *cpr6.* Data shown are the mean values ± SD (n ≥ 10). **(b)** Effects of TBB on hormone levels. Quantification of indole-acetic acid (IAA) in WT Arabidopsis roots (±TBB) (left panel), and of salicylic acid (SA) in WT and *NahG* roots (± TBB) (right panel). Hormones were quantified in 10-day-old roots after 16 h of TBB treatments (10 µM). Data shown are the mean values (±SE) of 10 biological replicates. **(c)** Quantification of the number of lateral roots in TBB-treated plants. Five-day-old plants were incubated with 10 µM TBB for 16 h and then transferred to plates without TBB. The number of lateral roots was counted 5 days after removing theTBB. *Abbreviations:* Wild-type plants (WT), SA HYDROXYLASE mutant (*NahG*), and *npr1-1 (NONEXPRESSER OF PATHOGENESIS-RELATED PROTEIN1)* mutant. Statistical analysis was performed using Student's *t*-test at p≤ 0.05. Asterisk (*) indicates statistically significant differences in comparison to the corresponding control plants.

Interplay between CK2 activity and SA-triggered transcriptional responses

We have previously reported that the basic machinery for polar auxin transport (PIN protein family and protein kinase PINOID) was misregulated in CK2mut plants (Marques-Bueno et al., 2011a). To study the contribution of SA, if any, to this misregulation, we performed a time-course study of *PIN* and *PID* expression in Arabidosis WT plants incubated with 0.25 mM salicylic acid (Figure 16a). Transcript levels were measured in roots by quantitative RT-PCR. Our results show that exogenous SA down-regulates *PIN1*, *PIN4* and *PIN7* and up-regulates *PID*, and that those effects remained for as long as 48H. *PIN2* and *PIN3* showed a bimodal response to SA, with transient up-regulation at the beginning of the treatment (Figure 16a). Moreover, a time-course study of *PIN/PID* expression in Dex-treated CK2mut roots revealed that *PIN2, PIN4* and *PIN7* were up-regulated in CK2mut plants, in spite of the elevated SA content of this mutant. On the other hand, *PIN1* and *PID* expression showed similar responses in CK2mut or WT + SA plants (down-regulation for *PIN1* and up-regulation for *PID*) (Figure 16b).

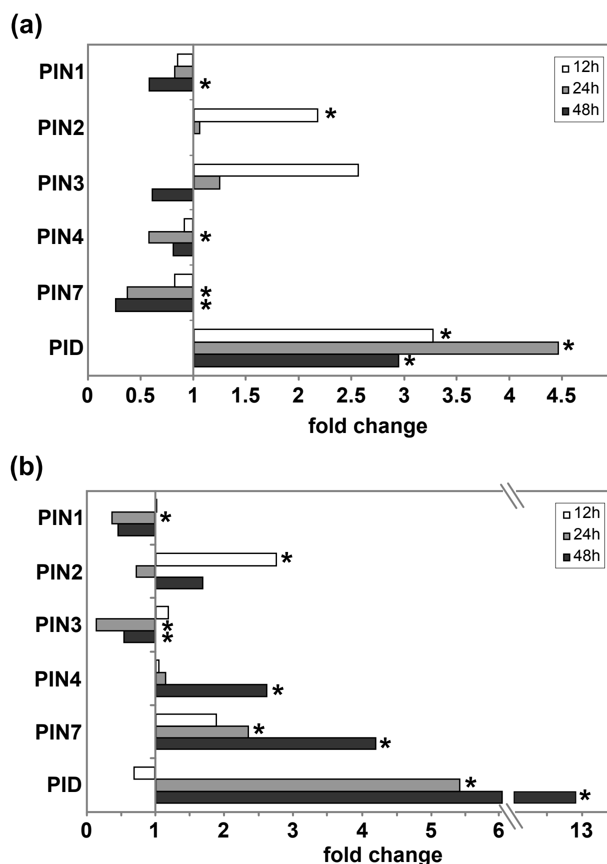

Figure 16. Influence of salicylic acid on *PIN* and *PID* expression.
Fold changes of *PIN* and *PID* transcript levels in Arabidopsis WT plants incubated with 0.25 mM salicylic acid (SA) **(a)** or in CK2mut plants treated with Dex **(b)** for the indicated times. Transcript levels were measured by quantitative RT-PCR in roots and normalized to those of *EF-1-α* gene. Mean values of three biological replicates were obtained, with standard deviations always ≤30%. The data are represented as fold changes in SA-treated or Dex-treated plants versus their respective controls. Asterisks (*) indicate statistical significant differences of treated plants versus untreated plants, using the Student's *t*-test ($p \leq 0.05$). Statistical significance was assigned to a fold-change value of 2.

To further study the influence of SA content and CK2 activity on *PIN* and *PID* expression, we used the SA-defective *sid2* mutant and the CK2mutx*sid2* double mutant. In an independent experiment, *PIN* and *PID* transcript levels were measured in all the conditions and lines shown in Table 4. The results are shown as fold changes of gene expression (in Dex-treated versus untreated roots for CK2mut, *sid2* x CK2mut and *sid2*; versus WT roots for WT+SA and $CK2^{OE}$). Dex inductions and SA treatments were carried out for 48 H in these experiments. Interestingly, our data show that *PIN1* and *PIN3* fold-changes were similar in CK2mut roots and SA-treated plants, and showed a tendency to increase in the CK2mut x *sid2* double mutant (which contains less amounts of SA) (statistical analyses of these data, using the Students' *t*-test can be seen in Table S1 of the digital version of the manuscript). These results strongly suggest that SA is sufficient to repress *PIN1* and *PIN3* expression, and that *PIN1* and *PIN3* down-regulation in CK2mut roots is a consequence of the high SA content in this mutant. To the contrary, *PIN4* and *PIN7,* which were also repressed by exogenous SA, appeared strongly up-regulated in CK2mut roots, revealing a CK2-dependent mechanism underlying their response to SA. Concordantly, *PIN4* and *PIN7* transcript levels were similar in CK2mut and CK2mut x *sid2* mutants. On the other hand, *PID* transcript levels were much higher in CK2mut roots than in SA-treated WT roots, and they did not decrease in the CK2mut x *sid2* double mutant. Thus, although SA is sufficient to increase *PID* transcription, additional mechanisms, involving CK2 activity, might come into play in order to explain the high *PID* transcript levels found in CK2mut roots.

Taken together, these results show that most of the genes involved in auxin-efflux transport are transcriptionally responsive to SA, but that the mechanism underlying this response is very complex and exhibits differential characteristics between genes. In some cases, the SA-triggered response is independent of CK2 activity (*PIN1* and *PIN3*), whereas in others it is CK2-dependent, and depletion of CK2 activity either changes the response (giving the opposite effect, such as in *PIN4* and *PIN7*) or enhances it (*PID*). Table 4 also shows that constitutive overexpression of CK2 ($CK2^{OE}$ plants) does not significantly affect *PIN* and *PID* transcript levels.

Table 4. Fold-changes of *PIN* and *PINOID* (PID) gene expression in different Arabidopsis lines and conditions.
Transcript levels were measured by quantitative RT-PCR in roots of 7-day-old seedlings. Values were normalized to those of *EF-1-α* gene, and mean values of three biological replicates were obtained, with standard deviations always ≤30%. The results are shown as fold changes of gene expression (in Dex-treated versus untreated roots for CK2mut, *sid2* x CK2mut and *sid2*; versus WT roots for WT+SA and $CK2^{OE}$). Statistical analyses were performed between pairs of conditions, using the Student's *t*-test at p≤0.05 (the *p*-values are shown in Table S1 of the digital version of the manuscript). Compared conditions are denoted with the same letter, and capital letters indicate statistically significant differences whereas lower letters indicate no significant differences. Fold-changes in $CK2^{OE}$ plants were not compared with the rest of conditions because these plants do not exhibit changes in endogenous SA levels. $CK2^{OE}$ plants did not showed statistically significant changes in *PIN*/*PID* expression as compared to their control (WT plants) (Student's *t*-test, p≤0.05).

	$CK2^{OE}$	WT + SA	CK2mut	CK2mutx*sid2*	*sid2*
PIN1	1.61	0.59 ^{ab}	0.48 ^{aC}	0.66 ^{bCd}	0.85 ^{d}
PIN2	1.54	1.00 ^{Ab}	2.74 ^{AC}	1.13 ^{bCd}	1.16 ^{d}
PIN3	1.47	0.62 ^{aB}	0.69 ^{aC}	0.98 ^{BCd}	1.28 ^{d}
PIN4	1.27	0.82 ^{AB}	3.43 ^{Ac}	3.55 ^{BcD}	1.13 ^{D}
PIN7	1.58	0.27 ^{AB}	3.85 ^{Ac}	4.45 ^{BcD}	0.95 ^{D}
PID	1.10	2.94 ^{AB}	17.29 ^{Ac}	18.30 ^{BcD}	1.04 ^{D}

CK2-encoding genes are transcriptionally regulated by SA in Arabidopsis

To get more insight about the mutual influence between CK2, SA and auxin, we investigated the transcriptional response of Arabidopsis CK2-encoding genes to exogenous SA. Our results show that all the CK2α- and CK2β- encoding genes were overexpressed in roots of Arabidopsis seedlings incubated with SA. In particular, *CK2αA*, *CK2β1* and *CK2β3* were overexpressed 2.21-, 2.73- and 2.74-fold, respectively (Table 5). Moreover, CK2-encoding genes were down-regulated in the SA-defective *NahG* mutant and in the SA-signalling *npr1-1* mutant, and were slightly up-regulated in the SA-overproducing *cpr6* mutant. Statistical analyses of the data shown in Table 5 (ANOVA, p≤0.05) showed that the fold changes of CK2-encoding genes expression were significantly different between the different conditions and genotypes. Additional statistical analyses between pairs of conditions, performed by the Student's *t*-test (*p*-values shown in Table S1), corroborated the above conclusions.

Taken together, these results support the idea that the CK2-encoding genes are transcriptionally regulated by SA. Moreover, they revealed the existence of a regulatory feed-back loop between SA and CK2, in which SA mediates up-regulation of CK2-encoding genes whereas CK2 activity, in its turn, limits SA accumulation. Moreover, overexpression of CK2 does not alter this regulatory loop.

Table 5. Regulation of CK2-encoding gene expression by salicylic acid (SA).
Transcript levels of CK2-encoding genes were measured in WT plants incubated with 0.25 mM SA for 48 H (WT +SA) and in SA-biosynthetic and SA-signalling mutants. Values were obtained by quantitative RT-PCR in 7-day-old roots and normalized to those of *EF-1-α* gene. Mean values of three biological replicates are shown as fold-changes of transcript levels versus those in WT roots, with standard deviations always ≤30%. Statistical analyses to assess differences in gene expression between the different lines and conditions were carried out for each gene, using One-way ANOVA (p≤0.05). The expression changes were statistically significant for all genes. Pairs of conditions were also compared by the Student's *t*-test and the *p*-values are shown in Table S1 (of the digital version of the manuscript). *Abbreviations: CK2αA* and *CK2αB*: Arabidopsis CK2α-encoding genes. *CK2β1-4*: Arabidopsis CK2β-encoding genes. *npr1-1 (NONEXPRESSER OF PATHOGENESIS-RELATED PROTEIN1)* (Durrant and Dong, 2004)*:* Arabidopsis mutant impaired in SA-signalling; *cpr1* and *cpr6 (CONSTITUTIVE EXPRESSER OF PR1)* (Clarke *et al.*, 2000)*:* Arabidopsis mutants with constitutive high levels of SA; *NahG (SA HYDROXYLASE)* (Delaney *et al.*, 1994): SA-defective Arabidopsis mutant.

	CK2αA	***CK2αB***	***CK2β1***	***CK2β2***	***CK2β3***	***CK2β4***
WT + SA	2.21	2.01	2.73	1.96	2.74	1.91
cpr1	1.24	1.22	0.90	1.04	1.14	1.09
cpr6	1.5	1.77	1.59	1.31	1.56	1.58
npr1-1	0.59	0.61	0.39	0.51	0.6	0.62
NahG	0.58	0.5	0.54	0.48	0.52	0.48
***p*-value**	**0.034**	**0.013**	**0.012**	**0.012**	**0.000**	**0.007**

Genome-wide expression changes in CK2mut seedlings of genes involved in SA-signalling

Genome-wide expression profiling in CK2mut seedlings was obtained using ATH1 Affymetrix microarrays, as previously reported (Marques-Bueno et al., 2011a; Moreno-Romero et al., 2012). We analyzed the expression changes of genes involved in SA-signalling. The results are shown in Table S2 of the digital version of the manuscript, with the genes grouped according to their biological function. The complete array of data can be found at NASCARRAYS-642 (http://affymetrix.arabidopsis.info/).

An important number of genes encoding transcription factors (TFs) show significant fold changes: two members of the TGA-type (bZip family), three members of the WRKY family, nine members of the myb family and one member of the Dof-type zinc finger domain-containing protein. Fourteen out of the fifteen genes were up-regulated, and one gene was down-regulated. Thus, depletion of CK2 activity has a profound impact on the expression of transcription factors that mediate the SA responses.

Moreover, the genes encoding both isochorismate synthase 1 (*SID2* gene) and phenylalanine ammonia-lyase1 (*PAL1* gene) were up-regulated. These two enzymes participate in SA biosynthesis by alternative pathways, and their up-regulation can explain the high SA content found in CK2mut seedlings. Moreover, other authors have demonstrated that *SID2* is, in its turn, up-regulated by SA (Wildermuth et al., 2001), and that its promoter contains *cis*-elements specific for families of TFs that appear up-regulated in the CK2 mutant. On the other hand, *NPR4,* which is considered a SA receptor and that might play a role in regulating NPR1 stability (Zhang et al., 2006; Fu and Dong, 2013), is also up-regulated in CK2mut seedlings. NPR1 is an important co-transcription factor in SA-signalling functions. Thus, all these data support the idea that both SA homeostasis and signalling are disturbed in CK2mut seedlings.

Additionally, other SA-responsive genes misregulated in CK2mut seedlings encode proteins involved in defence, cell protection (against oxidative stress, for example), signal transduction (protein kinases and phosphatases), or they have putative structural roles (glycine-rich and proline-rich proteins). Most of the genes were up-regulated.

In silico promoter analysis of auxin-responsive genes

It is well known that the transcriptional responses to auxin are primarily mediated by *cis*-regulatory Auxin Response Elements (AREs), which are recognized by *trans*-factors called Auxin Response Factors (ARFs) (Ulmasov et al., 1999). Although ARE sequences are sufficient to confer auxin responsiveness, they are often found as composite elements in the natural promoter context (Ulmasov et al., 1995) or in association to other *cis*-elements recognized by different families of transcription factors (Berendzen et al., 2012). In concordance with these findings, putative MYB related elements (MREs) or bZIP response elements (ZREs) have been found to modulate transcriptional auxin responses (Heinekamp et al., 2004; Shin et al., 2007). MREs and ZREs are also involved in responses to biotic and

abiotic stresses. On the other hand, the WRKY plant-specific family of transcription factors has a prominent role in the SA-triggered responses, particularly through NPR1, and in SA biosynthesis through the shikimate pathway (Wildermuth et al., 2001; Yu et al., 2001; Wang et al., 2006).

We analyzed the promoters (2,000 bp) and 5'-untranslated regions (UTR) of *AUX1*, *PID*, and five members of the *PIN* gene family, for the presence of *cis*-elements specific to *trans*-acting factors of the ARF, WRKY, and bZip families. The results are shown in Figure S3 and in Table S3 of the digital version of the manuscript. As expected, all these promoters contained at least one ARE element; in many cases, the ARE box was repeated several times (up to 6 times in the *PIN3* promoter, including one copy in the 5'-UTR region). Moreover, all these promoters contain many copies of the W-box, which is present either as a single motif or in close proximity to other regulatory elements (either an ARE element, another W-box, or an *as-1* element). The *as-1* element is recognized by the TGA family of TFs (bZip type), and it confers response to SA and other hormones, auxin among them. Several members of the TGA family have been reported as CK2 substrates in plants (Kang and Klessig, 2005). The *as-1* element occurs with less frequency than the others *cis*-acting elements in the promoters analyzed, and it is absent from the *PIN1* and *PIN3* regulatory regions. The *as-1* element was found as a single motif or in close proximity to AREs, W-boxes, or another copy of *as-1*.

It has been postulated, that those bipartite and tripartite organizations of *cis*-elements might have functional significance in the modulation of gene transcriptional responses (Berendzen et al., 2012). In the case of auxin–mediated responses, bZIP- and MYB-related binding sites are potential AuxRE-coupling elements in auxin-mediated transcription. The promoters of the *PIN* and *PID* genes show significant differences among them, both in abundance and distribution of auxin- and SA-responsive elements. Although experimental analysis is needed to test the functionality of the *cis*-elements, those differences might account for the differential qualitative and quantitative responses to SA measured for *PIN* and *PID* genes in this work.

Discussion

Whereas depletion of CK2 activity is lethal for Arabidopsis plants (Moreno-Romero et al., 2008), constitutive overexpression of a CK2 catalytic subunit is not deleterious, but, on the contrary, entails some advantages for plant development, such as faster growth of the root system. Thus, it is interesting to note that, unlike in mammals (Piazza et al., 2012), CK2α-subunit overexpression in plants does not produce neoplasia, providing support to the idea that plant cells have a development plasticity that enables growth optimisation in a variety of conditions.

Very little is known about the signalling pathways in which plant CK2 participates. We previously showed that CK2 loss-of-function mutants (CK2mut plants) have shorter roots and are impaired in lateral root formation. In this work we show that those are salicylic acid-mediated effects. Several data support our conclusion: 1) CK2 loss-of-function mutants have enhanced levels of SA; in particular, roots of CK2mut seedlings show increments of 3.7-fold as compared to the WT levels; 2) both, Arabidopsis WT plants incubated with exogenous SA, and Arabidopsis *cpr* mutants (which contain high constitutive SA levels), show the same root phenotypes as CK2mut seedlings; and 3) partial depletion of CK2 activity (using genetic of pharmacological tools) in SA-defective and SA-signalling mutants (*sid2*, *NahG, npr1*) resulted in less severe root phenotypes. Moreover, these findings reveal the existence of a negative regulatory point in the SA biosynthesis that is bypassed in CK2-defective seedlings. In addition, we demonstrate that CK2-encoding genes are transcriptionally up-regulated by SA, and that the NPR1-mediated pathway is involved in this regulation. Taken together, these data strongly suggest the existence of an autoregulatory feed-back loop between CK2 and SA. Analysis of transcript profiles in CK2mut seedlings revealed up-regulation of *SID2* and *PAL1* genes, involved in two alternative SA-biosynthesis pathways, as well as of many transcription factors belonging to families involved in the transcriptional regulation of SA-responsive genes (Qin et al., 1994; Maleck et al., 2000; Krawczyk et al., 2002). It has been previously reported that CK2 mediates post-translational regulation of transcription factors belonging to the TGA family, which recognize the SA-responsive *as-1* element (Kang and Klessig, 2005). These findings support the idea that CK2 activity is required for both SA signalling and homeostasis.

SA levels can also influence auxin polar transport in plants (Du et al., 2013). Stimulation of SA biosynthesis and the subsequent SA accumulation, as it occurs during biotic stress, inhibits auxin polar transport, and this appears to be a plant defence

mechanism to hinder pathogens from growth and reproduction (Wang et al., 2007). As CK2mut seedlings accumulate such high levels of SA (which was concomitant with constitutive up-regulation of *PR-1*), and show up-regulation of *PIN2*, *PIN4* and *PIN7*, as well as of *PID* (Marques-Bueno et al., 2011a; Moreno-Romero et al., 2012), we decided to investigate the role, if any, of SA in the transcriptional regulation of *PIN* and *PID* genes. Surprisingly, Arabidopsis WT plants incubated with SA showed significant down-regulation of all the members of the *PIN* gene family, with the exception of *PIN2*. SA-mediated repression of *PIN7* was previously reported by other authors (Wang et al., 2007), and now this SA-mediated effect can be extended to most of the *PIN* family members, suggesting that this might be the mechanism underlying inhibition of auxin transport by SA. Moreover, these results also show that the high SA content in CK2mut roots is not able to mediate repression of *PIN4* and *PIN7* genes, and, thus, that this particular SA-signalling pathway is impaired in CK2-defective plants. We propose that the SA-mediated transcriptional regulation of *PIN4* and *PIN7* is CK2-dependent. CK2 activity might be required for transcriptional regulation and/or post-translational modification of particular TFs involved in *PIN* transcriptional control. Analysis of the 5'-regulatory sequences in *PIN* promoters showed the presence of many copies of *cis*-acting elements that mediate both SA- and auxin-responses, in particular of W-boxes.

On the other hand, our results also show that SA is sufficient to up-regulate *PID* transcription. However, CK2mut roots show much higher *PID* transcript levels than SA-treated WT plants, suggesting that at least two mechanisms converge into *PID* transcriptional regulation, one CK2-dependent and another CK2-independent.

In conclusion, this work presents evidence that CK2 mediates SA responses by affecting both SA biosynthesis and SA-signalling pathway. Overexpression of CK2 does not have a significant impact on SA-mediated effects, indicating that an excess of CK2 activity does not produce an imbalance in the CK2/SA mutual influence. To the contrary, loss-of-function of CK2 mutants presents defects in SA accumulation and in SA-mediated root phenotypes. Very importantly, loss-of-function of CK2 activity also impairs the SA-signalling pathway that links SA and auxin transport, a mechanism exploited by pathogens to bypass plant defences.

Chapter 2: Protein kinase CK2 maintains the "dark state" of the protein core complex phot1/NPH3, a central modulator of the phototrophic response

Summary

Plant stems grow towards the direction of light to ensure its efficient capture necessary to achieve photosynthesis and development. This is called positive phototropism and is initiated by autophosphorylation of membrane-associated blue-light photoreceptors, the phototropins (phot). Phot1 is the substrate of a CUL3-Ring E3-ubiquitin ligase, which contains the phototropic transducer NPH3 as a substrate adaptor ($CRL3^{NPH3}$). It has been proposed that $CRL3^{NPH3}$-mediated phot1 ubiquitination is a mechanism to modulate phot1 internalization and turnover in response to unidirectional BL, and that NPH3 signalling capacity is controlled by its phosphorylation state. In this work we show that protein kinase CK2 interacts with several members of the plant specific NPH3/RPT2 protein family, including NPH3 itself. We also demonstrate that CK2 phosphorylates NPH3 *in vitro*, and that inhibition of CK2 activity *in vivo* prevents NPH3 re-phosphorylation during light to dark transition of Arabidopsis seedlings. Furthermore, we found that phot1 is constitutively ubiquitinated in CK2-depleted plants, supporting the idea that CK2-mediated phosphorylation of NPH3 inhibits the activity of the $CRL3^{NPH3}$ complex. On the other hand, CK2-defective plants exhibited depletion of phot1 from the plasma membrane, which is in agreement with the aphotropic phenotype of plants depleted of CK2 activity. Contrary to what was previously reported, we found that light-induced phot1 movement from the plasma membrane to the cytoplasm was independent on the activity of the $CRL3^{NPH3}$ complex. Moreover, ubiquitin-independent phot1 internalization occurred both under dark and light conditions in the absence of CK2. Thus, CK2 also regulates phot1 movement by yet unidentified mechanisms.

Introduction

Plants, as sessile organisms, have developed the ability to sense and adapt to changes in their surrounding environment. Particularly, plants have evolved a wide range of photoreceptors that perceive variations in the direction of light, as well as in its quality, intensity and duration, allowing plants' optimal growth and development in the ever-changing light conditions. Red (R) and far-red (FR) light are detected by the phytochromes (phys), UV-B light by the UVR8 receptor and UV-A/blue light (BL) by three families of photoreceptos: phototropins (phots), cryptochromes (crys), and the members of the ZTL/FKF1/LKP2 family (see (Hohm et al., 2013) for a review). Downstream light signalling involves complex protein regulatory networks notably regulated by posttranslational protein modifications. For instance, in the case of photomorphogenesis and circadian rhythms regulation, phosphorylation and ubiquitination act cooperatively to fine-tune responses to light inputs (Ni et al., 2014; Fujiwara et al., 2008).

Hypocotyl (the embryonic stem) phototropism, or reorientation of hypocotyl growth toward the light, represents an adaptive response to ensure plant photosynthetic capacity. The unilateral blue light (BL) is sensed by membrane-associated photoreceptors called phototropins (phot1 and phot2) that are Ser/Thr protein kinases. Blue light perception by phototropins is followed by the activation of a signal transduction pathway leading to the formation of asymmetric auxin gradients necessary for organ bending (Esmon et al., 2006). phot2 is specialized in the phototropic response under high intensity BL, whereas phot1 is involved in both low and high BL-driven phototropism (Sakai et al., 2000). In dark grown seedlings, phot1 is located at the inner surface of the plasma membrane (Sakamoto and Briggs, 2002), but BL irradiation induces a rapid but incomplete movement of phot1 to the cytoplasm (Han et al., 2008; Kaiserli et al., 2009; Sakamoto and Briggs, 2002; Wan et al., 2008). Both phot1 kinase activity and BL-induced phot1 autophosphorylation are necessary for phot1 internalization via the clathrin-dependent endocytic pathway (Kaiserli et al., 2009; Roberts et al., 2011). Phot1 interaction with the phototropic signal transducer NPH3 is necessary for early phototropic signalling (Motchoulski and Liscum, 1999). NPH3 is a BTB-NPH3 containing protein that belongs to the largely unknown plant-specific NRL (for NPH3/RPT2) family (Stogios et al., 2005; Motchoulski and Liscum, 1999; Pedmale et al., 2010). Phot1 is a substrate of the $CRL3^{NPH3}$ complex, a CUL3-based E3 complex that contains NPH3 as a substrate adapter. The ubiquitination pattern of phot1 and its functional significance depends on the intensity of the blue-light. Under low light intensities, phot1 is

mono/multiubiquitinated, a modification that has been proposed to target the receptor for its light induced internalization (Roberts et al., 2011). Additionally, under high-light conditions, phot1 is also polyubiquitinated, inducing its 26S proteasome–dependent degradation, probably as a means of receptor desensitization (Roberts et al., 2011).

In addition, BL modifies the NPH3 phosphorylation state, which might modulate the BL-induced signal transduction pathway. NPH3 is phosphorylated in darkness, which is believed to correspond to the inactive state of the protein. Following BL irradiation, NPH3 is dephosphorylated in a phot1-dependent manner, which likely corresponds to the active form of the protein (Pedmale and Liscum, 2007). Importantly, phot1 itself is not the kinase that phosphorylates NPH3. It was proposed that dephosphorylated NPH3 is incorporated into the $CRL3^{NPH3}$ complex, which ubiquitinates phot1 (Roberts et al., 2011). Because dephosphorylated NPH3 is rapidly phosphorylated when seedlings are transferred from light to dark conditions, Pedmale and Liscum, (2007) proposed that the protein kinase that phosphorylates NPH3 is “constitutively” present and active. One such kinase that fulfills this requirement is the protein kinase CK2.

Indeed, CK2 has extensively been involved in photomorphogenesis and circadian rhythms in Arabidopsis (for a revision see Mulekar and Huq, (2013)), although it is not a light activated kinase (Moreno-Romero et al., 2011). CK2 is a highly pleiotropic and evolutionary conserved dual specificity protein kinase, with the minimum phosphorylation consensus sequence motif Ser/Thr–Xaa–Xaa–Acidic, where X represents any non-basic amino acid (Pinna, 2002). The CK2 holoenzyme has a tetrameric structure composed of two catalytic (α) and two regulatory (β) subunits (Niefind et al., 1998; Litchfield, 2003) but, both subunits can also be found as monomeric active forms (Filhol et al., 2004). Several light signaling- or clock-related transcription factors have been shown to be phosphorylated by CK2 *in vitro* and/or *in vivo*. For instance CK2-mediated phosphorylation of HY5 and HFR1 in their N-terminus reduces their affinity for COP1, resulting in higher protein stability (Osterlund et al., 2000; Saijo et al., 2003; Mulekar and Huq, 2013; Hardtke et al., 2000; Park et al., 2008). Moreover, phosphorylated HY5 has a lower DNA-binding capacity in vitro (Hardtke et al., 2000). On the contrary, phosphorylation of PIF1 by CK2 promotes its degradation through the 26S proteasome system (Bu, Zhu, Dennis, et al., 2011). Mulekar and Huq, (2013) suggested that CK2 could also phosphorylate PIF3 (and other PIF proteins), as PIF3 is phosphorylated under both dark and light conditions (Ni et al., 2013) and some of the phosphorylation sites correspond to CK2 consensus sequences.

Here, we show that CK2 interacts with several members of the NRL protein family, including NPH3. We show that CK2 phosphorylates NPH3 *in vitro* and that CK2 activity is required for NPH3 phosphorylation *in vivo* in light to dark transition experiments. Furthermore, inactivation of CK2 activity promotes NPH3-mediated phot1 ubiquitination in the dark. Consistently, CK2 inhibition abolished the phototropic response of the hypocotyl. We also show that CK2 depletion produces phot1 relocation from the plasma membrane to the cytosol both in dark and light conditions. Surprisingly, this effect is independent of NPH3, suggesting that CK2 has a dual role in phototropism: i) on one side, CK2 controls the phosphorylation status of NPH3, which might regulate its incorporation into the $CRL3^{NPH3}$ complex and phot1 ubiquitination, and ii) on the other side, CK2 controls phot1 localization in a process that is independent of NPH3.

Experimental procedures

Plant material, plant growth and phenotype analysis

Arabidopsis thaliana wild type (Col-0) and Arabidopsis mutants used in this study were generated in Col-0 genetic background and had been described previously elsewhere: CK2mut (Moreno-Romero et al., 2008), *phot1-5* (Huala et al., 1997), *phot1-5*PHOT1::phot1-GFP (Sakamoto and Briggs, 2002), *nph3-6phot1-5*PHOT1::phot1-GFP (Roberts et al., 2011). Seeds were surface sterilized and then cold-treated (4ºC) for 3 d in the dark and illuminated with red light for 2h prior germination at 22±2ºC in culture chambers. Seedlings were grown on Petri plates containing 1% (w/v) agar-solidified Murashige and Skoog (MS) medium, or in 15mL of half-strenght liquid MS medium in 250-mL Erlenmeyer flasks with continuous shaking (50 rpm). Hypocotyl phototropic responses were measured in dark-grown 5-d-old seedlings that were exposed to unilateral blue-light for the last 24h. Expression of the *CK2mut* transgene was induced with 1µM dexamethasone (DEX) (Sigma, www.sigmaaldrich.com) dissolved in ethanol (30mM stock solution) and inhibition of CK2 activity with 4,5,6,7-tetrabromobenzotriazol (TBB) (Calbiochem, www.emdmillipore.com) dissolved in DMSO (20 mM stock solution) at the indicated concentrations. Both treatments were perfomed 24 h prior to blue-light stimulus and the drug was kept in the medium until the end of the experiment. Blue-light was obtained by filtering light through one layer of blue acrylic (195 Zenith blue, LEE Filters, www.leefilters.com), and the different fluence rates were obtained by changing the distance between the light source and the plant material. The

angle of hypocotyl curvature was measured with the ImageJ software (rsb.info.nih.gov/ij). *Nicotiana benthamiana* plants, used for transient expression experiments, were grown in the greenhouse under 16-h-light/8-h-dark conditions.

Gene constructs

CK2α-encoding sequence was amplified by RT-PCR, using specific primers based on the *Nicotiana tabacum CKA3* (NtCKA3) cDNA sequence (GenBank/EMBL bank accession no. AJ438263). ENP, NPY3 and NPH3 full-length coding sequences (as described in TAIR8; www.Arabidopsis.org) and their corresponding SID sequences were obtained by PCR amplification using the appropriate primers (Table S4 of the printed version of the manuscript) and 7-d-old Arabidopsis seedlings cDNA as template. Gateway compatible PCR products were introduced to pDONOR221 vector (Life technologies, www.lifetechnologies.com) and subsequently recombined to tag-containing destination vectors used for expression.

For yeast two-hybrid (Y2H) interaction assays, GAL4-AD-CK2α, GAL4-AD-NPH3, GAL4-AD-sid–NPH3 and GAL4-DBD-CK2α were obtained using pACT2-AD-GW (Shimoda et al., 2008) and pGBKT7-DBD-GW (Rajagopala and Uetz, 2011) vectors, respectively. For subcellular localization, using transient expression in *N. benthamiana* leaves, GFP-ENP, GFP-sid ENP, GFP-NPY3, GFP-sid NPY3, GFP-NPH3 and GFP-sid NPH3 constructs were generated using the pMDC43 vector (Curtis and Grossniklaus, 2003). For BIFC assay, we used the binary vectors pYFN43 and pYFC43 (Belda-Palazón et al., 2012) to obtain cYFP-CK2α, nYFP-ENP, nYFP-NPH3, cYFP-NPY3 and nYFP-CK2α constructs. Strep-tagged NPH3 was constructed by recombination with the pEU-E01-STREP-GW vector, modified from the pEU-E01 of the EndexT Technology from Cell Free Sciencies (CFS, www.cfsciences.com). Briefly, the pEU-E01 was amplified by PCR using the following primers: pEU-STREP-HpaI-Fw (tcttttcaaattgaggatgagaccaagaagccatatggatatcttggtgatgtagata gg) and pEU-STREP-HpaI-Rev (ccggtttggttaacagaggttccggataactcgagctcggtacctgtccgcggtcg) to introduce a STREP-tag II (MAS-WSHPQFEK; IBA-lifescience) and a unique HpaI site between the EcorV and XhoI sites of the pEU-E01 vector. The primers were then 5'phosphorylated for ligation purposes. The methylated template DNA was digested by the DpnI enzyme (NEB, www.neb.com) and ligated (Quick Ligase, NEB). The resulting plasmid was digested with HpaI, ligated with the gateway cassette A (Life Technologies) and

transformed into ccd survival cell (Life Technologies) to produce the pEU-E01-STREP-GW vector.

Yeast-two-hybrid analysis

Yeast two-hybrid screening was performed by Hybrigenics Services, S.A.S., Paris, France (www.hybrigenics-services.com). The coding sequence for the full-lenght *Nicotiana tabacum* CK2A3 (GenBank accession number gi: 45238336) was PCR-amplified and cloned into pB27 as a C-terminal fusion to LexA (N-LexA- ck2A3-C). The construct was checked by sequencing and used as a bait to screen a random-primed *Arabidopsis thaliana* seedlings cDNA library constructed into pP6. pB27 and pP6 derive from the original pBTM116 (Vojtek and Hollenberg, 1995) and pGADGH (Bartel et al., 1993) plasmids, respectively.

55 million clones (5-fold the complexity of the library) were screened using a mating approach with YHGX13 (Y187 ade2-101::loxP-kanMX-loxP, matα) and L40ΔGal4 (mata) yeast strains as previously described (Fromont-Racine et al., 1997). 169 His+ colonies were selected on a medium lacking tryptophan, leucine and histidine, and supplemented with 0.5 mM 3-aminotriazole to handle bait autoactivation. The prey fragments of the positive clones were amplified by PCR and sequenced at their 5’ and 3’ junctions. The resulting sequences were used to identify the corresponding interacting proteins in the GenBank database (NCBI) using a fully automated procedure. A confidence score (PBS, for Predicted Biological Score) was attributed to each interaction as previously described (Formstecher et al., 2005).

Validation of the interaction between CK2α and full-lenght NPH3 was performed by mating AH109 (mata) yeast strain containing the pACT2-AD constructs and Y187 (matα), the yeast strain containing the pGBKT7-DBD-CK2α gene construct. Mating reactions were plated in SD/DO-Trp-Leu to select for diploids and positive colonies were transferred to SD/DO-Trp-Leu-His to select for diploids in which protein interaction was occurring.

BIFc and subcellular localization

The abaxial air spaces of *N. benthamiana* leaves were co-infiltrated with a mixture of *Agrobacterium tumefaciens* GV3101/pMP90 harbouring the appropriate constructs and of *A. tumefaciens* C58C1 expressing the p19 protein of the tomato bushy stunt virus to suppress gene silencing (Voinnet et al., 2003) (Plant Bioscience Limited, www.pbltechnology.com), according to the procedure described in Belda-Palazón et al., (2012). For BIFC,

Agrobacterium strains containing the fluorescent translational fusion constructs and the p19 plasmid were mixed at OD_{600} 0.4:0.4:0.4, and for subcellular localization analysis at 0.5:0.5. Visualization was performed at 2 to 4 days post-infiltration.

NPH3 expression, purification and in vitro CK2 phosphorylation assay

Strep-NPH3 protein was synthesized using wheat germ cell-free extracts (EndexT Technology, CFS), according to the manufacturer's instructions. Soluble protein was obtained by the large-scale bilayer reaction for protein translation carried out at 16ºC for 20h, using 1.5% Brij58 detergent in the reaction mixture. Strep-NPH3 was purified from the soluble fraction using Strep-tactin gravity flow columns (IBA-lifesciences*,* www.iba-lifesciences.com*).* Protein elution was performed with a buffer containing 2.5mM desthiobiotin.

In vitro CK2 phosphorylation assays were carried out with 600 ng of strep-NPH3 or β-casein (as a positive control) and 250 U of human recombinant heterotetrameric CK2 (hrCK2) (Calbiochem), at 30ºC during 1h with the following buffer: 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$ and 200 µM ATP. The final reaction volume was 40µl, and the reaction was stopped by addition of protein loading buffer. TBB dissoved in DMSO (2 µM) was used as CK2 inhibitor.

Extraction of microsomal proteins and total proteins

Protein extracts were prepared from 4d-old Arabidopsis etiolated seedlings grown in liquid medium. For microsomal extracts, seedlings were incubated with 20µM TBB or DMSO for 2h, exposed to white light (50µmol·m-2·s-1) for 30 min, and then placed back in the dark for 30min or 1h, without removing TBB from the medium. Control plants were grown in the dark during the whole experiment. Plant tissues were ground in ice-cold microsomal extraction buffer (50mM HEPES, pH 7.4, 500 mM sucrose, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, complete mini EDTA-Free Protease inhibitor mixture tablets (Roche, www.roche.com), and PhosSTOP phosphatase Inhibitor cocktail tablets (Roche)) followed by two centrifugation steps at 13000xg to remove cell debris. The resultant supernatant was subjected to ultracentrifugation at 100,000g for 90 minutes. Pelleted microsomal membranes were resuspended in microsomal extraction buffer. All the extractions were achieved under dim red light at 4ºC. For total protein extracts, plant treatment procedure was the same as above,

except that seedlings were only exposed to 1h light. Plant tissues were ground in extraction buffer (50 mM HEPES, pH 7.8, 300 mM sucrose, 150 mM NaCl, 10 mM potassium acetate, 5 mM EDTA, 1% Triton X-100 and complete mini EDTA-free protease inhibitor mixture tablets (Roche)), followed by centrifugation at 10000xg. Protein concentration in the extracts was determined by Bradford's colorimetric method (Bio-Rad, www.bio-rad.com).

Immunoprecipitation and antibodies

Anti-GFP immunoprecipitation was carried out using µMACS technology (Miltenyi Biotech, www.miltenyibiotec.com) following the manufacturer's instructions for native elution by pH shift. In brief, 250 µg of total protein extracts were loaded to anti-GFP-microbeads columns and the bound proteins were eluted with TEA buffer (0.1M Triethylamine pH 11.8, 0.1% Triton X-100), and collected in tubes containing 1M MES, pH 3. 15 µl of immunoprecipitated proteins was used for analysis.

Immunoblots were performed using the following antibody dilutions: anti-phospho-(Ser/Thr) CK2 Substrate (P-S/T^{3}-100) (Cell Signalling Technology, www.cellsignal.com), 1:1000; anti-NPH3 (Motchoulski and Liscum, 1999; Pedmale and Liscum, 2007),1:10000; anti-GFP (Roche), 1:2000; anti-strep (Amersham pharma-biotech, www.gelifesciences.com),1:5000; anti-ubiquitin P4D1 (Santa Cruz Biotechnology, www.scbt.com), 1:100; anti-rabbit secondary antibody (Promega, www.promega.com), 1:5000; and anti-mouse secondary antibody (GE Healthcare, www.gelifesciences.com),1:5000.

Microscopy

Confocal fluorescence images from hypocotyls of 4-d-old Arabidopsis etiolated seedlings were acquired on a Zeiss LSM 700 microscope (www.zeiss.com). Etiolated seedlings were treated with 20µM TBB or DMSO in half-strenght liquid MS medium, either in complete darkness or exposed to white light (50 µmol·m-2·s-1) the last hour prior to visualization. Subcellular localization of GFP tagged proteins and reconstitution of the signal from split-YFP in BIFC assays was carried out using a Leica True Confocal Scanning (TCS) laser microscope (Leica SP5) (www.leica.com). GFP or YFP fluorescence visualization was achieved by sample excitation with a 488 nm laser and the spectral detection was set between 510 and 535 nm. Images were processed with LAS lite leica software and ImageJ.

Bioinformatic tools

Multiple Sequence Alignments were realized on the T-Coffee WEB server (Di Tommaso et al., 2011) using the homology extended mode (PSI-Coffee) recommended for remote homologs (Taly et al., 2011). Parameters were left to their defaults, *i.e.* the profiles built for each input sequence have been computed from the sequence of homologous proteins showing between 40 and 90 % of sequence identity in pairwise alignments. Prediction of putative CK2 phosphorylation residues was done with NetPhosK 1.0 Server using a threshold of 0.5 (Blom et al., 2004).

Results

CK2 interacts with NPH3 and NPH3-LIKE proteins

In order to identify novel substrates of plant protein kinase CK2, we performed a high-throughput yeast two-hybrid (Y2H) screen of a cDNA library from 7-d old Arabidopsis (*Arabidopsis thaliana*) seedlings, using a CK2 catalytic subunit (CK2α) as a bait. Out of 5.54 x 10^7 clones screened, we obtained 169 positive clones corresponding to 28 new putative CK2α-interacting proteins (Table S5 of the printed version of the manuscript). Among them, four members of the plant-specific NPH3/RPT2 family (or NRL family (Holland et al., 2009)) were found: ENP (for Enhancer of Pinoid, also named MACCHI-BOU 4 (MAB4) and NAKED PINS IN YUC MUTANTS 1 (NPY1)) (Treml et al., 2005; Furutani et al., 2007; Cheng et al., 2007), NPY3 (for NAKED PINS IN YUC MUTANTS 3, also named MAB4/ENP/NPY1-LIKE 2 (MEL2))(Cheng et al., 2008; Furutani et al., 2011), NPH3 (for NON-PHOTOTROPIC HYPOCOTYL 3, also named ROOT PHOTOTROPISM 3 (RPT3)) (Motchoulski and Liscum, 1999; Sakai et al., 2000), and a NPH3-LIKE protein of unknown function (AT5G66560). Figure 17a shows the conserved sequence features characteristic of the NRL protein family (Pedmale et al., 2010), and Figure 18b the sequence alignments of the four members of the family that interact with CK2. We noticed that the four members contain the N-terminal Broad-Complex/Tramtrack/ Bric-a-brac (BTB) domain and the centrally located NPH3 domain, but only NPH3 and NPH3-like proteins contain the C-terminal Coiled-Coil domain. The interaction site (thereafter SID, for Selected Interaction Domain) of each one of these four NRL proteins with CK2α was determined by sequencing, and it mapped to the C-terminal part of the polypeptide chain (Figure 17a and 17b, cyan boxes). The shortest

overlapping sequence between the four CK2-interacting NLR proteins contained 61 residues, located at the junction between the end of the NPH3 domain and its juxtaposed linker. We noticed that all the SIDs contained a highly conserved region of 26 amino acids, located at the C-terminal end of the NPH3 domain (Figure 17b). Interestingly, this region is conserved in all the members of the NLR family (Motchoulski and Liscum, 1999; Pedmale et al., 2010). The finding that four members of the same protein family were found in our yeast-two-hybrid screen and that they share overlapping and evolutionary conserved SID sequences suggested that this proteins might be true CK2 interactors as well as possible substrate. To confirm this hypothesis, we further studied the interaction between CK2α and the NRL protein members.

We first validated by yeast-two-hybrid, the interaction between CK2α and the full-length NPH3 protein, the founder member of the NRL family (Motchoulski and Liscum, 1999; Sakai et al., 2000) (Figure 17c). Next, in order to verify that these four members of the NRL family could be protein partners of CK2 *in planta*, we analyzed their subcellular localization as well as that of CK2α. To this end, we fused CK2, ENP, NPY3 and NPH3 with GFP and expressed them transiently in *N. benthamiana* epidermal cells. Figure S4a shows that CK2α is localized in the nucleus and nucleolus (as previously reported in Salinas et al., (2006)) and in the cytosol and at the cell periphery. ENP was found in endosomal compartments (Furutani et al., 2007), NPH3 at the plasma membrane (Motchoulski and Liscum, 1999) and NPY3 at the plasma membrane and partially in the cytosol (Furutani et al., 2011). Interestingly, NPH3 was not uniformly distributed within the plasma membrane, but in patches and membrane microdomains (Figure S4b). In addition, we also analysed the localization of the SID sequences fused to GFP (Figure S4a). We found that none of the SID domains was sufficient to target the corresponding NRL protein to their correct subcellular localization, indicating that additional protein domains are responsible for their localization into the cell. In the case of NPH3, the C-terminal coiled-coil domain (which is not present in the SID) has already been shown to be necessary for its membrane localization (Inoue, Kinoshita, Takemiya, et al., 2008).

Finally, we further confirmed that the full-length ENP, NPY3 and NPH3 interacted *in vivo* with CK2α by bimolecular fluorescence complementation analysis. CK2α and ENP interactions occurred in endosomal compartments, while it interacted with NPY3 and NPH3 at the plasma membrane (Figure 17d).

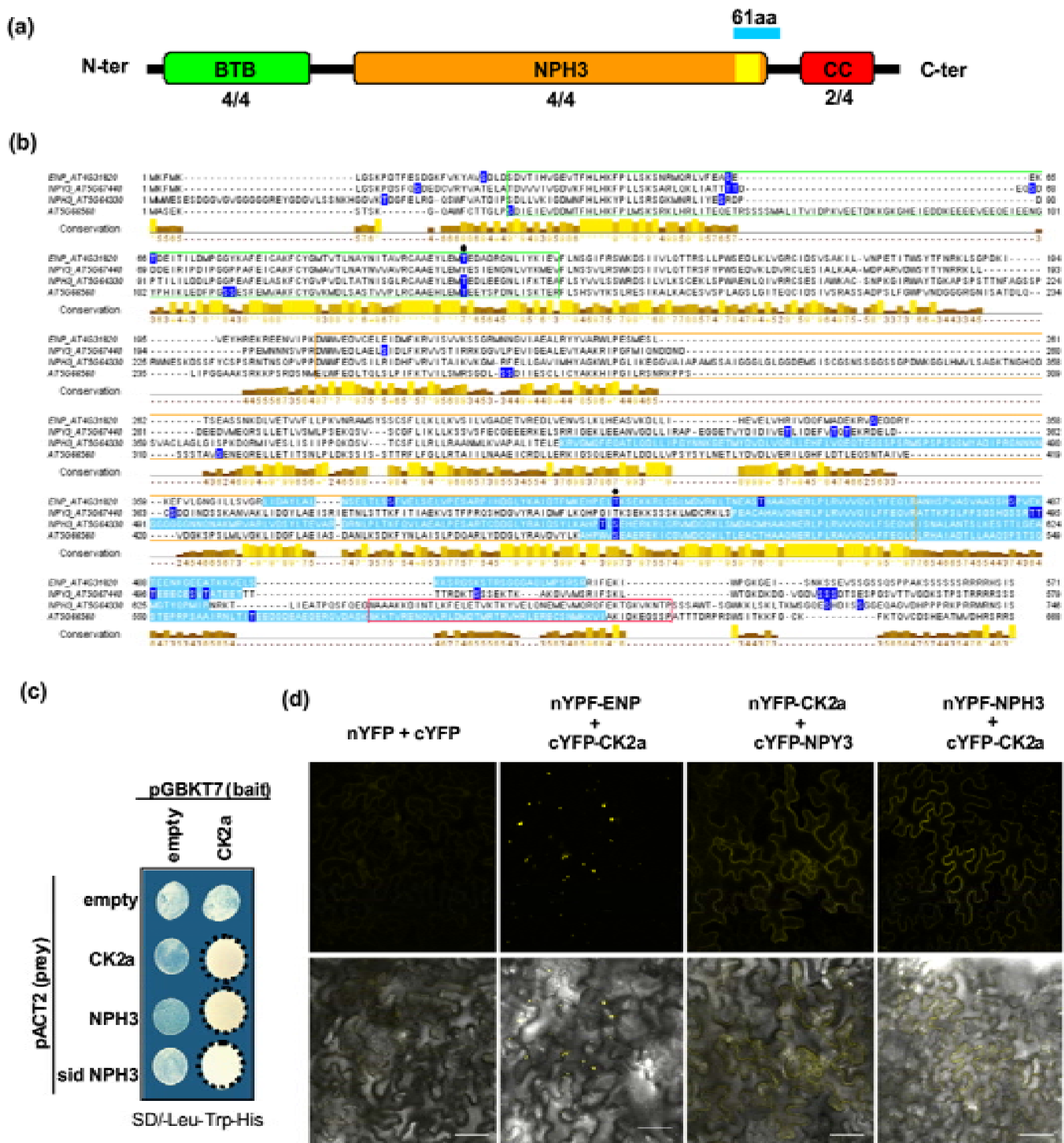

Figure 17. CK2 interacts with several members of the plant specific NPH3/RPT2 (NRL) protein family.
(a) Schematic representation of the conserved sequence features present in the four members of the NPH3/RPT2 (NRL) protein family that interact with CK2 in the Y2H screen. Ratios indicate the number of proteins containing each domain. The smallest interaction sequence with CK2α is depicted in cyan. The yellow box in the NPH3 domain corresponds to a highly conserved region found in all the NRL proteins analysed. *Abbreviations*: BTB, broad complex, tramtrack, bric a brac (green box); NPH3, non-phototropic hypocotyl 3 (orange box); CC, coiled-coil (red box). Domain sequences were defined as described in (The UniProt Consortium, 2014; Motchoulski and Liscum, 1999; Pedmale et al., 2010). **(b)** Sequence alignment of the NPH3/RPT2 proteins found to interact with CK2. The three characteristic domains of the NPH3/RPT2 proteins are squared using the same color code as in (a). The interaction sequences with CK2α (or SID, for Selected Interaction Domain) are hightlighted in cyan. Dark-blue boxes depict NetphosK prediction of putative CK2 phosphorylation sites (Ser and Thr residues). Black dots indicate predicted CK2 phosphorylation sites found in all but one of the NRL proteins analyzed. Column-wise conservation of the amino acids physico-chemical

CK2 modulates NPH3 phosphorylation and phot1 ubiquitination

All the NPH3/RPT2 prey proteins appear to contain multiple serine and threonine residues located in predicted CK2-consensus sequences (Figure 17b, dark blue boxes), suggesting that they might be CK2 substrates. To address this idea, we focused our studies on the NPH3 protein, as it is well established that its signaling capacity depends on its phosphorylation state (Pedmale and Liscum, 2007). We first performed *in vitro* phosphorylation assays using *in vitro*-transcribed/translated Strep-NPH3 as a substrate. We used recombinant human tetrameric CK2 (hrCK2αβ) because it had already been successfully used to phosphorylate plant proteins and is readily commercially available (Kang and Klessig, 2005; Tosoni et al., 2011; Tuteja et al., 2001). Phosphorylation was detected by immunoblotting with an anti-P-S/T^3-CK2 antibody that recognizes phosphorylated serine and threonine residues within the CK2 consensus sequence. As a control, phosphorylation of β-casein (an *in vitro* substrate of CK2) (Figure 18a), as well as CK2 autophosphorylation, were efficiently detected by the anti-P-S/T^3-CK2 antibody (Figure S5). Our experiment show that Strep-NPH3 was phosphorylated by recombinant human tetrameric CK2 (hrCK2αβ), and that this phosphorylation was prevented in the presence of 4,5,6,7-tetrabromobenzotriazol (TBB), a powerful and specific CK2 inhibitor (Sarno et al., 2001) (Figure 18a).

Figure 17 (continued)
properties within the four NPH3/RPT2 proteins shown in the alignment is represented as histograms. The conservation score (indicated below the histogram) ranges from 1 to 11 and is depicted as follow: the asterisk (*) indicates no amino acid change (score 11), the sign (+) is used when all the aa in the column have the same physico-chemical properties (score 10), and the numbers (1 to 9) indicate diferent degrees of conservation of the aa physico-chemical properties (Livingstone and Barton, 1993). Moreover, the histogram is depicted using a colour code, from dark brown for not conserved positions to yellow for conserved positions. *Abbreviations*: ENP, ENHANCER OF PINOID; NPY3, NAKED PINS IN YUC MUTANTS 3; NPH3, NON-PHOTOTROPIC HYPOCOTYL 3**. (c)** Validation of the interaction between CK2α and full-lenght NPH3 by the yeast two hybrid system (Y2H). After performing the corresponding mating reactions, protein interactions were checked by analysing the level of yeast growth in triple drop-out medium (SD/-Leu-Trp-His) plates. Dimerization of CK2α and interaction between CK2α and sid-NPH3 were included as positive controls. **(d)** *In vivo* interaction between CK2α and NPH3/RPT2 full-length proteins. We performed bimolecular fluorescence complementation (BiFC) assays with split yellow fluorescent protein (YFP) in agroinfiltrated *Nicotiana benthamiana* leaves. Reconstitution of YFP fluorescence was observed in all the interactions tested, at the subcellular compartment where the full lenght proteins are located **(see Figure S4):** ENP interacted with CK2α in endosomal compartments, NPH3 at the plasma membrane, and NPY3 at the plasma membrane and partially in the cytosol. Scale bar: 50 µm.

Next, we wondered whether CK2 might phosphorylate NPH3 *in vivo*. To assess this question, we analyzed the light-induced mobility shift of NPH3 on SDS-PAGE (Figure 18b). The mobility shift of NPH3 is phosphorylation-dependent, the high molecular weight band corresponding to the phosphorylated, signaling-incompetent (inactive) state that accumulates in the dark ($NPH3^{DS}$, dark state), while the low molecular weight band corresponds to the non-phosphorylated, signaling-competent (active) state that accumulates in the light ($NPH3^{LS}$, light state) (Pedmale and Liscum, 2007) (Figure 18b, dmso control). In dark-grown seedlings of mock treated plants (DMSO, dark), we confirmed that NPH3 had a low electrophoretic mobility compared to light treated plants (DMSO, light). Inhibition of CK2 by TBB in dark-grown seedlings induced a slight shift of NPH3 electrophoretic mobility (TBB, dark) that was only partial as compared to light treated plants (DMSO, light). This result suggests that CK2 might phosphorylate only few residues in NPH3 and that another kinase might also contribute to phosphorylation of NPH3 in dark conditions. Additionally, this result might indicate a slow turnover of the $NPH3^{DS}$ form under dark conditions, and thus a remaining pool of $NPH3^{DS}$ already phosphorylated by CK2 before its inhibition by TBB (which was carried out for the last 4h prior analysis). Light treatment produced the accumulation of the $NPH3^{LS}$ in both control plants (DMSO, light) and CK2-depleted plants (TBB, light), indicating that phot1-mediated NPH3 dephosphorylation is not dependent on CK2 activity. Next, to further analyse the involvement of CK2 in the dark phosphorylation of NPH3, we performed light-to-dark recovery experiments. In mock treated seedlings, light-to-dark transition produced a rapid shift from the low- to the high-molecular weight band, indicating recovery of the $NPH3^{DS}$. Interestingly, in TBB-treated plants no recovery of $NPH3^{DS}$ was observed when plants were transferred back to the dark. These results indicate that CK2 is required to phosphorylate NPH3 in the dark, and thus to modulate the light-induced changes of NPH3 activity. The absence $NPH3^{DS}$ in TBB-treated plants in light-to-dark experiments suggests that either CK2 is fully responsible for NPH3 phosphorylation in these conditions or that CK2 phosphorylates only a few residues of the NPH3 protein that are required for the recruitment of additional kinase activities.

It has been reported that NPH3 acts as a substrate adapter of a CUL3-based E3 complex ($CRL3^{NPH3}$ complex) (Roberts et al., 2011), thus participating in ubiquitin signaling. This complex ubiquitinates the membrane associated blue-light photoreceptor phot1 under exposure to BL (Roberts et al., 2011; Deng et al., 2014), conditions in wich NPH3 is found in its dephosphorylated, signaling-competent state. If CK2 is indeed involved in the maintenance of $NPH3^{DS}$, plants depleted of CK2 activity should exhibit constitutively active NPH3 within the $CRL3^{NPH3}$ complex, even in dark conditions. To check this hypothesis, we

analyzed the ubiquitination status of immunoprecipitated phot1-GFP, using *phot1-5*PHOT1::PHOT1-GFP etiolated seedlings treated with TBB. Ubiquitin moieties were detected by the P4D1 antibody which recognizes monoubiquitin and several forms of polyubiquitin chains (Haglund et al., 2003; Barberon et al., 2011). In mock-treated seedlings (dmso) in the dark, we found that phot1 was not ubiquitinated (Figure 18c). After 1h of white light treatment (50 µmol·m^{-2}·s^{-1}), we noticed the appearance of a high molecular weight smear, typical of ubiquitinated proteins. This result suggested that in these conditions phot1 was ubiquitinated, as reported for low- and high intensity BL (Roberts et al., 2011). Interestingly, when we incubated seedlings with the CK2 inhibitor TBB, phot1 was ubiquitinated in both dark and light conditions. This finding supports the idea that in CK2-defective plants, NPH3 is constitutively active, which induces phot1 ubiquitination.

The biological significance of light-induced phot1 ubiquitination still remains unclear, but it has been suggested that it might be an important step to transduce the phototropic signal, perhaps by regulating phot1 intracellular localization. It was proposed that phot1 internalization could induce the subsequent intracellular phosphorilation of phot1 substrates or, desensitize phot1 signaling by targeting the photoreceptor to degradation, or both (Kaiserli et al., 2009; Roberts et al., 2011; Liscum et al., 2014).

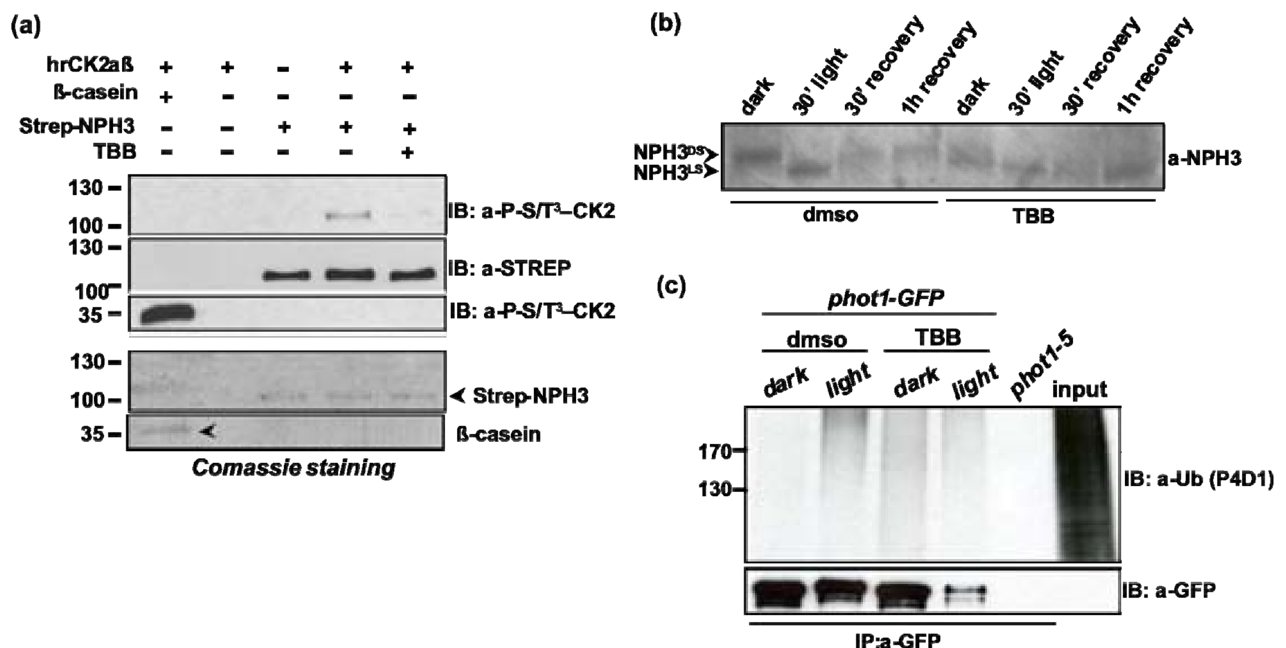

Figure 18. CK2 phosphorylates NPH3 and modulates phot1 ubiquitination.
(a) *In vitro* phosphorylation of Strep-NPH3 by protein kinase CK2. *In vitro* transcribed and translated Strep-NPH3 was used as a substrate of human recombinant CK2αβ (hrCK2αβ) in an *in vitro* phosphorylation assay (as described in Experimental procedures section). Phosphorylation reactions were then blotted and immunodetected with an anti-P-S/T^3-CK2 antibody that specifically recognizes phosphorylated serine and threonine residus at the CK2 consensus sequence. The CK2 inhibitor TBB

Phot1 subcellular localization is modulated by CK2 ina NPH3-independent manner

Next, we examined the subcellular localization of phot1 in plants with reduced CK2 activity. We focused on the elongation zone of hypocotyls because it was shown that this region is sufficient to trigger phototropic bending (Preuten et al., 2013; Yamamoto et al., 2014). In dark-grown control plants, phot1-GFP was found located at the cell surface, and it partially moved to the cytoplasm after white light treatment (Figure 19), as previously reported in response to BL (Wan et al., 2008; Sakamoto and Briggs, 2002; Kaiserli et al., 2009; Sullivan et al., 2010; Yamamoto et al., 2014). Interestingly, in dark-grown TBB-treated plants, phot1 showed a mottled distribution accompanied with the appearance of bubble-like (dark areas outlined by more intense signal) and punctate structures. Moreover, a partial diffusion to the cytoplasm was also observed. These severe alterations of phot1 localization in the dark produced by inhibition of CK2 are similar to those reported in response to BL (Sakamoto and Briggs, 2002; Wan et al., 2008). The same changes in phot1 localization were observed after exposure of TBB-treated plants to light. As a control, we examined the subcellular localization of the membrane marker Lti6b (Cutler et al., 2000). No deffects in Lti6b membrane localization were observed after light treatment nor after TBB treatment, indicating that the reported altered pattern is specific for phot1.

Figure 18 (continued)
was used to prove the specifity of the reaction and the *in vitro* substrate β-casein was used as a positive control. Loading of equal amounts of substrate in each reaction was controlled by α-STREP immunoblotting and by Comassie staining. **(b)** *In vivo* phosphorylation of NPH3 by CK2. Four-day-old etiolated wild-type *Arabidopsis* seedlings (Col-0) grown in liquid culture were illuminated for 30 minutes with white light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) (30’ light) and then put back in the dark for additional 30 min or 1h (30’ or 1h recovery). TBB (20 µM) or DMSO (TBB solvent) was added to the medium 2 h before the light treatment and maintained for the whole experiment. Plants grown in complete darkness were submitted to the same TBB/DMSO treatments (dark). Total microsomal proteins were extracted, separated by SDS-PAGE and immunoblotted with anti-NPH3 antibodies (α-*NHP3*). The mobility band shift of NPH3 indicates its phosphorylation state: the high molecular weight band corresponds to the phosphorylated form (or dark state NPH3, $NPH3^{DS}$), whereas the low molecular weight band corresponds to the dephosphorylated form (or light state NPH3, $NPH3^{LS}$) (Pedmale and Liscum, 2007). Note that TBB-treated plants are not able to accumulate the phosphorylated form of NPH3 after their transition from light to darkness. **(c)** phot1 ubiquitination in wild-type and TBB-treated plants. Four-day-old *phot1-5*PHOT1::phot1-GFP or *phot1-5* etiolated seedlings grown in liquid medium were illuminated for 1h with white light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$). TBB (20 µM) or DMSO (TBB solvent) was added to the medium 2 h before the light treatment. Total protein extracts were obtained, immunoprecipitated with anti-GFP antibodies and immunoblotted with either P4D1, which recognizes both mono-/multi- and polyubiquitinated proteins (upper panel), or anti-GFP antibodies (lower panel). Protein extracts from *phot1-5* mutant were used as a negative control of the immunoprecipitation reaction and as a *input* control. *Abbreviations:* IP, immunoprecipitation; IB, immunoblotting; Ub, ubiquitin.

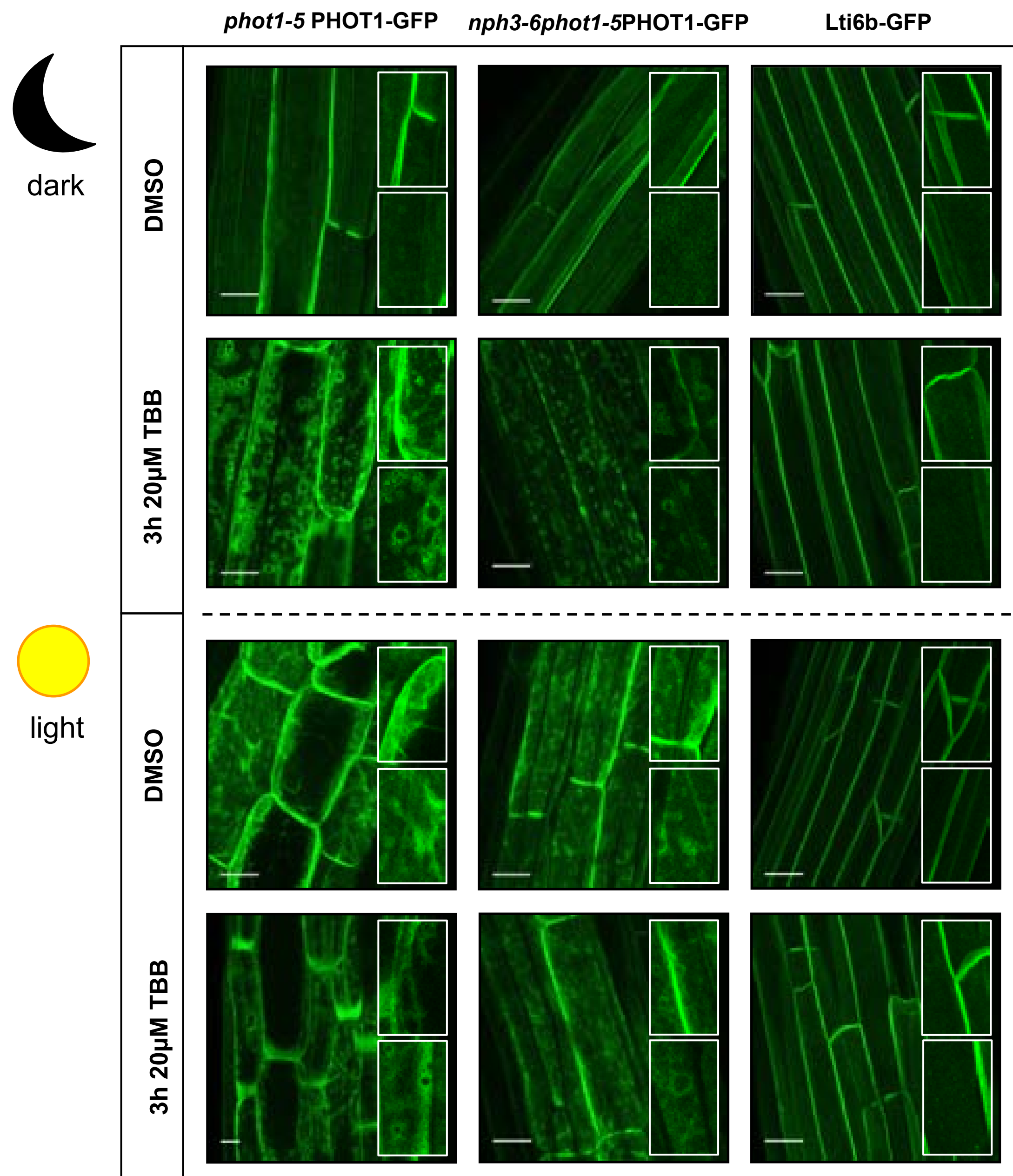

Figure 19. CK2 activity is required for proper phot1 localization at the plasma membrane. Subcellular localization of phot1-GFP (*phot1-5*PHOT1::phot1-GFP), phot1-GFP in *nph3* mutant background (*nph3-6phot1-5*PHOT1::phot1-GFP), and the membrane marker Lti6b-GFP are shown in the hypocotyl elongation zone of 4d-old etiolated seedlings. Plants were treated with either 20µM TBB or DMSO (TBB solvent) for 3h in the dark, or were light stimulated (50 µmol·m^{-2}·s^{-1}) during the last hour of TBB treatment. Insets show details of the membrane-cytosol interfaces (upper panel) and of the membrane surfaces (lower panel). Images correspond to maximum projections of z-stack of 30 µm in depth. Bar: 25 µm.

NPH3 activity is necessary to induce phot1 ubiquitination, which has been suggested to induce its plasma membrane to cytosol relocalization (Roberts et al., 2011; Liscum et al., 2014). We reasoned that in the absence of NPH3, phot1 movement from the plasma membrane should be abolished even after TBB or light treatments. To challenge this idea, we analysed the subcellular localization of phot1-GFP in the *nph3-6* mutant background (*nph3-6*PHOT1-GFP plants) (Figure 19). Similarly to the *nph3-6* mutant, these plants do not bend towards unilateral BL (data not shown). Surprisingly, the light induced movement of phot1 was observed in these plants, showing that phot1 localization dynamics is not controlled by NPH3. Thus, contrary to what was previously suggested (Roberts et al., 2011), phot1 movement seems independent of phot1 $CRL3^{NPH3}$-mediated ubiquitination. Furthermore, *nph3-6*PHOT1-GFP plants exhibited the same drastic defects in phot1 localization as NPH3 containing plants treated with TBB in the dark. Altogether, our results suggest that PHOT1 localization is not regulated by NPH3 but is dependent on CK2 activity. We propose that CK2 might have a dual role in phototropism signaling, a NPH3-dependent function in PHOT1 ubiquitination and a NPH3-independent function in controlling phot1 localization.

Inhibition of CK2 activity impairs the phototropic response

NPH3, as well as NPH1/PHOT1 encoding genes were uncovered in a screen for non-phototropic hypocotyl mutants. Several *nph3* loss-of-function mutants were identified and shown to be completely aphototropic (Liscum and Briggs, 1995; Liscum and Briggs, 1996; Motchoulski and Liscum, 1999). On the other hand, in a screen for auxin related phenotypes of CK2 defective plants, we previously reported defects on the phototropic bending of a dominant negative CK2 mutant in response to directional white light (Marques-Bueno et al., 2011a). To further characterize the phototropic phenotype of CK2 defective plants, we quantified the angle of curvature of etiolated hypocotyls in response to unilateral blue-light (BL), at two different fluence rates: low (0.4 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and moderate (4 $\mu mol \cdot m^{-2} \cdot s^{-1}$) BL intensities (Figure 20b – 20h). We found that both TBB-treated WT plants and CK2mut plants had a reduced or null capacity to bend towards the light source. While TBB-treated plants showed an intermediate bending phenotype in all the conditions tested (Figure 20a, 20c-d, 20f-g), a drastic effect was observed in CK2mut plants, with a high frequency of plants unable to orient the cotyledons toward the light (Figure 20a, 210d, 20h). Together, these results functionally implicate CK2 in the phototropic response.

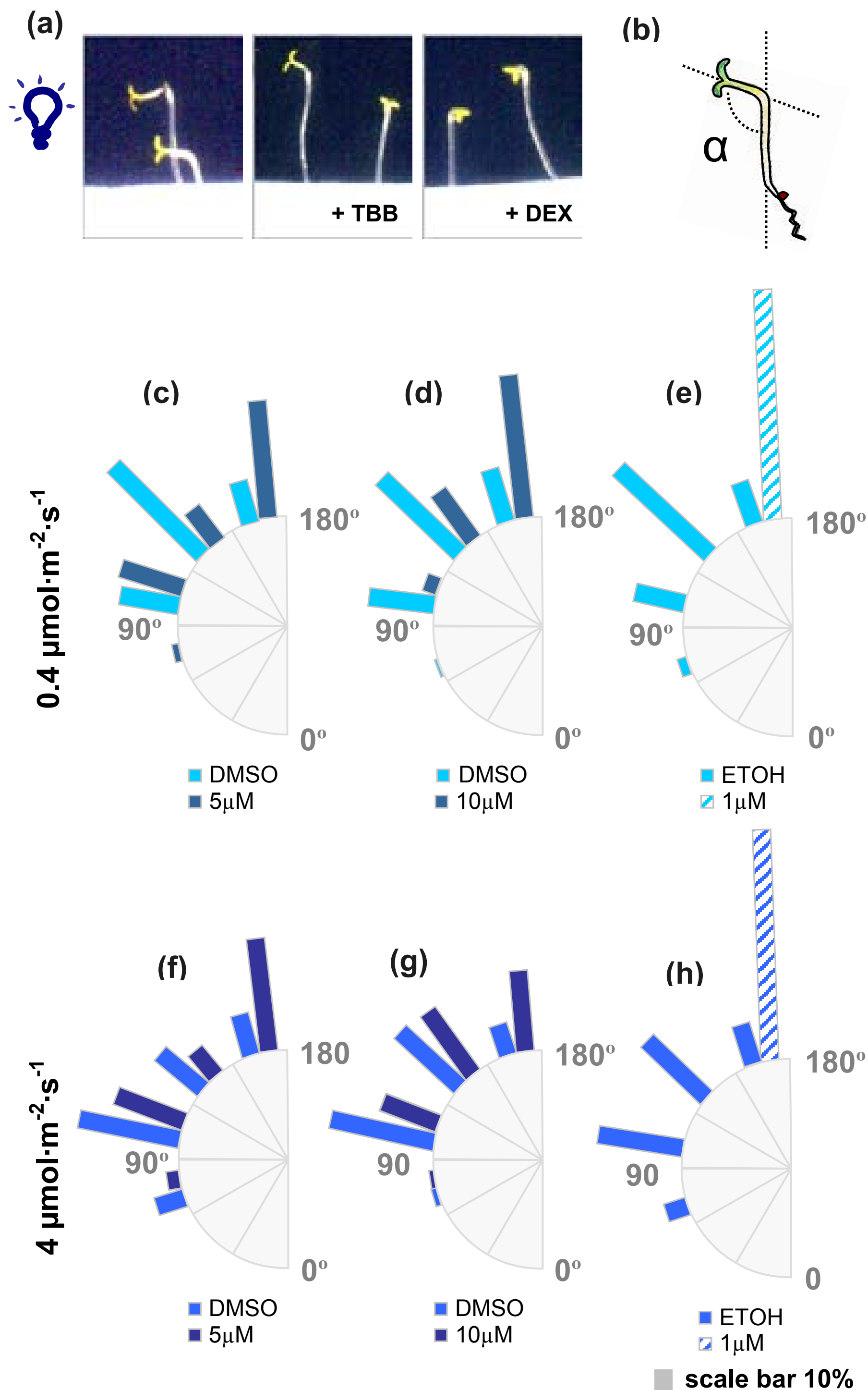

Figure 20. CK2 is required for normal phototropic response.
(a) Phototropic hypocotyl bending in response to unidirectional blue light (BL). Three-days-old WT and CK2mut seedlings grown in the dark were incubated respectively with 5µM TBB (WT+TBB) or 1µM DEX (CK2mut+Dex) for 24h prior to BL treatment. Control plants were treated with either DMSO (TBB solvent) or ethanol (Dex solvent). The bending phenotype was analysed 24h after light induction. **(b)** Schematic representation of the angle measured to quantify the phototropic curvature. 180º represents no bending. **(c-h)** Histograms of hypocotyl bending angles. The frequency of seedlings in each 30º interval is represented as the percentage of all plants analyzed. **(c-e)** Angle mesurements under low BL (0.4 µmol·m^{-2}·s^{-1}) in 5uM TBB-treated WT plants, n= 60-70 **(c)**, 10uM TBB-treated WT plants, n=100-120 **(d)** and 1uM DEX-induced CK2mut plants, n= 70-90 **(e)**. **(f-h)** Bending angles under moderate BL conditions (4 µmol·m^{-2}·s^{-1}); n= 50-70 **(f)**, n=100-120 **(g)** and n= 80-90 **(h)**. Experiments were perfomed as in **(a)**.

Discussion

NPH3 is phosphorylated by protein kinase CK2

Pedmale and Liscum, (2007) suggested that the kinase modulating NPH3 activity must be constitutively present and active, two characteristic traits of the protein kinase CK2 (Pinna, 2003; Moreno-Romero et al., 2011). We have shown that CK2 phosphorylates NPH3 *in vitro* and that CK2 is required for the rapid recovery of the phosphorylated dark state of NPH3, indicating that CK2 phosphorylates NPH3 *in vivo*. Moreover, we demonstrated that CK2 inactive plants are non-phototropic (Figure 20, (Marques-Bueno et al., 2011a), thus involving CK2 in the phototropic signalling pathway. This phenotype might seem in contradiction with the previous finding that NPH3 phosphomutants (in which serine were changed to alanine residues in several of the predicted phosphorylation sites) complement the aphototropic phenotype of the *nph3* mutant (Tsuchida-Mayama et al., 2008). However, the set of NPH3 phosphomutants that were generated in that study did not included mutations of potential CK2 phosphorylation sites. Thus, NPH3 phosphorylation by CK2 could be a prerequisite for the normal activity of NPH3 and the phototropic response. Similarly, the outcome of the phosphorylation of transcription factors involved in photomorphogenesis (HFR1 and PIF1) depends on the number and identity of the residues phosphorylated by CK2 (Bu, Zhu and Huq, 2011; Park et al., 2008; Bu, Zhu, Dennis, et al., 2011), even if other kinases are involved in their phosphorylation (Bu, Zhu, Dennis, et al., 2011; Bu, Zhu and Huq, 2011). Alternatively, CK2 might also contribute to the phototropism response via its NPH3-independent function on phot1 localization.

Taken together, our results indicate that CK2 is one of the kinases responsible of NPH3 phosphorylation, although our data suggest the involvement of additional kinases. Notably, NPH3 was identified as a putative substrate of the mitogen-activated protein kinase 3/6 complex in a high throughput screen of novel *in vivo* MAP kinase substrates (Liscum et al., 2014; Hoehenwarter et al., 2013). However, the function of these kinases in phototropism in general, and in NPH3 phosphorylation in particular, has not been studied yet.

CK2 modulates NPH3 ubiquitin signalling activity

Functionally, phosphorylation of NPH3 by CK2 is important to maintain NPH3 in its inactive state in the dark, demonstrated by the constituve ubiquitination of phot1 in CK2 inactive plants (Figure 19c). Phot1 ubiquitination has been proposed to promote phot1 internalization from the plasma membrane to the cytosol (Roberts et al., 2011). Our finding that phot1 is ubiquitinated both in dark and light conditions in CK2 depleted plants led us to postulate that this might result in phot1 depletion from the membrane, and thus an inability of plants to sense the light stimulus. As a consequence, CK2 inactive plants are aphototropic. Concordantly, plasma-membrane localization of phot1 was disrupted in TBB-treated plants (Figure 20). Interestingly, in the *nph3* mutant background phot1 still moves to the cytosol, indicating that light-induced phot1 relocation is independent of NPH3. Thus, as Roberts et al (2011) demonstrated that phot1 is not ubiquitinated in the absence of NPH3 we conclude that phot1 ubiquitination is not necessary to promote phot1 movement. Therefore, the presence/absence of ubiquitination in phot1 and the nature of this pattern could be important for phot1 signalling rather than localization (i.e. interaction and modification of the activity of known interacting partners such as the 14-3-3λ or ARF proteins (Sullivan et al., 2009), the PKS proteins (Lariguet et al., 2006; Demarsy et al., 2012) and the auxin transporter ABCB19 (Christie et al., 2011)), as well as for phot1 turnover. Additionally, the NPH3 essential role in phototropism could be due to the ubiquitination of other substrates that are themselves required for phototropism.

CK2 regulates phot1 subcellular localization

According to our results, phot1 internalization appears to be driven by a NPH3-independent mechanism and this pathway is altered in CK2-inactive plants. Mutant versions of phot1 with constitutive kinase activity, also showed internalization in dark conditions (Kaiserli et al., 2009). Whether CK2 could act directly by modulating phot1 activity is not known, but recent analysis of the phosphorylation status of phot1 showed that some residus of phot1 are phosphorylated in dark and light conditions (Deng et al., 2014). Particularly, S170 is maintained phosphorylated in dark and light conditions and thus, the action of a constitutive kinase seems necessary for this modification. Interestingly, this serine is found in a CK2 consensus sequence. Nonetheless, the importance of phot1 N-ter phosphorylation (and thus, the importance of S170 phosphorylation) is not clear, as it has been shown that both the LOV2 and kinase domains are sufficient to trigger phot1 functions and intracellular

movement (Kaiserli et al., 2009). Alternatively, CK2 depletion could influence the clathrin-dependent endocytosis of phot1 (Kaiserli et al., 2009; Roberts et al., 2011). In animal cells, CK2 is one of the most abundant protein kinase present in clathrin-coated vesicles (CCVs) (Korolchuk and Banting, 2002; Korolchuk et al., 2005) and controls several steps of endocytosis (Korolchuk and Banting, 2002; Korolchuk et al., 2005; Galovic et al., 2011). The ideas that CK2 could regulate phot1 localization either by directly controlling its activity or through the regulation of clathrin-mediated endocytosis will need further experimental validation.

Conclusion

In summary, our results suggest that CK2 has a dual function during the phototropic response. On one hand, CK2 phosphorylation of NPH3 in dark conditions is necessary to inhibit the activity of CRL3NPH3. This complex is involved in the ubiquitination of the photoreceptor phot1, thus providing new evidences of the role of CK2 in the crosstalk between phoshorylation and ubiquitination in light signalling pathways. Additionally, we have shown that CK2 interacts with several members of the NRL family, wich let us to postulate that CK2 could be a general modulator of the activity of this family of BTB-containing proteins. On the other hand, CK2 activity is necessary to maintain phot1 at the plasma membrane in dark conditions and thus, to ensure the proper localization of this photoreceptor. The way CK2 regulates phot1 localization is still unknown but it is independent on NPH3 activity.

Discussion

Role of CK2 in the salicylic acid signaling pathway

Salicylic acid (SA) is a well known phytohormone with an essential role in plant immunity. Increase in SA endogenous levels is one of the first responses upon pathogen infection, and produces an extense reprogramming of gene expression to accurately activate proper defense responses, both locally and sistemically. By contrast, much less is known about the role of SA in vegetative growth. Phenotypic analysis of Arabidopsis mutants or of transgenic plants affected in SA signalling provided the first evidences of the involvement of SA in the regulation of plant cell growth. For example, SA-depleted Arabidopsis *NahG* transgenic plants have a higher growth rate than wild-type plants (Abreu and Munné-Bosch, 2009), while mutants with constitutively high levels of SA, such as *cpr5* (Bowling et al., 1997), show a dwarf phenotype. Nonetheless, the effect of SA on root development has been poorly described. For instance, Arabidopsis plants overexpressing the auxin/SA-inducible OBP3 and OBP1 transcription factors, which belong to the DOF (DNA binding with one finger) family, show decreased growth rate of the main root (Kang and Singh, 2000; Skirycz et al., 2008). In addition, OBP3-overexpression mutants have increased root hair density (Kang and Singh, 2000), and OBP1-OE plants show reduced number of lateral roots (Skirycz et al., 2008).

In this thesis work, we provided new evidences of the role of SA in root development. Exogenous treatment of Arabidopsis wild-type plants with 0,25 mM SA reduces the growth rate of the main root and inhibits formation of lateral roots, and SA-accumulating mutants, such as *cpr1*, *cpr5* and *cpr6,* exhibit the same root phenotypes. We have also demonstrated that CK2mut plants and, specifically, the roots of these plants, accumulate high levels of SA,

and that hyperaccumulation of SA is responsible for the altered root phenotypes of these plants (i.e: reduced root lenght and absence of lateral roots). These phenotypes were partially rescued when the endogenous high SA content of *CK2mut* plants was reduced by crossing them with the SA-deficient mutant *sid2*.

Increase of SA biosynthesis seems to be a primary effect of CK2 depletion, as it was observed shortly after CK2 inhibition with TBB (4 hours) (data not shown). Thus, these results involve CK2 as a negative regulator of SA biosynthesis, either by directly modifying the activity of SA biosynthetic enzymes or by altering the expression of genes encoding those enzymes, or both. In support of the second hypothesis, we observed overexpression of the isochorimate synthase 1 (ICS1/SID2) gene in CK2mut plants. Several SA-induced transcription factors of the WRKY family also act as negative regulators of SA biosynthesis in non-infected conditions, by maintaining reduced expression of the ICS1 (Wang et al., 2006). Moreover, CK2 appear to be involved in a negative regulatory feedback loop aiming to maintain SA homoeostasis, as SA stimulates transcription of CK2-encoding genes. Upon pathogen infection, NPR1 is also part of a negative feedback loop, as NPR1 negatively regulates the expression of ICS1 while SA stimulates NPR1 activity and expression. SA-modulation of CK2 gene expression is in concordance with previous findings that demostrated that SA increases the nuclear activity of CK2 (Hidalgo et al., 2001). The authors also reported that nuclear CK2 activity is necessary for early SA-induced transcriptional activation.

How can SA affect vegetative growth? It has been suggested that the higher growth rate of SA-deficient Nahg plants might result from enhanced cell expansion, as demonstrated because NahG leaves were 2.8-fold larger than those of wild type plants, while no differences were reported in the number of epidermal cells between the two genotypes (Scott et al., 2004). On the other hand, SA negatively regulates the expression of cyclin D3 (CYCD3), which drives the G1/S transition of the cell cycle (Rivas-San Vicente and Plasencia, 2011). Additionally, the auxin/SA-induced OBP1 transcription factor is involved in the control of cell cycle entry at G1 phase, and modulates the expression of positive and negative regulators of cell cycle progression (Skirycz et al., 2008). Interestingly, OBP1 gene is overexpressed in CK2mut plants (Table S2). Moreover, we have previously shown that CK2mut plants have reduced levels of CYCB1;1 protein (a mitotic marker expressed from G2 to M phase of the cell cycle) at the root meristem and at the sites of initiation of lateral root primordia (Moreno-Romero et al., 2008; Marques-Bueno et al., 2011a). In addition, CK2 depletion produces cell cycle arrest both at G1 and G2 phases of the cell cycle (Moreno-

Romero et al., 2011). Whether or not this altered cell cycle pattern of CK2mut plants could be directly related to SA accumulation remains elusive. Alternatively, these effects on cell cycle could be indirectly mediated by SA repression of the auxin signalling pathway.

Often, interaction between two hormonal signaling pathways conveys at the transcriptional modulation of certain genes. SA has been shown to repress several genes involved in the auxin signaling pathway, among them the auxin importer AUX1 and the auxin exporter PIN7, as well as auxin receptors (TIR1 and AFB1) and some members of the Aux/IAA gene family that encode auxin signaling repressors (Wang et al., 2007). In this work we show that exogenous SA treatment of Arabidopsis roots repress the transcription of additional auxin transporters, such as PIN1, PIN3 and PIN4, whereas PIN2 expression is not afected by SA treatment. On the other hand, SA stimulates the transcription of the gene encoding the protein kinase PINOID, which regulates PIN subcellular localization. In concordance with these results, the accumulation of SA in roots of CK2mut plants also produces repression of PIN1 and PIN3 and stimulation of PINOID transcription. By contrast, the repressive efect of SA on PIN4 and PIN7 is abolished in CK2mut plants, and consequently the presence of active CK2 is required. A model summarizing the data obtained about the role of CK2 in SA signaling is shown in Figure 21.

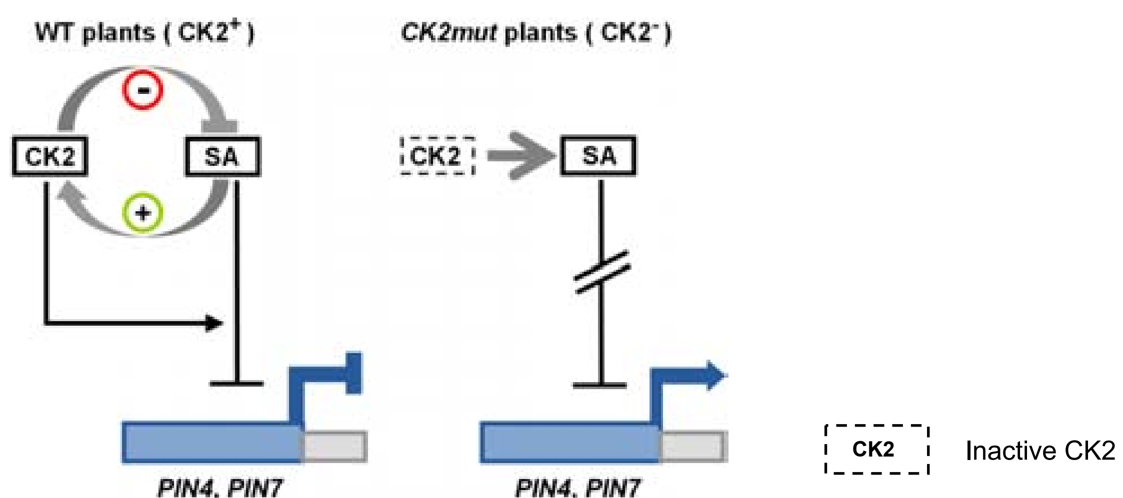

Figure 21. Proposed model for the interplay between CK2, salicylic acid and *PIN* transcription.
The model presents an autoregulatory feed-back loop between CK2 and salicylic acid (SA): CK2 activity negatively regulates SA biosynthesis, whereas CK2-encoding genes are transcriptionally up-regulated by SA. In addition, CK2 activity is also required for the SA-mediated transcriptional down-regulation of *PIN4* and *PIN7*. Thus, in wild-type plants high levels of SA repress *PIN4* and *PIN7* transcription (left), whereas depletion of CK2 activity (such as in CK2mut plants, right) is followed by the bypass of the negative regulatory point in the SA-signalling pathway.

The results presented in this work suggest that CK2 could modulate the activity or the expression of transcription factors (either positive or negative acting factors) that act downstream of SA to fine-tune the expression of PIN and PID genes. Expression of auxin-inducible genes, such as those coding for PINs and PID, is mostly regulated by the ARF transcription factors that recognize the auxin response *cis*-acting elements (AREs). Nonetheless, it has been reported that the amplitude of the auxin-induced responses is modulated by the presence of bZIP- and MYB-related binding sites in the promoters of these genes (Berendzen et al., 2012). The number and position of these additional regulatory moduls, specifically determine the transcriptional outcome. For this reason, we searched for the presence of SA-responsive *cis*-acting elements in the promoters of PINs and PID genes (Figure S3 and Table S3). We focused on the *as*-1 element and on the W boxes located at 3000 bp upstream of the transcription starting site and in the 5' UTR region. The *as*-1 element is recognized by the TGA family of transcription factors, and the W-boxes by the WRKY family of transcription factors. In addition, we also confirmed the presence of AREs in the promoters of these genes. As expected, all the promoters analysed contained one or more AREs within their sequences. Moreover, we found that all the promoters contained W-boxes, as a single motif or in combination with other W-boxes or *as-1* elements and AREs. The *as-1* elements were also found as single motifs or close to AREs. Interestingly, the promoters of PIN1 and PIN3 genes, which are repressed by SA treatment or by SA accumulation in CK2mut roots, do not contain any *as-1* element. Thus, the SA-mediated repression of these genes must be mediated by WRKY transcription factors. The existance of positive and negative WRKY transcription factors has already been reported (Wang et al., 2006). Interestingly, at least three WRKY factors are overexpressed in CK2mut plants (Table S2): WRKY 60, which acts as a negative regulator of defense responses, and WRKY 18 and 70, which act as negative regulators of basal SA biosynthesis (without pathogen infection) but as positive regulators of defense responses upon pathogen infection (Wang et al., 2006). Althought we can not exclude that WRKY factors are also involved in the modulation of PIN4 and PIN7, the presence of *as-1* elements in their promoters could explain its differential response to SA, i.e: SA-mediated repression but overexpression in CK2mut roots. It has been reported that CK2 phosphorylates several members of the TGA family of transcription factors and that this phosphorylation reduces their DNA-binding capacity (Kang and Klessig, 2005). Thus, loss of CK2 activity in CK2mut plants could enhance the binding capacity of the TGA factors at PIN4 and PIN7 promoters. Taken together, all this data involve SA-responsive elements as modulators of the expression of auxin responsive genes. However,

further characterization of the role of these elements in the promoters of PINs and PID should be performed.

Dong and colleagues (Wang et al., 2007) suggested that repression of the auxin signaling pathway by SA is a mechanism to inhibit pathogen growth and ensure plant resistance to pathogen infection. It would be interesting to analyse whether CK2mut plants, which can be classified as a SA-accumulating mutant, show a phenotype of increased pathogen resistance. Unfortunately, this is experimentally unfeasible, due to the nature of this mutant. The same authors suggest that part of the repressive effect on auxin-related gene transcription might be due to the stabilization of auxin repressors of the Aux/IAA family (Wang et al., 2007). Interestingly, we have preliminary results indicating stabilization of one of the members of the Aux/IAA protein family by short-term inhibition of CK2 in *Arabidopsis thaliana* (data not shown). Whether this observation is an indirect effect of hyperaccumulation of SA in CK2 defective plants or is a direct effect of the CK2-mediated regulation of AUX/IAA degradation should be further studied. Nonetheless, these results suggest that transcriptional repression of PIN1 and PIN3, either by exogenous SA or by inhibition of CK2 activity, could be mediated by stabilization of AUX/IAA repressors, thus impeding the binding of ARF transcription factors to their promoters.

PID expression was stimulated by exogenous SA in roots, as well as by CK2 inhibion in CK2mut plants. Moreover, this is concomitant with accumulation of the PID protein, as visualized with a PID::PID-GUS construct (data not shown) and by Western blots (Marques-Bueno et al., 2011a). In both cases, wt + SA plants and CK2mut plants, the spatial pattern of PID expression change as compared to wt plants, i.e: PID-GUS signal shifts from the root meristem to the root elongation and diferentiation zone (Marques-Bueno et al., 2011a).

In addition to its role as a transcriptional repressor of the auxin signaling pathway, it has been recently reported that SA interferes with auxin transport by inhibition of clathrin-mediated endocitosis of PIN1 and PIN2 (Du et al., 2013). We have previously shown that CK2mut plants have strong deffects on auxin polar transport, and show mislocalization of PIN4 and PIN7 (Marques-Bueno et al., 2011a). Again, it would be interesting to analyze if the high levels of SA in the mutant are responsible for PIN4 and PIN7 mislocalization. If so, this will be an additional example of the differential regulation of PIN proteins by SA.

Finally, it has been recently demonstrated that SA is involved in DNA damage responses (Yan et al., 2013). Although not mentioned in the results part of this thesis work, I briefly participated, at the beginning of my thesis work, in the study of the role of CK2 in DNA

damage responses. I performed some experiments aimed to check the maintenance of genomic stability in CK2mut plants, and the results obtained were part of my master degree and are published in (Moreno-Romero et al., 2012). In brief, we reported that CK2mut plants are hypersensitive to a wide range of genotoxic agents producing double-strand DNA breaks, but that these plants have a higher efficiency to repair DNA damage. These apparently contradictory results were explained by enhanced genomic instability in CK2mut plants. Yan and coworkers suggest that activation of DNA damage responses by SA is an intrinsic component of the plant immune responses (Yan et al., 2013). SA produces enhanced DNA damage and enhanced DNA repair by modulating the expression of genes involved in the DNA repair machinery. Thus, the high levels of SA in CK2mut plants could be responsible for their enhanced efficiency in DNA repair.

In summary, we have shown that CK2 is necessary to maintain SA homeostasis. Disturbance of SA byosinthesis and/or SA signaling in CK2mutant plants could be responsible (at least in part) of the wide range of altered phenotypes and altered signaling pathways observed in these plants, such as auxin signaling and transport and DNA repair. Moreover, prolonged exposure to high SA levels, as those found in CK2mut plants, might produce cytotoxicity and, finally, cell death.

Dual role of CK2 in phototropism

Protein CK2 is a highly pleiotropic protein kinase involved in a large amount of signalling pathways that are far from being completely characterized. An important aspect in the functional characterization of a protein kinase is the identification of its substrate(s), which is also the first step to ascribe the kinase to a particular signaling pathway. With the purpose to obtain new insights into the CK2 interaction network, we performed a high-throughput Y2H screen using CK2α as bait. Among the 28 new putative CK2 substrates found (Table S5), we identified NPH3, the founder member of the NPH3/RPT2 (NRL) protein family. NPH3 is an essencial component of the phototropic signaling pathway. The signalling capacity of NPH3 proteins depends on its phosphorylation status (Pedmale and Liscum, 2007). For this reason, together with the fact that CK2mutant plants are aphototropic (this work and (Marques-Bueno et al., 2011a)), we considered NPH3 as a bona fide CK2 substrate and further studied this interaction. Indeed, our results show that CK2 phosphorylates NPH3 *in vitro* and that CK2 activity is necessary to fully acomplish the

phosphorylated state of NPH3 *in vivo*, which occurs in the dark. Whether CK2 is the only kinase that phosphorylates NPH3 or it acts in concert with other kinases, has not been solved in this work. Mapping the *in vivo* phosphorylation sites of NPH3 would help to elucidate this question. Recently, the involvement of a MAPK3/6 complex in NPH3 phosphorylation has been suggested (Hoehenwarter et al., 2013)

Under light conditions, dephosphorylated active NPH3 acts as a substrate adaptor within a CUL3-based E3 complex ($CRL3^{NPH3}$ complex) (Roberts et al., 2011), thus participating in the ubiquitination pathway. One of the known substrates of this complex is the membrane associated blue light photoreceptor phot1. We have found that phot1 is constitutively ubiquitinated (both in dark and light conditions) in CK2-inactive plants, suggesting that in these plants NPH3 is constitutively active. Thus, CK2 phosphorylation seems to be necessary to maintain the phosphorylated inactive state of NPH3 in the dark.

In wild-type plants, mono-/multi-ubiquitination of phot1 upon blue light irradiation at low light intensities modulates its clathrin-dependent internalization (Roberts et al., 2011; Kaiserli et al., 2009). In addition, at high blue light intensities phot1 is polyubiquitinated and targeted for its subsequent degradation via the 26S proteasome (Roberts et al., 2011). However, our results show that light-induced phot1 internalization is independent of its ubiquitination status, as internalization occurs in the absence of NPH3. Thus, it is unlikely that ubiquitination modulates the intracellular trafficking of phot1. Instead, phot1 ubiquitination might be important to regulate phot1 kinase activity, phot1 interaction with partners or substrates, and/or phot1 turnover. Moreover, we show that CK2 inhibition produces a massive internalization of phot 1, even in dark conditions. This produces a depletion of phot 1 in the plasma membrane, leading to plants unable to sense the light stimulus, which can explain the non-phototropic phenotype of CK2 mutant plants. Additionally, the phot1 internalization observed in these plants is also independent of ubiquitination.

In summary, CK2 activity seems to be necessary to repress the activity of the CRL^{NPH3} complex in the dark, and to retain phot1 associated to the plasma membrane. Thus, CK2 ensures proper light absortion to induce the phototropic response. Both phot1 and NPH3 activities are essential to properly activate the phototropic signaling cascade that lead to asymetric auxin distribution and hypocotyl bending towards the light source. A detailed scheme of the proposed model of the dual role of CK2 in Arabidopsis phototropism is depicted in Figure 22.

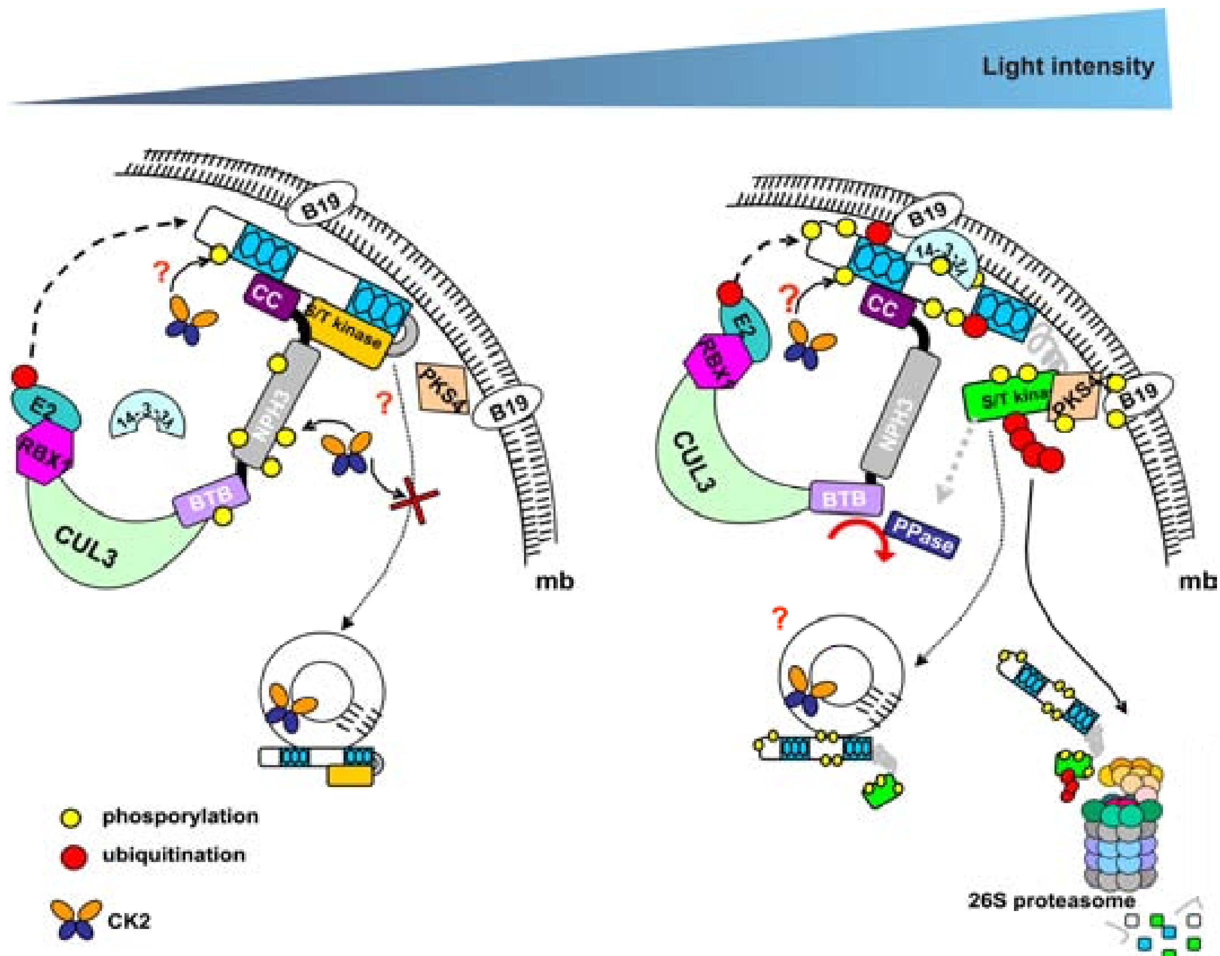

Figure 22. Proposed model of the dual role of CK2 in phototropism.
In dark conditions or in cells at the shaded side of the hypocotyl (left pannel), CK2 phosphorylates NPH3 and inhibits the activity of the CRL^{NPH3} complex. Under these conditions, phot1 is found associated to the plasma membrane in its inactive state. CK2 activity is necessary to retain phot1 at the plasma membrane, by a yet unknown mechanism. Upon light irradiation or in cells at the illuminated side of the hypocotyl (right pannel), light sensing by phot1 produces its activation and autophosphorylation. Phot1 dependent activation of a protein phosphatase mediates NPH3 dephosphorylation and thus, activation of the CRL^{NPH3} complex. The CRL^{NPH3} complex ubiquitinates phot1 and modulates its kinase activity (i.e. phosphorylation of PKS4 and ABCB19 (B19) and turnover. On the other hand, phot1 is internalized by an unknown mechanism, independent of the NPH3-mediated ubiquitination. The cytoplasmic phot1 fraction could be necessary to mediate intracellular transduction of the phototropic signal. CUL3 (cullin3), RBX1 and the E2 ligase constitute the cullin3 ring E3 ubiquitin ligase complex.
Abbreviations: mb, plasma membrane, PPase, phosphatase.

In a scenario where CK2 regulates phot1 subcellular localization by a NPH3-independent mechanism, what could be the specific process(es) targeted by CK2? Other authors have shown that treatment of phot1-GFP etiolated seedlings with oryzalin and 1-butanol, two compounds that disturb microtubules polymerization, produced partial internalization of phot1-GFP in the dark (Sullivan et al., 2009). Interestingly, among the CK2 interacting partners that we found in the Y2H screen, we identified the protein WAVE5, a member of the SCAR/WAVE family of proteins. SCAR proteins are part of the WAVE regulatory complex that controls actin cytoskeletal dynamics in the cell, by stimulating the activity of the Arp2/3 complex at distinct membrane sites (Zhang et al., 2008). The Arp2/3 complex facilitates the first step in the assembly of actin filaments, called nucleation, which is defined as the formation of a stable multimer of actin monomers (Firat-Karalar and Welch, 2011). In addition, in the same Y2H screen, we also identified MAP65-6, a microtubule-associated protein (Struk and Dhonukshe, 2014). These findings suggest that CK2 might function as a regulator of the cytoskeleton dynamics, which is responsible for the subcellular localization of proteins such as phot1. In animals, it has been shown that the WAVE complex links diverse membrane-localized receptor or membrane-associated proteins to the actin cytoskeleton (Chen et al., 2014).

As previously mentioned, phot1 endocytosis is a clathrin-dependent process. Interestingly, mammalian CK2 has been found in clathrin-coated vesicles, and it has been shown to modulate vesicle formation by phosphorylation of vesicles' structural proteins (Korolchuk and Banting, 2002; Korolchuk et al., 2005). These findings allow us to hypothesize that plant CK2 could also be involved in vesicle formation and thus, in clathrin-dependent endocytosis of phot1 (Kaiserli et al., 2009; Roberts et al., 2011); i.e: CK2 activity would inhibit this process. A role of CK2 in vesicle trafficking is also supported by the finding that CK2 interacts with SEC3A protein (data from our Y2H assay). SEC3A is a component of the exocyst complex, which is involved in tethering vesicles to the plasma membrane during regulated or polarized secretion (Hála et al., 2008). It was recently reported that the exocyst complex plays a role in polar auxin transport (PAT) by modulating the subcellular localization of PIN1 and PIN2 in Arabidopsis roots (Drdová et al., 2013). It is worth noting that CK2mut plants and TBB-treated plants have altered polar auxin transport and the auxin transporters PIN4 and PIN7 are mislocalized (Marques-Bueno et al., 2011a).

Does CK2 modulate lateral auxin distribution in phototropism?

Former members of the lab hypothesized that CK2 might modulate the activity of protein modules composed by an AGC kinase and a NPH3-like protein, which are involved in auxin transport and signalling (Cheng et al., 2007; Marques-Bueno et al., 2011b). Concordantly, we have identified ENP and NPY3 as CK2-interacting proteins. These two members of the NPH3/RPT2 (NRL) protein family are key regulators of auxin fluxes and PIN localization during organogenesis and root gravitropism (Cheng et al., 2008; Furutani et al., 2007; Furutani et al., 2014; Furutani et al., 2011; Cheng et al., 2007; Li et al., 2011), acting in concert with the AGC kinase PINOID. By analogy, it was suggested that the module formed by phot1 and NPH3 is involved in the regulation of auxin signaling and transport during phototropism (Cheng et al., 2007). In the present work, we have shown that CK2 modulates the signaling capacity of NPH3 by maintaining its phosphorylated dark state.

Phototropic bending in response to unilateral BL is mediated by asymetric auxin distribution between the lit and the shaded side of the hypocotyl (Esmon et al., 2006). The activity of $CRL3^{NPH3}$ is indispensable for the bending phenotype, as *nph3* mutants show null phototropic bending (Liscum and Briggs, 1995; Liscum and Briggs, 1996) likely due to absence of lateral auxin distribution (Haga et al., 2005). To date, the only known substrate of the $CRL3^{NPH3}$ complex is the photoreceptor phot1, though its direct involvement in auxin transport is still unclear. Potential aditional targets of the CRL^{NPH3} complex could be the members of the PIN protein family. Indeed, other members of the NPH3 family, among them ENP and NPY3, have been also directly linked to polar auxin transport by regulating the subcellular localization of PIN proteins (Furutani et al., 2007; Furutani et al., 2011), althought the molecular mechanism by wich they do this function is not known.

PIN1, PIN3, and PIN7 might act redundantly in the positive phototropic response in the hypocotyl (Sakai and Haga, 2012; Haga and Sakai, 2012), but whether they play a role in the formation of lateral auxin gradients has not been demonstrated. By contrast, changes in the subcellular localization of PIN2 have been directly linked to the negative phototropic curvature of roots in response to unidirectional blue-light (BL). Interestingly, the mechanism driving the light-modulated PIN2 subcellular re-localization depends on the presence of the NPH3/phot1 module (Wan et al., 2012). Several studies have reported that PIN2 is located at the plasma membrane in light conditions but, in the dark, it moves to the lytic vacuole for degradation (Wan et al., 2012; Laxmi et al., 2008; Kleine-Vehn et al., 2008). Upon gravistimulation, asymetric degradation of PIN2 in the upper and lower parts of the root is

necessary to create auxin gradients and the root curvature (Abas et al., 2006; Kleine-Vehn et al., 2008; Baster et al., 2013). Gravity-stimulated PIN2 trafficking to the vacuole requires the sorting activity of the retromer components SNX1 and VPS29. The retromer complex regulates the rate of PIN2 translocation from the prevacuolar compartments (PVC) to the vacuole, or its recycling to the early endosome (EE)/trans Golgi Network (TGN) (Kleine-Vehn et al., 2008; Jaillais et al., 2006). Wan and colleagues (Wan et al., 2012) proposed that during the root phototropic response NPH3 acts in the retromer pathway as a switch to determine PIN2 trafficking between PVCs and EEs/TGNs. Considering the actual knowledge about the role of NPH3 in ubiquitin signalling, it seems plausible to think that this posttranslational modification determines the direction of PIN2 trafficking in response to light, i.e: recycling to the plasma membrane or moving into the vacuole for degradation. Indeed, ubiquitination of PIN2 (as well as of other plasma membrane proteins) acts as a sorting signal to enter the endocytic pathway, which modulates PIN2 intracellular dynamics and turnover (Korbei and Luschnig, 2013; Luschnig and Vert, 2014). Specifically, lysine63-linked poliubiquitination targets PIN2 for its vacuolar degradation (Leitner et al., 2012) during root gravitropic response (Leitner et al., 2012; Abas et al., 2006). On the other hand, it has been suggested that the COP9 signalosome modulates the dark-induced vacuolar degradation of PIN2 (Laxmi et al., 2008). Thus, ubiquitination and subsequent protein degradation seems to be an important mechanism to modulate auxin-mediated tropic responses. Although PIN ubiquitination has not been reported as a part of the hypocotyl phototropic response, auxin accumulation in the elongation zone of the hypocotyl is facilitated by reduction of PIN3 abundance in and below this region of the hypocotyl (Christie et al., 2011), thus suggesting conserved mechanisms between the regulation of root and hypocotyl tropic responses.

In addition to PIN proteins, indirect modulators of auxin transport could also be substrates of $CRL3^{NPH3}$. Recently, it has been reported that NPH3 interacts with Arabidopsis Gβ (AGB1) (Kansup et al., 2014), a G-protein that has been involved in auxin transport in the root (Mudgil et al., 2009). Identification of additional CRL^{NPH3} targets would give new clues about the essential role of this complex in phototropism.

Plant CK2 at the crosstalk of phosphorylation and ubiquitination in...

...light signalling pathways

Different authors have shown that CK2 activity in mammals and yeast is involved in ubiquitin-mediated protein degradation. On one hand, CK2 phosphorylation promotes ubiquitination by increasing the activity of ubiquitin ligases (Papaleo et al., 2011; Sadowski et al., 2007) or by creating phosphodegrons (Tsuchiya et al., 2010; Chakraborty et al., 2011; Watanabe et al., 2005; Scaglioni et al., 2008). On the other hand, CK2 phosphorylation can prevent ubiquitination, acting as a protective mark in some particular substrates (van Tiel et al., 2012; Parsons et al., 2010). In plants, CK2 seems to be a key player in the phospho-dependent regulation of protein degradation in light signalling pathways. CK2 phosphorylation of the positive transcription factors HY5 and HFR1 induces their stabilization by preventing interaction with COP1 (Hardtke et al., 2000; Park et al., 2008), whereas CK2-dependent phosphorylation of the negative regulator PIF1 induces its degradation via the 26S proteasome system (Bu, Zhu, Dennis, et al., 2011). Together, the balance between these factors modulate the changes of gene transcription that direct de-etiolation (Huq, 2006). Interestingly, autophosphorylation of the β4 regulatory subunit of Arabidopsis CK2 stimulates its ubiquitination and degradation by the proteasome (Perales et al., 2006), which is important for the modulation of the circadian clock activity.

In this work, we propose a model in which the CK2-dependent phosphorylation of the phototropic transducer NPH3 inhibits its activity as a substrate adaptor in a Cullin3 ring E3 ubiquitin ligase complex. As such, we provide another example of the role of CK2 in the phosphorylation-ubiquitination crosstalk in light signalling.

... organogenesis (and other pathways?)

We have shown that CK2 interacts with ENP and NPY3, two members of the NRL protein family that, as the NPH3 protein, are characterized by the presence of a N-terminal BTB domain. BTB-containing proteins have been described as substrate adaptors of Cul3 ring complexes in different organisms, including plants (Willems et al., 2004; van den Heuvel, 2004; Geyer et al., 2003; Hua and Vierstra, 2011). Considering the wide variety of cellular

process in which the NRL proteins are involved, together with the severe developmental and signalling defects shown by CK2 mutant plants (Marques-Bueno et al., 2011a; Moreno-Romero et al., 2008; Mulekar and Huq, 2013; Mulekar et al., 2012; Armengot et al., 2014), it is tempting to speculate that CK2 could be a general modulator of the NRL family and, thus, of the phosphorylation-ubiquitination crosstalk involved in a wide variety of signalling pathways.

In support of this widespread/unespecific role of CK2 in ubiquitination is the finding that CK2 interacts with the protein CAND1 (cullin-associated and neddylation-dissociated 1) in our Y2H screen (Table S5). Cand1 is an evolutionary conserved protein. We found that AtCAND1 and its human homolog contain two conserved serine/threonine residues which fall into the CK2 consensus phosphorylation sequences (data not shown). Interestingly, these two residues have been found phosphorylated *in vivo* in human CAND1 using a high throughput mass spectrometry assay (Daub et al., 2008). In Arabidopsis, CAND1 interacts with CUL1 and modulates the assembly/disassembly of SCF complexes, which mediate, for example, the degradation of AUX/IAA repressors (Zhang et al., 2008). Moreover, in Drosophila CAND1 regulates the activity of Cullin3-dependent E3 ligases (Kim et al., 2010).

... SA signalling pathway

There are several examples about the role of ubiquitination in SA-dependent defense responses. An interesting example is the ubiquitin-mediated degradation of NPR1, a master regulator of the SA-induced transcriptional reprogramming in innate immunity (Spoel et al., 2009). NPR1 and its homologs NPR3 and NPR4 are also BTB containing proteins, but NPR3 and NPR4 are also SA receptors (Fu et al., 2012). They interact with Cul3 and act as substrate adaptors within the CRL3NPR3 and CRL3NPR4 complexes, respectively. Both complexes target NPR1 for its degradation via the 26S proteasome in a SA-dependent manner (Yan and Dong, 2014). It would be interesting to test whether CK2 can modulate the activity of these receptors as it does with NPH3. Moreover, NPR1 ubiquitination is driven by phosphorylation. Perhaps, CK2 could contribute to the creation of this phosphodegron (Spoel et al., 2009).

An additional example can be found in the role of the CPR1 protein in the ubiquitin pathway. CPR1 is an F-box protein that acts as a substrate adaptor of a SCF E3 ubiquitin ligase complex (Gou et al., 2012). This complex targets the leucine-rich repeat domain (NLRs) type of immune receptors for its degradation by the proteasome, thus, avoiding

unwarranted autoimmune responses due to high concentrations of these receptors (Gou et al., 2012). The Arabidopsis *cpr1* mutant shows increased endogenous SA content and root phenotypes similar to those of CK2mut plants, thus, suggesting a possible functional link between CK2 and CPR1.

Concluding remarks

Protein kinase CK2 is a highly pleotropic protein kinase, involved in a long list of putative signalling pathways. In addition to its known role in some particular pathways, CK2 could be considered as a master regulator of regulators. CK2 might modulate the activity of regulatory proteins acting upstream of specific signalling pathways, and thus, be indirectly involved in the regulation of many signalling cascades. Some examples can be found in the list of new putative substrates presented in this work. Among them, CAND1 and SEC3A could be good candidates.

With this thesis project, we have contributed to get new insights about the far-known but incomplete knowledge of CK2 in SA signalling pathways. Additionally, we have obtained evidences about a new role of CK2 in light-regulated pathways, such as the modulation of plant phototropism. Moreover, we also uncovered a key role of plant CK2 in the crosstalk between phosphorylation and ubiquitination, a quite well studied role of CK2 in mammals but mostly unknown in plants. I would deeply encourage future researchers on plant CK2 to further analyse this latter role.

Conclusions

1. Several homozygous lines of transgenic Arabidopsis plants constitutively overexpressing an active catalytic subunit of protein kinase CK2 ($CK2^{OE}$) were obtained. The phenotypic analysis showed that CK2α overexpression did not affect their root architecture, but increased the rate of root growth. $CK2^{OE}$ plants showed enhanced length of the main root and increased number of laterals roots.

2. CK2 mediates salicylic acid (SA) responses by affecting SA biosynthesis and SA-signalling:

   2.1. The dominant negative mutant of CK2 (*CK2mut*), as well as TBB-treated wild-type plants, contain increased levels of SA in roots. In addition, several genes encoding enzymes of the SA biosynthesis pathways were overexpressed in CK2mut plants, indicating that CK2 is a negative regulator of SA biosynthesis.

   2.2. SA stimulates the expression of CK2 subunits in a NPR1-dependent manner. Thus, we propose a negative regulatory feedback loop between SA biosynthesis and CK2 activity, necessary to maintain SA homeostasis.

   2.3. We conclude that the high SA levels in roots of CK2mut plants are responsible for the previously reported altered root phenotypes of these plants. Several evidencies support this conclusion: i) SA-treated wild-type plants and SA-accumulating mutants show the same root phenotypes as CK2mut plants, and ii) depletion of CK2 activity in SA-defective and SA-signalling mutants produced partial recovery of the WT root phenotypes.

   2.4. SA induces transcriptional repression of genes encoding the auxin transporters PIN1-PIN4 and PIN7, and stimulates transcription of protein kinase PINOID, indicating a cross-talk between SA- and auxin-signalling pathways, However, the repressive effect of SA on PIN4 and PIN7 expression requires a functional CK2.

3. By performing a high throughput Y2H screen using CK2α as bait, we identified 28 new putative substrates of CK2 in plants. These putative substrates are proteins involved in a wide variety of biological processes, such as polar auxin transport, cytoeskeleton dynamics, vesicle exocytosis and ubiquitination. In particular, four members of the plant specific NPH3/RPT2 (NRL) protein family were identified as CK2-interacting proteins, including its founder member NPH3 (NON-PHOTOTROPIC HYPOCOTIL 3), an essential component of the phototropic signaling pathway.

4. We propose that CK2 plays a dual role in the hypocotyl phototropic signaling pathway in Arabidopsis, based on the following results:

   4.1. CK2 phoshorylates NPH3 *in vitro*, and CK2 activity is necessary to fully accomplish the phosphorylated dark state of NPH3 *in vivo*.
   4.2. CK2-mediated phosphorylation of NPH3 in the dark is necessary to maintain its inactive state and, thus, to repress its signaling capacity as a substrate adaptor of a culin3 ring E3 ligase complex ($CRL3^{NPH3}$).
   4.3. The blue-light photoreceptor phototropin 1, the only $CRL3^{NPH3}$ substrate known to date, is constitutively ubiquitinated in both dark and light conditions in CK2-depleted plants, supporting the idea of a constitutive activation of the $CRL3^{NPH3}$ complex in the absence of CK2.
   4.4. CK2 activity is necessary to maintain phot1 at the plasma membrane in dark conditions, which could explain the non-phototropic phenotype of CK2-defective plants.
   4.5. Contrary to what has been reported by other authors, light-induced phot1 internalization in wild-type plants is independent of NPH3, and thus, of its ubiquitination status. The same is true in CK2-defective plants.

5. The role played by CK2 in phototropism reported in this work, together with additional data published by other authors, lead us to postulate that CK2 might be a modulator of the crosstalk between phosphorylation and ubiquitination in light signalling pathways. It will be worth to investigate whether this conclusion might be extended to other signaling pathways, both in plants and mammals, since the high pleiotropic nature of this enzyme favors the idea of its function in basic processes affecting multitude of pathways.

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Supplemental information

Supplemental figures

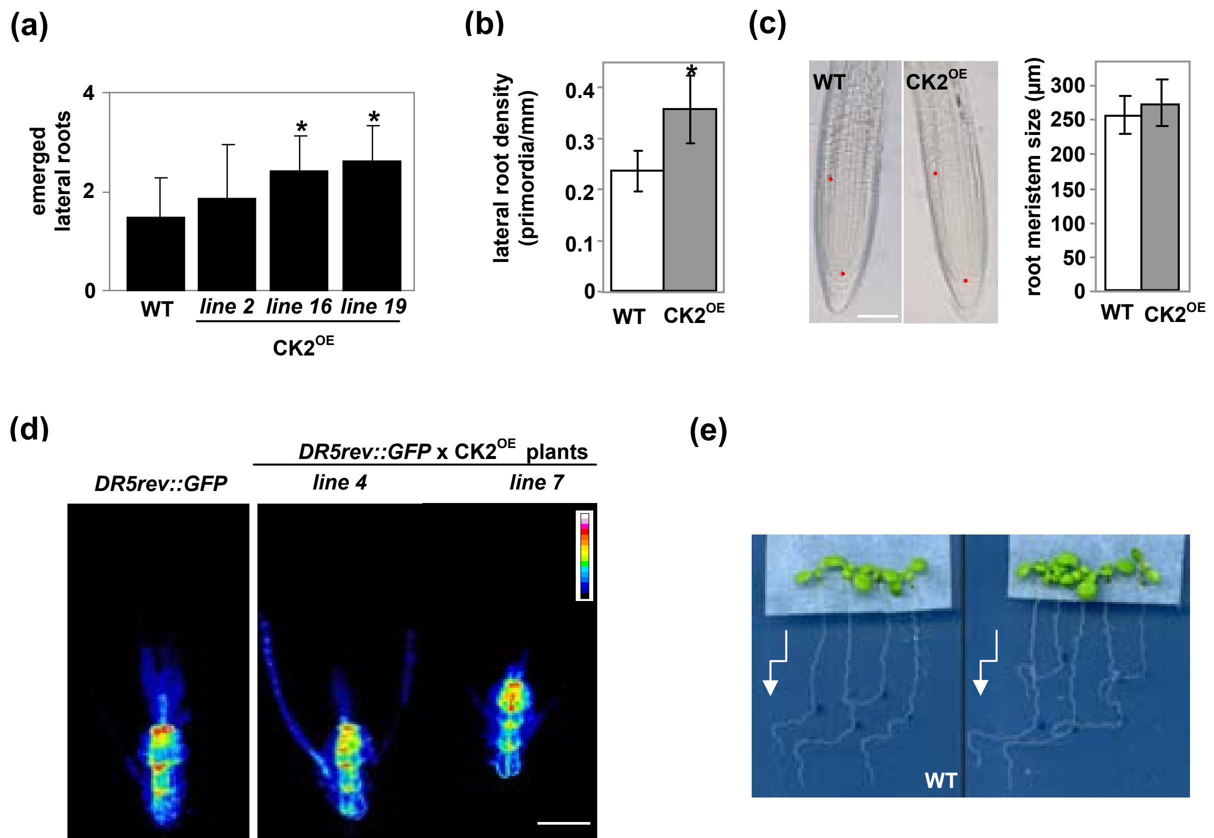

Figure S1. Lateral root density, meristem size, auxin distribution, and gravitropic response in CK2^{OE} roots.

(a) Number of emerged lateral roots in six-day-old Arabidopsis seedlings (WT and different CK2^{OE} lines). Mean values (±SD) are represented (n=10-25). **(b)** Lateral root densities, measured as number of root primordia per mm, in 5-day-old CK2^{OE}3 seedlings. Mean values (±SD) are shown (n≥25). The experiment was carried out three times with similar results. **(c)** Root meristem sizes of 5-day-old CK2^{OE}3 seedlings. Red dots in the pictures mark the meristem boundaries. Scale bar: 100 µm. **(d)** Expression of *DR5::GFP* reporter in CK2^{OE}3 roots, recorded by confocal microscopy as in (Marques-Bueno et al., 2011a). Scale bar: 50 µm. **(e)** Root gravitropic response in CK2^{OE}3 seedlings. The changes in the gravitropic vector (carried out twice) are indicated by the connecting arrow. Statistical analyses were performed using Student's *t*-test at p≤0.05 and significant differences are marked by asterisk (*). WT: wild-type; CK2^{OE}, CK2-overexpressing plants.

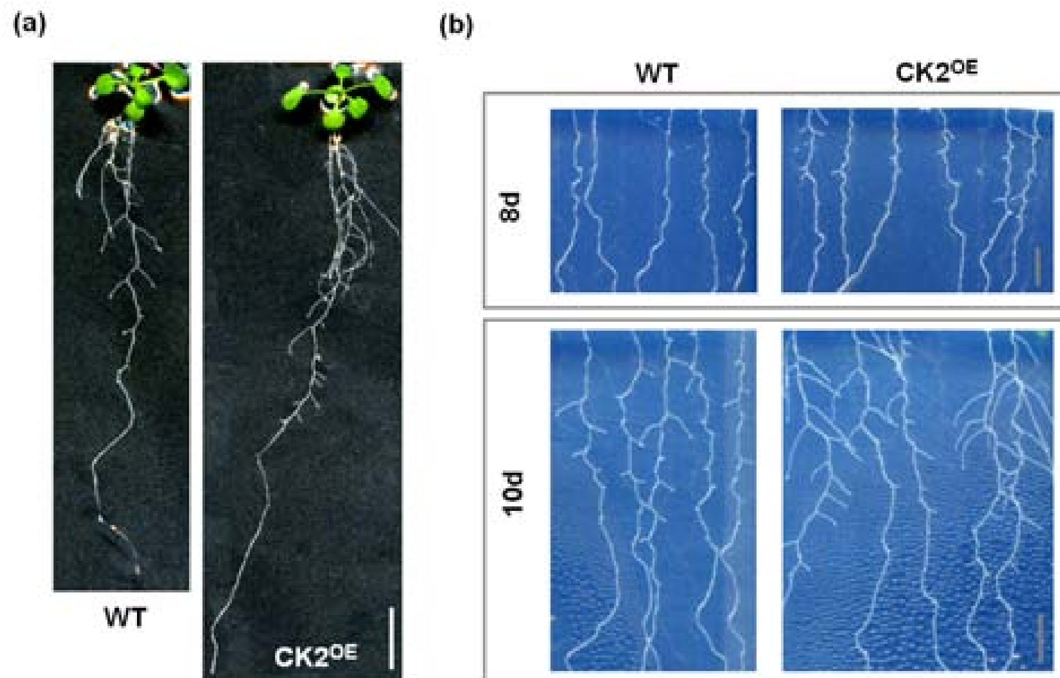

Figure S2. Root development of CK2α-overexpressing plants.
(a) Phenotype of 13d-old Arabidopsis seedlings. $CK2^{OE}$ plants exhibit slightly longer primary roots and increased number of lateral roots. Scale bar: 1cm. **(b)** Detail of lateral roots in 8d-old and 10d-old Arabidopsis seedlings. The number of emerged lateral roots is higher in 8d-old $CK2^{OE}$ seedlings than in WT plants and the number and length of emerged lateral roots is increased in 10d-old $CK2^{OE}$ plants. Scale bars: 0.5 cm. *Abbreviations:* WT, wild-type plants; $CK2^{OE}$, CK2α-overexpressing plants.

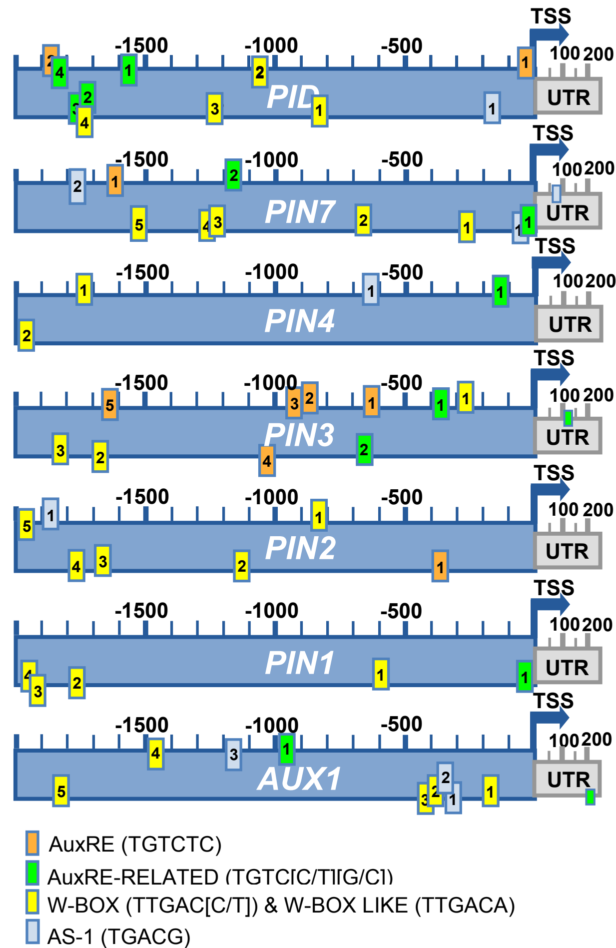

Figure S3. *Cis*-element organizations in gene promoters of *PINs*, *AUX1* and *PID*.
The -2,000 bp promoter sequences and the 5'-UTRs of five members of the *PIN* gene family (*PIN1*, *PIN2*, *PIN3*, *PIN 4* and *PIN7*), as well as of *PINOID* and *AUX1* genes, are plotted in the 5' to 3' orientation. The location of specific *as-1*, W-box, and ARE *cis*-elements is shown for each gene, using a color code. The motifs in the promoter region are in numerical order according to their proximity to the transcription start site. The exact positions of the motifs are shown in Table S3. *Abbreviations*: TSS, transcription start site; UTR, untranslated region.

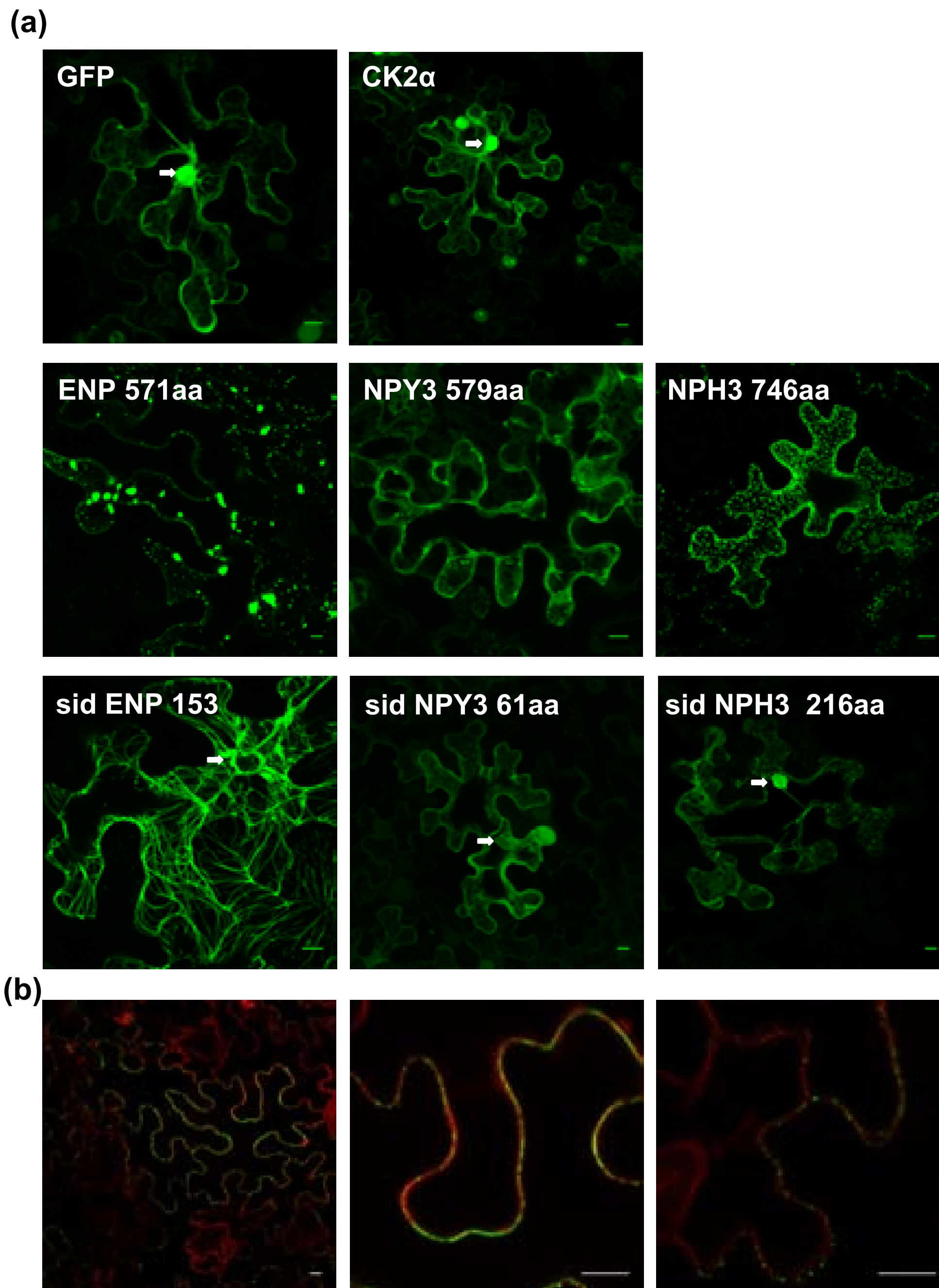

Figure S4. Subcellular localization of full-length ENP, NPY3 and NPH3 and of their respective selected interaction domains (SID).

(a) N-terminal GFP-tagged full-lenght ENP, NPY3 and NPH3 and their respective SID sequences were transiently expressed in *N benthamiana* leaves by agroinfiltration. The lenght of each protein (aa, aminoacids) is shown. The full lenght proteins were found located at the same subcellular compartment than previously reported in Arabidopsis: ENP in endosomal compartments (Furutani et al., 2007), NPH3 at the plasma membrane (Motchoulski and Liscum, 1999) and NPY3 at the plasma membrane and partially at the citosol (Furutani et al., 2011). Note that NPH3 is not uniformly distributed throught the plasma membrane. To the contrary, localization of the GFP-tagged SID domains showed significant differences as compared to their full-length counterparts: sid-ENP was located in the cytoskeleton, and sid-NPH3 and sid-NPY3 were located in the plasma membrane, the cytosol and the nucleus. Arrows indicate nuclear localization. Subcellular localization of GFP and CK2α are also shown. Images correspond to maximum projections of z-stack of 15 µm in depth. Bar: 10 µm. **(b)** Detail of the subcellular localization of NPH3. Left pannel shows a single section of GFP-NPH3 expressing cells, where discontinous localization of NPH3 within the membrane can be seen. Magnification details of the membrane are shown in the middle and right pannels. Patches and dotted-like distribution of NPH3 can be observed. Membranes were stained with FM4-64 (red). Bar: 10 µm.

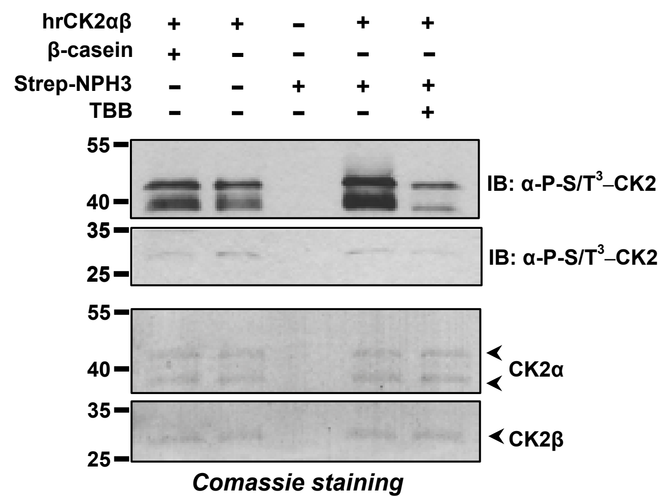

Figure S5. Autophosphorylation of CK2 subunits.
Anti-Phospho-S/T^{3}-CK2 antibodies efficiently recognize the autophosphoryled forms of CK2α and CK2β subunits of human recombinant CK2αβ that was used in the phosphorylation assays in Figure 2A. Moreover, the CK2 specific inhibitor TBB reduces the level of autophosphorylation of the kinase subunits. Equal amounts of kinase were used in each reaction as visualized by Comassie staining. *Abbreviation*: IB, immunoblotting.

Supplemental tables

Table S4. List of primers used for cloning into the entry vector pDONOR221.

GENE NAME	PRIMER NAME	SEQUENCE
Full lenght coding sequence		
ENP (AT4G31820)	attB1-ENP	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAGTTCATGAAGCTAGG
	attB2-ENP	GGGGACCACTTTGTACAAGAAAGCTGGGTTCACGATATCGAATGTCTGC
NPY3 (AT5G67440)	attB1-NPY3	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAGTTTATGAAACTTG
	attB2-NPY3	GGGGACCACTTTGTACAAGAAAGCTGGGTTCAAGACGATGACCTTCTC
NPH3 (AT5G64330)	attB1-NPH3	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGATGTGGGAATCTGAGAG
	attB2-NPH3	GGGGACCACTTTGTACAAGAAAGCTGGGTTCATGAAATTGAGTTCCTCC
NtCK2A3 (AJ438263)	attB1-CKA3	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCAAAAGCTCGTGTTTACACCG
	attB2-CKA3	GGGGACCACTTTGTACAAGAAAGCTGGGTCTACTGCGTCCTCATCCTGCTATTC
Selected Interaction Domain (SID)		
ENP (AT4G31820)	attB1-sid ENP	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTTGATTGATGCTTATCTCGC
	attB2-sid ENP	GGGGACCACTTTGTACAAGAAAGCTGGGTCTACCTTGACCTCGACGGCATCA
NPY3 (AT5G67440)	attB1-sid NPY3	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCCGGAGGCTTGTGCTCACGC
	attB2-sid NPY3	GGGGACCACTTTGTACAAGAAAGCTGGGTCTACGTCTCCTCCGTGGCTGTGA
NPH3 (AT5G64330)	attB1-sid NPH3	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAAACGCGTAGGGATGCAATT
	attB2-sid NPH3	GGGGACCACTTTGTACAAGAAAGCTGGGT CTATGGGATCATAGGCTGATACG

Table S5. List of CK2-interacting proteins identified in a Y2H screen

	Protein	**Arabidopsis Genome Iniciative Code**	**Nº of clones[a]**	**SID[b]**
1	SEC3A	AT1G47550	2	3-152
2	MAP65-6	AT2G01910	4	212-442
3	MC4	AT1G79340	1	11-236
4	CAND1	AT2G02560	1	301-765
5	D111/ G-patch domain-containing	AT3G09850	1	294-781
6	ENP *	AT4G31820	3	374-726
7	F18C1.6	AT3G05670	3	263-426
8	F18G18.100	AT5G25360	1	25-169
9	F25P17.1	AT2G24690	2	98-414
10	F8F6.230	AT5G04020	3	39-158
11	K18I23.16	AT5G05360	6	33-163
12	K1F13.23	AT5G66560	12	480-619
13	MZN1.26	AT5G58720	1	92-384
14	***NPH3****	AT5G64330	2	419-633
15	NPY3*	AT5G67440	7	412-472
16	T19C21.6	AT2G38450	11	49-117
17	T1O24.8	AT2G43340	5	1-84
18	T28P16.10	AT2G31410	1	2-199
19	centromeric protein- related	AT4G32190	1	430-637
20	hypothetical prot ein	AT3G53540	7	203-606
21	hypothetical protein	AT3G15770	3	65-162
22	ribosomal protein S5 family protein	AT1G64880	1	306-512
23	transcription-coupled DNA repair	AT5G28740	3	291-524
24	transcriptionfactor	AT3G14180	2	168-403
25	ubiquitin thiolesterase	AT4G01037	1	150-460
26	WAVE5	AT4G18600	1	238-569
27	F9K20.10	AT1G78850	1	200-441
28	F9L1.29	AT1G15350	1	11-154

[a]Number of positive clones whose nucleotide sequence could be assigned to annotated genes in *Arabidopsis thaliana* databases. [b]The Selected Interaction Domain (SID) defines the minimum sequence necessary for the interaction with the bait, identified by sequence comparison of the different clones found for an interacting protein. * The interaction has been validated in planta (this work). ***NPH3*** has been shown to be phosphorylated by CK2α (this work).

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