Universitat Autònoma de Barcelona

# Functional implications of protein kinase CK2 in F-actin dynamics and in the cross-talk between auxin and salicylic acid signalling pathways in Arabidopsis thaliana

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*Ai miei genitori,*
*Giuseppa e Giovanni*

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# *Summary*

Protein kinase CK2 is a pleiotropic Ser/Thr kinase, vital for all eukaryotes. It is involved in the regulation of many plant signalling pathways, including responses to various hormones and to abiotic stresses. Continuing the work previously carried out in the laboratory of Dra. C. Martínez (UAB), we have addressed the study of the role of CK2 in three plant biological processes.

**Role of CK2 in plants phototropisms**. A dominant negative mutant of CK2 (CK2mut) obtained by former members of the laboratory showed phenotype traits linked to alterations in auxin dependent processes and defects in shoot phototropism. Here, we confirm the previous findings of a loss of phototropic response in hypocotyls and we show for the first time an increase of the root phototropic curvature under blue light. Furthermore, studies of auxin distribution in blue-light illuminated seedlings showed a dramatic increase of auxin after CK2 depletion. We also studied the intracellular distribution of PIN-FORMED2 (PIN2) in CK2mut seedlings, grown under dark and light conditions using the reporter PIN2-GFP. We found that PIN2 internalization into vacuolar compartments, which normally occurs in wild type seedlings when transferred to darkness, was absent in CK2mut seedlings under the same conditions. By using brefeldin A (BFA), an inhibitor of the intracellular protein transport, we confirmed that PIN2 is not transported to vacuolar compartments in

CK2mut seedlings but that PIN2 recycling pathway towards the PM is not affected. Thus, we propose that CK2 activity is necessary for PIN2 trafficking, which is part of the cellular response linked to phototropisms.

**Role of CK2 in the crosstalk between salicylic acid and auxin signalling pathways**. CK2mut seedlings exhibited widespread changes in the expression of auxin-related genes (Marquès-Bueno et al., 2011a). In order to better understand auxin-signalling regulation in CK2-defective plants we performed histochemical and fluorimetric assays of Arabidopsis seedlings containing a translational fusion of the auxin repressor AXR3 with GUS (HS::AXR3.3NT-GUS) under the control of a heat-shock inducible promoter. We demonstrated that inhibition of CK2 resulted in over-stabilization of the AXR3 repressor, which could not be reverted by treatments with exogenous auxin. As CK2mut seedlings contain high levels of salicylic acid (SA), which are responsible for the altered root phenotype (shorter roots and absence of lateral root formation), we then studied the cross-talk between auxin and SA pathways. To do that, we crossed the HS::AXR3.3NT-GUS line with the NahG transgenic line, which is unable to accumulate SA. We demonstrated that the over-stabilization of AXR3 in CK2-defective plants is mediated by SA.

**Role of CK2 in the dynamics of actin cytoskeleton**. *In vivo* confocal imaging of actin architecture was performed by using the actin reporter GFP-FABD2. Our results show that CK2-depleted seedlings showed strong disorganization of the actin network, the main affected characteristics being, the collapse of F-actin bundles, the loss of the central nucleus position within the cell, and deformation of cell's shape. Studies of actin turnover in Arabidopsis seedlings

confirmed that the lack of CK2 activity strongly affects polymerisation of actin filaments and their polar distribution. F-actin turnover is regulated by actin binding proteins, among them the family of actin depolymerisation factors (ADFs), which promote severing and actin depolymerisation. Recently it was shown that the Arabidopsis casein Kinase 1-like protein 2 (CKL2) regulates actin filament stability by phosphorylation of ADF4. In our study, we found some putative predicted phosphorylation sites for CK2 in ADF4. Thus, we can speculate that CK2 might be involved in the regulation of the ADF4 actin filament disassembly activity, probably localized around the nuclear envelope where the actin filaments are stabilized.

# Introduction

Phosphorylation is one of the most common post-translational modifications of proteins responsible for the signal transmission (Garcia-Garcia et al., 2016). It provides a sensitive, rapid and dynamic way to regulate protein activity, stability and turnover or to modulate protein-protein interactions and sub-cellular localisations (Hunter, 2012). The antagonistic action of two types of enzymes regulates the reversible process of phosphorylation: protein kinases and protein phosphatases. Protein kinases catalyze the transfer of a γ-phosphoryl group from ATP (or GTP) to a specific amino acid of the protein substrates, principally serine, threonine, tyrosine and histidine. Conversely, the phosphate groups are hydrolysed from the protein substrates by the Ser/Thr, Tyr or dual specificity phosphatases. Protein kinases are classified as “conventional” protein kinases, which include the above mentioned Ser/Thr and Tyr kinases, and as “atypical” potein kinases, a small group of kinases that do not have a clear sequence similarity with the other kinases, but they show kinase activity (Manning et al., 2002). Genomic analysis in the model plant *Arabidopsis thaliana* revealed that the number of genes that encode protein kinases amounts to 4% of its whole genome (Champion et al., 2004). Plant kinomes are significantly larger than those of other eukaryotes (i.e: protein kinase encoding genes in yeast is ~1.8% and in Homo sapiens is ~1.7%, versus the 4% in Arabidopsis), which is

probably due to events of gene and genome duplications. This functional redundancy plays an important role in plant genetic robustness, since, due to the sessile nature of plants, a large number of protein kinases might help to quickly adapt to changing environmental conditions (Champion et al., 2004). Moreover, it has been reported that about 30% of the whole nuclear proteins encoded by the *A. thaliana* genome require phosphorylation. If this is true, each kinase from *A. thaliana* is expected to phosphorylate an average of 7 proteins. This is probably an underestimation because many proteins are regulated by phosphorylation at multiple sites, and closely related protein kinases might possess overlapping specificities. Furthermore, some protein kinases phosphorylate specifically only one or few substrates (Champion et al., 2004). In order to meet the need of phosphorylation of so many proteins, the cells contain kinases which are able to phosphorylate multiple substrates, and protein kinase CK2 is a good example of this type of kinases.

# 1. Protein Kinase CK2

## *1.1. Biological roles of CK2, CK2 substrates and functions*

CK2 (formerly called casein kinase II), is an ubiquitous, pleiotropic, serine/threonine protein kinase, highly conserved in all eukaryotes. It is essential for cell viability and, despite it was one of the first protein kinase identified in animals (Burnett and Kennedy, 1954), its biological role has not been yet fully characterized, due to its high pleiotropy. CK2 has been largely studied in several plants species, such as maize (Riera 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). It has been found involved in different important processes, such as protein translation, chromatin structure or cell cycle regulation (Dennis and Browning, 2009; Moreno-Romero et al., 2012, 2011). Furthermore, CK2 activity modulates the responses to several hormones (Marquès-Bueno et al., 2011a; Mulekar et al., 2012), light signalling and circadian clock (Bu et al., 2011; Sugano et al., 1999). More than 300 substrates of CK2 have been identified in animals, where it is considered one of the major kinases contributing to the mammalian phosphoproteome. In plants, the number of identified CK2 substrates is much lower than in animals, and they are summarized in Table 1. Among them, we can find transcription factors (TFs) involved in the transduction of photomorphogenic signalling and of circadian rhythms, proteins of the basic machinery for DNA transcription and RNA translation, structural proteins of the proteosome, storage proteins of seeds, proteins related to stress responses, proteins involved in the synthesis of ATP and lipids, nuclear matrix proteins, nucleoid proteins and enzymes involved in lysine catabolism, and viral proteins. Analysis of plants with altered CK2 activity has revealed that plant CK2 regulates both plant growth and responses to certain environmental stimuli. CK2-mediated phosphorylation enhances the light-induced degradation of the phytochrome interacting factor (PIF1) to promote photomorphogenesis (Bu et al., 2011). CK2 is also involved in cell cycle control (Espunya et al., 1999, 2005), positively regulating cell proliferation as well as the suppression of apoptosis in normal mammalian cells (Trembley et al., 2010).

**Table 1. List of plant CK2 substrates.**

Adapted from Vilela et al. (2015).

| Name | Type | Species | Role | References |
|---|---|---|---|---|
| **Light-signal transduction pathway and circadian clock** | | | | |
| AT-1 | DNA binding factor | Pea | Binds to ATI-box elements in light regulated promoters | Datta and Cashmore (1989) |
| ATBP-1 | DNA binding factor | Pea | Binds to ATI-box elements in light regulated promoters | Tjaden and Coruzzi (1994) |
| GBF1 | bZIP TF | *Arabidopsis* | Binds to G-box elements in light regulated promoters | Klimczak et al. (1995) |
| Opaque2 | bZIP TF | Maize | Circadian clock regulated | Ciceri et al. (1997) |
| CCA1 | Myb-related TF | *Arabidopsis* | Circadian clock regulator | Sugano et al. (1998) |
| LHY, OsLHY | Myb-related TF | *Arabidopsis*, Rice | Circadian clock regulator | Sugano et al. (1998); Ogiso et al. (2010) |
| HY5 | bZIP TF | *Arabidopsis* | Promotes photomorphogenesis | Hardtke et al. (2000) |
| HFR1 | bHLH TF | *Arabidopsis* | Promotes photomorphogenesis | Park et al. (2008) |
| PIF1 | Phytochrome interacting factor | *Arabidopsis* | Represses photomorphogenesis | Bu et al. (2011) |
| **Abiotic and biotic stress** | | | | |
| ZmSnRK2/ZmOST1 | Protein kinase | Maize | ABA signaling | Vilela et al. (2015) |
| Rabl7,ZmLEA5cERD14, TAS-14 | LEA proteins | Maize, *Arabidopsis*, tomato, wheat | Stress responsive proteins | Plana et al. (1991); Liu et al. (2014); Alsheikh et al. (2003); Godoy et al. (1994) |
| TsDHN1,2 | Dehydrins | *Thellungiella salsuginea* | Stress responsive proteins | Rahman et al. (2011) |
| EmBP-2/ZmBZ-1 | bZIP TF | Maize | Activates transcription of the abscisic acid-inducible gene rab28 | Nieva et al. (2005) |
| TGA2 | bZIP TF | *Arabidopsis* | Binds to promoter of salicilic-induced genes | Kang and Klessig (2005) |
| OREB1 | ABRE binding factor | Rice | Binds to ABRE (ABA responsive Elements) | Hong et al. (2011) |
| p23 | co-chaperone protein | *Arabidopsis* | Plant response to Salicihc acid | Tosoni et al. (2011) |
| PCS | phytochelatin synthase | *Arabidopsis* | Synthesis of heavy metal-binding peptides | Wang et al., 2009 |
| **Chromatin associated and nuclear proteins** | | | | |
| lamin-like protein | lamina matrix protein | Pea | Nuclear stability, chromatin organization | Li and Roux (1992) |
| MFP1 | coil-coil protein | Tomato *Allium cepa* | Structural roles in nuclear matrix and chloroplast | Meier et al. (1996); Samaniego et al. (2006) |
| NopA64/nopA61 | nucleolin-like phosphoproteins | *Allium cepa* | Located in nucleolus | de Cárcer et al. (1997) |
| P-proteins | Ribosomal proteins | Maize | Complex with 60S ribosomal subunits | Bailey-Serres et al. (1997) |
| DNA helicase I | DNA helicase I | Pea | DNA transcription | Tuteja et al. (2001) |
| DNA topoisomerase I | DNA topoisomerase I | Pea | DNA transcription | Tuteja et al. (2003) |
| HMGB proteins | High mobility group B proteins | Maize, *Arabidopsis* | Chromatin associated proteins | Stemmer et al. (2002) |
| SSRP1 | structure-specific recognition protein | Maize | Chromatin associated proteins | Krohn et al. (2003) |
| eIF2ab/3c/4b/5 | elongation initiation factors | *Arabidopsis*, maize, wheat | Translation initiation | Dennis and Browning (2009) |
| Histone deacetilase 2B | Histone deacetilase | *Arabidopsis* | Chromatin remodeling enzyme | Dennis and Browning (2009) |
| **Chloroplast machinery** | | | | |
| Chloroplast RNPs/28RNP/ p34/RNP29,33 | Ribonucleoproteins | Spinach, *Arabidopsis* | RNA binding proteins involved in chloroplast RNA processing and stabilization | Kanekatsu et al. (1993, 1995); Lisitsky and Schuster (1995); Reiland et al. (2009) |
| CP29 | photosystem II subunit | Maize | Light harvesting complex import | Testi et al. (1996) |
| TOC159 | preprotein receptor | *Arabidopsis* | Nuclear-encoded chloroplast preproteins from the cytosol | Agne et al. (2010) |
| SIG1/SIG6 | plastid sigma factors | *Arabidopsis* | Gene-regulatory proteins for promoter binding and transcription initiation | Schweer et al. (2010) |
| Alb3 | Thylakoid membrane protein | *Arabidopsis* | Thylakoid biogenesis | Schonberg et al. (2014) |
| **Other** | | | | |
| CFOCF1-ATPase | Chloroplast ATP synthase (b subunit) | Spinach | ATP synthesis | Kanekatsu et al. (1998) |
| C2 | subunit of the 20S proteasome | Rice | Protein degradation of ubiquitinated proteins | Umeda et al. (1997) |
| gpl00/gp96 | Glycyrrhizin (GL)-Binding Protein (gp100) | Soybean | Lipoxygenase that catalyzes the oxygenation of unsaturated fatty acids | Ohtsuki et al. (1994, 1995) |
| β-Conglycinin α Subunit | β-Conglycinin α Subunit | Soybean | storage protein | Ralet et al. (1999) |
| calreticulin | Calreticulin | Spinach | $Ca^{2+}$ binding protein | Baldan et al. (1996) |
| apyrase | apyrase | Pea | ATP hydrolysis | Hsieh et al. (2000) |

### *1.2. Structural features of CK2*

CK2 most often appears in eukaryotic cells as a heterotetramer complex of 130-150 kDa (Figure 1) consisting of two catalytic subunits (CK2α; Mr = 36-44 kDa) and two regulatory subunits (CK2β, Mr = 24-26 kDa) (Litchfield, 2003). Crystallographic analysis of the human heterotetrameric CK2 structure showed a characteristic butterfly shape, with a central building block of β-β dimer, interacting with the two α subunits by their C-terminal domain. The two α subunits are the most distal components of the tetramer, and they do not interact with each other (Niefind et al., 2009). CK2 subunits are highly dynamic and able to interact with other partners and to participate in the transient formation of specific multimolecular complexes (Filhol et al., 2004).

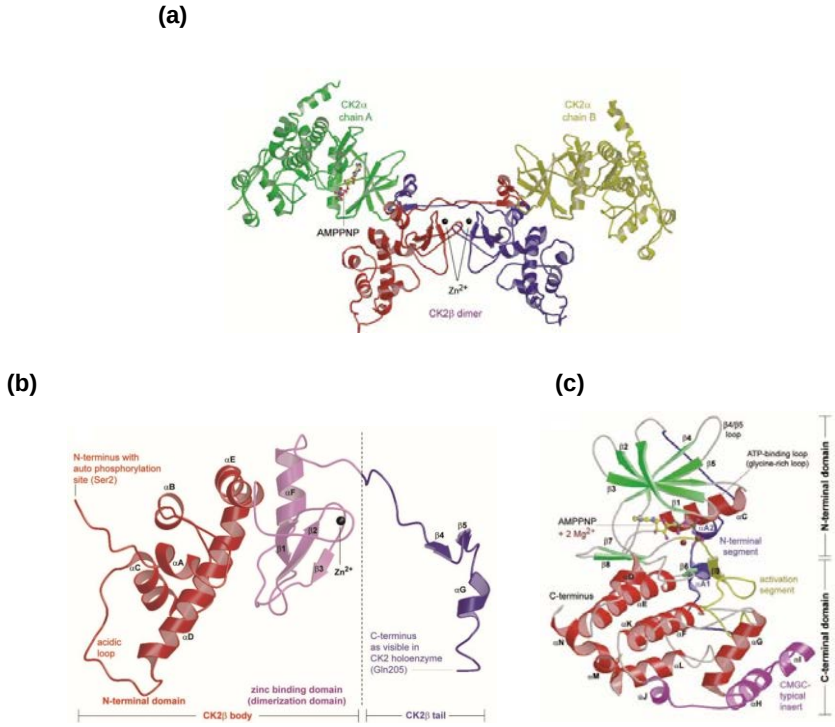



**Figure 1. Three-dimensional structure of protein kinase CK2.**

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

## *1.3. The catalytic subunit of CK2*

The catalytic subunit of CK2 is required for cell viability and it is highly evolutionary conserved in eukaryotes. This was demonstrated by experiments with *Saccharomices cerevisiae*, where simultaneous disruption of the two genes encoding CK2α subunits was lethal, and the phenotype was rescued by transformation with CK2α subunit from human or from *Caenorhabditis elegans* (Padmanabha et al., 1990). The amino acidic sequence of CK2α polypeptides show a high degree

of identity among the different plant forms (>90%), but this level decreases down to 50% when compared to those of animals and yeast (Espunya et al., 2005).The first three-dimensional structure of CK2α was obtained from *Zea mays*, in the presence of ATP and Mg2+ (Figure 1c), and has been helpful to understand some of the unique features of CK2 (Niefind et al., 1998). The CK2α structure contains a catalytic core composed of two major folding domains harbouring the active site in between. The N-terminal domain, which is the smaller domain, consists of an antiparallel β-sheet accompanied by a single long α-helix (helix αC), which is important for its function rather than for structural reasons. The C-terminal domain is folded as an α-helix. There are some important structural differences between CK2α and the catalytic subunit of other kinases, which explain the enzymatic characteristics of CK2. One of the specific features is the absence of serine and threonine amino acidic residues in the activation loop. In other kinases the phosphorylation of these residues determines a conformational change in the activation segment that stabilizes the protein in an “open conformation”, and activates its enzymatic activity. This phosphorylation has never been reported for CK2α and this supports the idea of CK2α as a constitutively active subunit (Niefind et al., 2009). This active state is maintained by intramolecular interactions between the N-terminal domain, the activation segment and the αC helix (Niefind et al., 1998). The three-dimensional structure (Figure 1c) shows these stabilizing contacts.

Another feature of CK2α is the presence of a cluster of basic residues (a region rich in lysine) placed at the beginning of the αC helix. The lysine basic stretch is implicated in the inhibition of CK2 by heparin (Vaglio et al., 1996) and it might contain a potential nuclear

localisation signal (NLS). It also has a crucial function in the recognition of CK2 substrates and confers to the enzyme its acidophilic character (Roher et al., 2001). Potential substrates of CK2 must contain multiple acidic residues located downstream of the phosphorylable amino acid (serine being preferred over threonine, and more rarely tyrosine). The minimal consensus sequence recognized by CK2 is X-S/T-X-X-E/D/pS, where X represents acidic amino acid residues, although variations from this consensus sequence have been also observed (Meggio and Pinna, 2003). The presence of a negative charge at position n+3, n+1 and n+2 in the substrates is crucial for CK2 phosphorylation. Another requirement is the absence of proline residues at position n+1. CK2 ability to phosphorylate tyrosine residues was first identified in *Saccharomices cerevisiae* (Wilson et al., 1997) and, later on, was also found in animals (Vilk et al., 2008). Furthermore, CK2 exhibits the ability to autophosphorylate on tyrosine residues (Donella-Deana et al., 2001). For this reason CK2 displays dual-specificity kinase activity in mammalian cells, which adds complexity to its study and increases the number of possible substrates and functions.

Unlike most protein kinases, CK2 can use both ATP and GTP as a phosphate donor, with similar Km (Meggio and Pinna, 2003). The Lys68 residue is localized nearby to this Lys basic stretch and is essential for the binding of ATP. Indeed, deletion of this residue inhibits CK2α activity. Conversely, Phe176 is an important conserved residue in nearly all kinases, with the only exception of CK2. In CK2α, Phe176 is replaced by a tryptophan and this substitution is important for the maintenance of the rigid “open” active state of the catalytic subunit (Battistutta, 2009) and also for the CK2 ability to use both ATP and GTP as phosphate donors (Niefind et al., 1998). The knowledge

about the structure of CK2α has been very useful to design specific inhibitors of CK2 activity, which are strategic tools in the study of CK2 functions. CK2 inhibitors are important for human health due to the anti-apoptotic nature of CK2 (Trembley et al., 2009) and due to its is high expression level in several cancer types.

### *1.4. The regulatory subunit*

The regulatory subunit of CK2, CK2β, is also highly conserved in eukaryotes through the evolutionary process but to a lesser extent than the catalytic subunit (Boldyreff et al. 1993; Maridor et al. 1991). The crystallographic structure of CK2β shows two distinguishable structural domains, one rich in α helix and the other rich in β sheets. Moreover, two unstructured tails, one at the N-terminal and the other at the C-terminal end of the protein polypeptide chain (Figure 1b) have been identified. A zinc finger domain, consisting of a $Zn^{2+}$ atom coordinated to four cysteine residues, has been found to be responsible for the formation of the CK2β-CK2β dimer, which is stabilized by hydrophobic interactions. An acidic tail is present at the terminal part of the tetramer in the N-terminal domain that can interact with polyamines and/or regulatory proteins of CK2 (Chantalat et al., 1999). A sequence with homology to the “destruction box” of the cyclins is located close to this acidic domain and might have a role in the stability of the protein. A loop rich in basic residues at the C-terminal domain is needed to establish interactions with the N-terminal domain of the catalytic subunit CK2α (Niefind et al., 2009). The plant’s β subunits show several peculiar characteristics not found in their animals counterparts, including the presence of an extra N-terminal extension of about 90 amino acids, a shorter C-terminal domain, and an acidic loop at the N-terminal domain with a poorly conserved amino

acid sequence (Riera et al., 2011; Velez-Bermudez et al., 2011). The three-dimensional structures of the CK2β subunit and of the CK2 holoenzyme from plants are not yet available.

## *1.5. Regulation of protein kinase CK2 activity*

Several mechanisms modulate the activity of the catalytic subunit of CK2 and contribute to its specificity for particular substrates (Litchfield, 2003). CK2 activity is independent of secondary messengers. In most cases, the tetrameric structure of CK2 shows higher catalytic activity than the monomer CK2α, and this is due to the interaction with the regulatory subunit, β. Only in the case of calmodulin the tetrameric form is less active than the monomeric form. Beside the regulation of the catalytic activity of CK2α, another interesting aspect of the β regulatory subunit is the modulation of the substrate specificity (Meggio et al., 1992). In the human genome, two genes encode the α subunit, which generates three different isoforms (α, $\alpha^{I}$ and $\alpha^{II}$), and one gene encodes the β subunit (Allende and Allende, 1995; Shi et al., 2001). In plants both α and β subunits are encoded by multigene families and due to this, the number of possible tetrameric combinations increases. In Arabidopsis, four genes encode the α subunit (αA, αB, αC and αcp) and four encode the β subunit (β1, β2, β3 and β4) (Espunya et al., 2005; Riera et al., 2001). The presence of multiple isoforms of CK2α/β subunits, the tissue distribution and the cellular and subcellular localisation of these subunits are also important for the substrate specificity of CK2 and for the CK2 regulation (Faust and Montenarh, 2000). In Arabidopsis, the catalytic subunits αA, αB, αC are localized in the nucleus, predominantly in the nucleolus, and the αcp is located only in the chloroplast. The

regulatory subunits β1 and β3 are located in the nucleus and in the cytosol, the β2 is located only in the nucleus and the β4 exclusively in the cytosol (Salinas et al., 2006). Each plant isoform has specific functions (Riera et al., 2001). In Arabidopsis, for example, only the β3 and β4 subunits seem to be involved in the regulation of circadian-clock (Sugano et al., 1999) and other different examples have also been found in tobacco (Espunya et al., 2005) and in maize (Riera et al., 2001). Furthermore, it has been found that co-expression of CK2α isoforms in maize with the four CK2β subunits modifies the subcellular localisation of the CK2 isoforms, causing their shift from the nucleolus to the nucleus and to cytoplasmatic aggregates. This suggests that CK2β subunits are regulators of CK2 localisation and consequentially of its functional diversification (Vélez-Bermúdez et al., 2015). Furthermore, both α and β, have been detected as free forms in different cellular compartments. They seem to be functionally independent from the ones associated to the heterotetramer and, thus, also the dynamic exchange of the subunits between the holoenzyme and the monomers might represent a form of regulation of CK2 activity. CK2β can also interact with other proteins in the absence of the catalytic subunit, acting as an anchoring element and/or docking platform for protein substrates and effectors (Bibby and Litchfield, 2005; Pinna, 2002).

A recently proposed mechanism of regulation is called “regulation-by-aggregation”, based on the higher-order interactions between CK2 tetramers (Schnitzler et al., 2014).

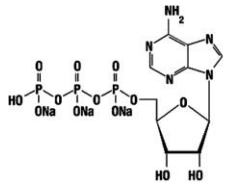
## 1.6. Strategies to study protein kinase CK2 function through its inhibition

### 1.6.1. Chemical inhibition of CK2

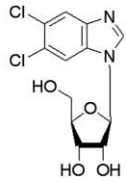
Two types of CK2 inhibitors are known nowadays: the classical ATP-competitive inhibitors, which are the most used, and the non-competitive inhibitors. To the first class belongs the 5,6-dichloro-1-(β-D-ribofuranosil)-benzamidazol (DRB) (Zandomeni et al., 1986), one of the earliest CK2 inhibitors developed. However, DRB inhibits also the protein kinase CK1 as well as other kinases (Meggio, Shugar, and Pinna 1990). Other CK2 inhibitors are reviewed in Battistutta, (2009). They are classified as anthraquinones, xanthenones, indoloquinazolines, coumarins, flavonoids, tyrphostins and pyrazolotriazines derivates. These inhibitors have a low inhibitory capacity or are not specific for CK2 (Sarno et al., 2003) except the anthraquinone quinalinzarin, which is highly specific for CK2 (Pagano et al., 2008). Nowadays, the most used ATP-competitive inhibitors are derivatives of DRB, and were obtained by substituting the DRB radicals with bromide atoms (Figure 2a), generating the tetrabrombo-benzimidazole (TBBz) and the 4,5,6,7-tetrabromobenzatriazol (TBB). Both compounds, TBBz and TBB, are stronger and more specific than DRB. Later another powerful compound even more specific than TBBz and TBB was identified, the 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT). These three compounds are highly specific 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). Comparative

studies demonstrated that the most effective and selective inhibitor of CK2 activity was the TBB (Pagano et al., 2008), which has been used in this work. Previous studies conducted in our lab showed that TBB inhibition produced effects similar to the genetic inhibition of CK2, which is described below.

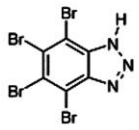
**(a)**



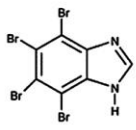
**ATP**



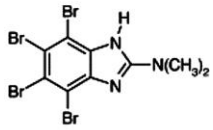
**DRB**
$IC_{50}$ = 13.0 μM
$K_i$ = 4.5 μM



**TBB**
$IC_{50}$ = 0.5 μM
$K_i$ = 0.4 μM



**TBBz**
$IC_{50}$ = 0.5 μM
$K_i$ = 0.7 μM



**DMAT**
$IC_{50}$ = 0.14 μM
$K_i$ = 0.04 μM

**(b)**

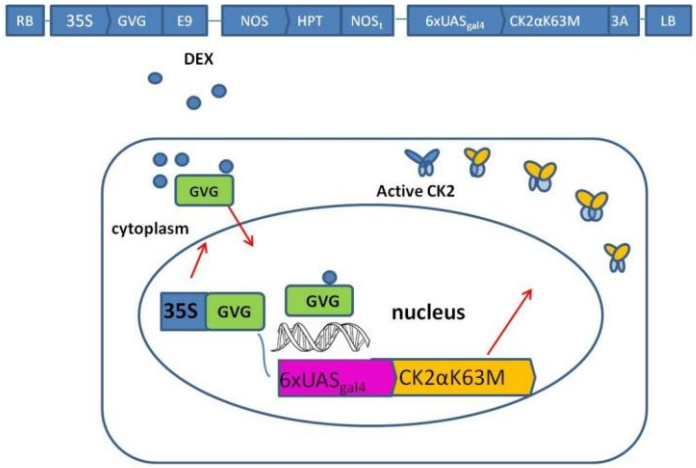


**Figure 2. Strategies of inhibition of CK2 activity.**

**(a)** Chemical structure of ATP and of the commercially available ATP-competitive CK2 inhibitors. Adapted from Duncan et al. (2008). **(b)** Scheme of the construct used to transform Arabidopsis plants for the genetic inhibition of CK2, and working mechanism of the dominant negative mutant of CK2 (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 synthase; 6XUASgal, promoter regulated by GVG; CK2αK63M, tobacco CK2α subunit with the inactivating K63M mutation; 3A, polyadenylation sequence of pea rbc-3A; L, left end of the T-DNA.

### *1.6.2. Genetic inhibition of CK2*

Several genetic strategies have been used for the *in vivo* inhibition of CK2. Among them, 1) use of antisense RNAs and of siRNAs to reduce the intracellular levels of CK2α; 2) generation of knockout mutants, and 3) generation of catalytically inactive mutants. However, the results from these genetic strategies were of difficult interpretation, likely due to remaining basal levels of CK2 activity caused by the facts that CK2 is highly expressed and that its activity is "constitutive" (Ruzzene and Pinna, 2010). Furthermore, an added difficulty arise from the partial functional redundancy of the multiple isoforms of CK2, by which the loss of activity of one isoform might be compensated other. For instance, a mouse knockout in one of the CK2α subunits generates a sterile but viable animal (Xu et al., 1999), whereas the disruption of both catalytic subunits in *S. cerevisiae* generates a lethal phenotype (Padmanabha et al., 1990). Based on these considerations, former members of our lab obtained a dominant negative mutant of CK2 in *Arabidopsis thaliana*, which has been used to increase our knowledge of the role of CK2 in plant systems, in particular in Arabidopsis development. This strategy was previously used in mammalian cells, and it is based on the elimination of CK2

activity by overexpression of a catalytic inactive CK2α subunit. This inactive subunit can still interact with the regulatory β subunits and with the CK2 substrates, competing with the endogenous active α subunits and producing a overall reduction of CK2 activity (Lebrin et al., 2001; Vilk et al., 1999) (Figure 2b). The catalytic inactive α subunit was obtained by site-directed mutagenesis of the *CKA3* gene of *Nicotiana tabacum* (GeneBank/EMBL accession no. AJ438263), substituting the lysine K63 by a methionine (Espunya et al., 2005; Moreno-Romero et al., 2008). This mutagenesis abolishes the phosphotransferase activity of the kinase. Moreover, to avoid the lethal effects of constitutive expression of the transgene, the mutated open reading frame (CK2mut) was cloned under a dexamethasone (Dex) inducible promoter. Dex is an analogue of glucorticoids that binds to the kimeric GVG transcription factor (Aoyama and Chua, 1997), whose DNA-encoding sequence is located in the same construct. The GVG is constitutively expressed in plant cells and accumulates in the cytoplasm. After binding to the inductor (Dex), GVG migrates to the nucleus where it recognizes the Gal4 6xUAS promoter and activates the expression of the *CK2mut* transgene. This construct was stably introduced in the Arabidopsis genome with *Agrobacterium tumefaciens,* and homozygous plants for the transgene were obtained.

Figure 2b shows a scheme of the working mechanism of the dominant negative mutant of CK2 (CK2mut line).

Studies performed with CK2mut seedlings revealed severe developmental defects (Moreno-Romero et al., 2008), such as depigmented and non-expanded cotyledons, larger and depigmented hypocotyls and shorter roots. CK2mut seedlings grown in darkness showed a partial de-etiolated phenotype. These developmental defects could be explained by the alterations in cell expansion and in cell-cycle progression. Moreover, prolonged induction of the transgene was lethal, confirming the vital function of CK2 in plants. Transient induction of the CK2mut transgene showed defects in lateral roots formation and in root growth (Moreno-Romero et al., 2008). Some of the altered phenotypes described are typically regulated by the phytohormone auxin (Moreno-Romero and Martínez, 2008). Indeed, CK2 was found to regulate auxin signalling and, more specifically, auxin transport (Marquès-Bueno et al., 2011a, 2011b). CK2mut plants exhibit reduced rootward auxin transport, and misregulation of gene expression, protein abundance and subcellular localisation of some members of the PIN-formed protein family, which function as auxin exporters (Marquès-Bueno et al., 2011a). Additionally, the regulatory kinase PINOID (PID) was misexpressed in CK2mut seedlings. Another important auxin-related phenotype identified in CK2mut hypocotyls is the lack of phototropic response (Marquès-Bueno et al., 2011a).

## 2. Overview Of Phototropism

Light is an environmental signal that modulates plant photomorphogenesis, contributing to plants adaptation to environmental conditions. Went and Cholodny (1928) defined positive hypocotyl phototropism as the tendency of hypocotyls to grow towards the light source, in contrast to the negative phototropism of the root. Plants perceive the light via multiple sensory systems called photoreceptors. The photoreceptors cover a wide range of the light spectrum, from near-UVB (280–315 nm) to far-red (FR) (~750 nm) (Briggs and Lin, 2012). With respect to the root phototropism, only phytochromes (red light photoreceptors) and phototropins (blue light photoreceptors) are relevant. Experiments conducted by Naundorf, (1940) showed the strong effects of blue light in root negative bending. It has been shown that blue light (BL) 320-500 nm and ultraviolet light (UV-A) are the most efficient wavelengths to induce phototropism.

Phytochromes (PHYs) were first discovered in the 1960s (Siegelman and Hendricks, 2006) and they are part of the red (R)/far-red (FR) (600–750 nm) photoreceptors family. In *Arabidopsis thaliana*, five members of this family (phyA-phyE) were found (Briggs, 2001). Their activation/inactivation is due to conformational changes of two photoconvertible isoforms. On the other hand, UV-A/Blue light (BL) is detected by three families of photoreceptors: cryptochromes (crys), phototropins (phots), and members of the ZTL/FKF1/LKP2 family (Zoltowski and Imaizumi, 2014).

Phototropins mainly regulate hypocotyl and root phototropisms. In Arabidopsis, two members of this family, phototropin 1 (Phot1) and phototropin 2 (Phot2), are known (Christie, 2007). Phot1 functions primarily as the photoreceptor for root and hypocotyl phototropisms (Kutschera and Briggs, 2012) over a broad range of blue light intensities (Christie et al., 2015), whereas Phot2 works only in hypocotyl phototropism and under conditions of high light intensity (Christie et al., 2015). The polypeptide chains of phototropins contain two functional domains: an N-terminal photosensory domain and a C-terminal serine/threonine kinase domain (Figure 3). The N-terminal region contains two repeated light-oxygen-voltage (LOV) domains, LOV1 and LOV2. These LOV domains bind non-covalently to two flavin mononucleotide (FMN) molecules that function as blue-light sensors (Christie et al., 1999). In darkness, LOV domains absorbs near 447 nm, and illumination with blue light initiates a photochemical reaction that results in the formation of a covalent adduct between carbon 4 of the flavin chromophore and a conserved cysteine residue within the LOV domain.

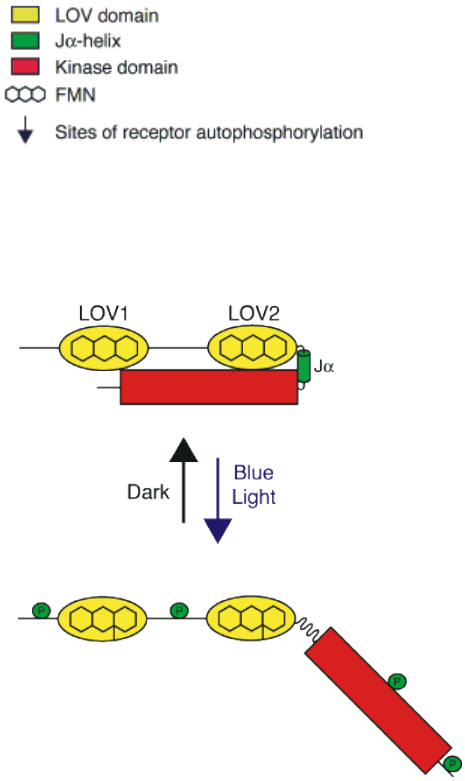



**Figure 3. Phototropin structure and activation by light.**

Phototropin in dark is in an unphosphorylated and inactive state (upper panel). After blue light irradiation, the main light sensor LOV2 perceives the light and this results in the unfolding of the Jα-helix and the activation of the C-terminal kinasic domain that leads to autophosphorylation (lower panel). Adapted from Christie, (2007).

The FMN-cysteynil adduct formation produces a spectral species with a maximal absorption at 390 nm, which is reversible to the dark form (Christie, 2007; Salomon et al., 2000). The LOV2 domain acts as a repressor of the phototropin kinase activity, located at the C-terminal end of the polypeptide chain (Matsuoka and Tokutomi, 2005). In darkness, the linker region (Jα-helix) between the LOV2 and the C-terminal domain interacts with the LOV2 domain. Under blue-light irradiation, the Jα-helix becomes disordered and its interaction with the LOV2 domain is disrupted upon cysteinyl adduct formation

(Harper, 2003), resulting in the unfolding of the helix. This structural change leads to phototropin self-phosphorylation on some specific conserved serine residues (Ser-849 and Ser-851) that are located within the phototropin kinase activation (Inoue et al., 2008; Kaiserli et al., 2009). Once the phototropin C-terminal kinase domain has been activated, it is able to phosphorylate its substrates. Both Phot1 and Phot2 have hydrophilic nature, but in darkness they are associated to the plasma membrane. Upon BL irradiation, Phot1 dissociates from the plasma membrane and it is internalized into the cytosol via clathrin-mediated endocytosis (Roberts et al., 2011; Sakamoto and Briggs, 2002; Sullivan et al., 2010; Wan et al., 2008). Phot1 kinase domain and the phosphorylation at Ser-851 are required for the Phot1 internalization (Kaiserli et al., 2009).

Phytochrome A can regulate the intracellular distribution of Phot1, preventing its internalization after blue light induction (Han et al., 2008). Nevertheless, a direct interaction between them seems unlikely, because PhyA is localized in the root cap (Pratt and Coleman, 1974) and Phot1 is localized in the upper part of the root (closer to the soil surface), (Sakamoto and Briggs, 2002; Wan et al., 2008), implying that the site of blue light perception is the root transition zone (Wan et al., 2012). Reorientation of root growth during the light avoidance response might require a local response in the transition zone mediated by Phot1 (Zhang et al., 2014).

## *2.1. Downstream signalling proteins involved in the phototropic response*

Two members of the plant NPH3/RPT2 family and three members of the phytochrome kinase substrate family (PKS) have been

identified as downstream signalling proteins in the phototropic response (Inada et al., 2004; Lariguet et al., 2006).

Protein non phototropic hypocotyl 3 (NPH3) is part of the NPH3/RPT2 (or NRL) protein family. Its structure contains a central located NPH3 domain and two protein-protein interaction domains, located at the two ends of the polypeptide chain, the BTB/POZ domain at the N-terminus and the coiled-coil domain at the C-terminus. *nph3* loss-of-function mutants lack the phototropic response under any light condition and show defects in root phototropism. This proves the essential role of NPH3 in the Phot1- and Phot2-induced light transduction signal (Liscum and Briggs, 1996; Wan et al., 2012). NPH3 acts upstream of the asymmetric auxin distribution triggered by light (Haga, 2005; Pedmale and Liscum, 2007). NPH3 is localized at the plasma membrane and it interacts with Phot1 and Phot2 both *in vivo* and *in vitro* (de Carbonnel et al., 2010). In particular, NPH3 interacts with the N-terminal photosensory domain of Phot1 through its C-terminal end (Motchoulski, 1999). NPH3 is phosphorylated in darkness but it is not a substrate of Phot1, and it is dephosphorylated within few minutes after blue light illumination in a Phot1-dependent manner. NPH3 interacts with CULLIN3 (CUL3), forming the $CRL^{NPH3}$ complex, where NPH3 acts as a substrate adaptor (Roberts et al., 2011). This interaction is required for the mono-/multi- and polyubiquitination of Phot1 proteins under blue light irradiation.

Root phototropism2 (RPT2) is also a member of the NPH3/RPT2 protein family and it works as a signal transducer of phototropins. It contains a BTB/POZ domain at the N-terminal region and a coiled-coil domain at the C-terminal region (Sakai et al., 2000; Wan et al., 2012). RPT2 is localized at the plasma membrane and it interacts through its N-terminal end with the N-terminal domain of Phot1 and through its C-

terminal end with NPH3 (Inada et al., 2004; Motchoulski, 1999). Mutants *rpt2* lack the root phototropic response and show defects in hypocotyl phototropism under high-intensity blue light (where Phot1 and Phot2 are both active), but they show normal response under low-intensity blue light, when Phot1 is the main photoreceptor (Sakai et al., 2000).

The phytochrome kinase substrate (PKS) family are signal transducers of phototropism. There are four members in Arabidopsis, PKS1–PKS4. PKS1, PKS2 and PKS4 localize at the plasma membrane and interact with Phot1, Phot2 and NPH3 (de Carbonnel et al., 2010; Lariguet et al., 2006). PKSs are involved in Phot1-mediated blue light effects, including hypocotyl and root phototropisms. PKS1 is essential for root negative phototropism and its expression is dependent on the phytochrome PhyA (Boccalandro et al., 2007).

### *2.2. Auxin transport*

Auxin (chemically indole-3-acetic acid, IAA) is a phytohormone identified by Fritz Went in 1926 (Went, 1945). It is a central regulator of plant growth and development, which modulates apical dominance, root gravitropism, root architecture and flower formation, among others (Santner and Estelle, 2009). Tryptophan is the main precursor for the biosynthesis of IAA, (Zhao, 2010). Auxin is synthesized in young and developing tissues and is asymmetrically distributed within plant tissues, where it forms local maxima and concentration gradients. Many aspects of auxin action depend on this differential distribution. IAA transport depends on a combination of two pathways, a long-distance pathway, in which IAA is transported rapidly from the site of synthesis to the rest of the plant through the phloem, and a

short-distance pathway, which is a slow, cell-to-cell auxin transport, also called polar auxin transport (PAT).

According to the chemiosmotic hypothesis, the ionic nature of IAA and the different pH inside and outside the cells (7 and 5.5, respectively), drives the passive transport of auxin towards the cytoplasm (Goldsmith, 1977). In order to facilitate the auxin efflux to the outside, the existence of PM-associated auxin-efflux carriers was hypothesized. Later on, three families of transmembrane proteins were identified: the PIN-FORMED family of proteins (PIN), the AUXIN-RESISTANT/LIKE AUX1 (AUX1/LAX), and some members of the ATP-binding cassette (ABC) superfamily. The PIN-FORMED (PIN) protein family are efflux carriers, and eight members have identified in Arabidopsis, belonging to two subfamilies: 1) those having a long hydrophilic loop that are localized at the PM (PIN1, PIN2, PIN3, PIN4 and PIN7) and 2) those having a short hydrophilic loop that are localized at the membrane of the endoplasmic reticulum (ER) (PIN5, PIN6 and PIN8) (Bennett, 2015; Křeček et al., 2009).
There is a correlation between PIN localisation and auxin distribution. A map derived from many different studies shows PIN localisation in the root and its correlation with auxin fluxes (Figure 4).

### *2.3. Regulation of PIN localisation*

The PIN proteins are integral membrane proteins synthesized at the endoplasmic reticulum (ER). The subtype 2 (PIN5, PIN6 and PIN8) are retained at the ER, whereas the subtype 1 (PIN1, PIN2, PIN3, PIN4, and PIN 7) are secreted to the Golgi cisternae, reach the

*trans*-Golgi network/early endosome continuum (TGN/EE), and they are finally targeted to particular domains of the plasma membrane (PM) (Richter et al., 2007, 2009). Secretion of *de novo* synthesized PINs to the PM is apolar, so that their final polar localisation requires re-internalization into the TGN/EE compartments, followed by their final specific targeting to the PM (Dhonukshe et al., 2014; Murphy et al., 2005; Richter et al., 2011). Endocytosis from the PM is controlled by clathrin-mediated vesicle trafficking, and the process is inhibited by auxin treatment (Robert et al., 2010). There are two types of endosomes, early and late endosomes. The early endosomes are responsible for recycling proteins from endosomes towards the PM (Lam et al., 2007) whereas late endosomes (multivesicular bodies MVB or prevacuolar compartment PVC) are involved in targeting to protein degradation (Grunewald and Friml, 2010; Robinson et al., 2008). Cycling of PIN proteins from endosomes to the PM is controlled by ADP-ribosylation factors (ARF), which are small soluble guanine-nucleotide-binding proteins. They can bind weakly to the membrane in their inactive GDP-bound form, whereas the active GTP- bound form binds tightly to the membrane.

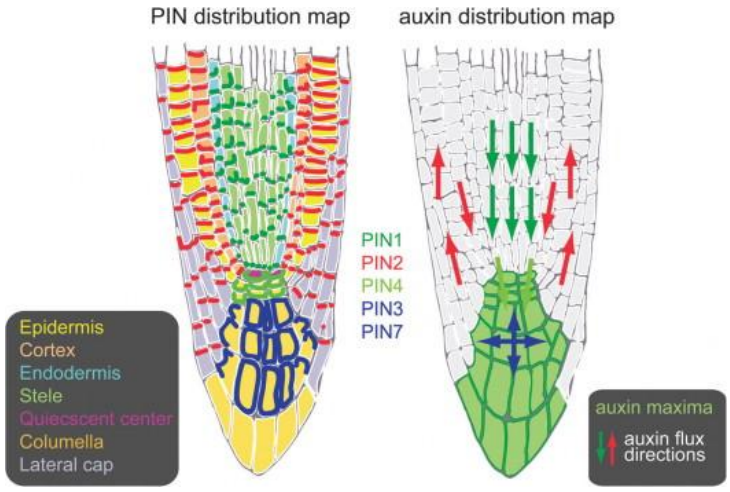



**Figure 4. PIN proteins localisation in Arabidopsis root and auxin fluxes.**

Schematic representation of the PIN protein distribution. In the stele PIN1 shows preferential rootward polarization, whereas PIN2 in epidermal cells shows shootward localisation. PIN3 and PIN7 are apolar localized in gravity-sensing columella cells but they polarize after variations of environmental stimuli. Polar distribution of PIN proteins determines the direction of auxin flux. The presumed spatial accumulation of auxin in the root tip is showed in the right. Adapted from Rosquete, Barbez and Kleine-Vehn, (2012).

The guanine nucleotide exchange factors (GEFs) catalyze the exchange GDP/GTP, and the GTPase-activating proteins (GAPs) stimulate ARF-bound GTP hydrolysis and, hence, the return of ARF to its inactive GDP-bound state (Donaldson and Jackson, 2000). In plants, only two families of ARF-GEF have been identified, the BIG and the GBF families (Cox et al., 2004). GNOM is a very well-studied ARF-GEF member of the GBF family. It is sensitive to brefeldin A (BFA) a fungal toxin that inhibits the recycling process. GNOM localizes in the early endosomes and it is involved in the targeting of the PIN proteins back to the PM, but not in their endocytosis (Geldner, 2004). BFA treatments produce PIN accumulation in the so-called BFA compartments (Muday et al., 2003). GNOM regulates PIN

recycling to the basal side of the cells while their apical targeting is GNOM-independent (Kleine-Vehn et al., 2008c). Polar distribution of PIN proteins also depends on PIN phosphorylation (Huang et al., 2010; Kleine-Vehn et al., 2009). The Ser/Thr protein kinase PINOID (PID) phosphorylates the hydrophilic loop of the PIN proteins (Michniewicz et al., 2007), regulating the movement from the basal to the apical cell side in a GNOM-independent manner (Kleine-Vehn et al., 2009). Protein phosphatase 2A (PP2A) reverts PID-dependent phosphorylation activity and promotes the basal localisation of PIN proteins in a GNOM-dependent pathway (Kleine-Vehn et al., 2009).

### *2.4. Vacuolar sorting of PIN proteins*

The PIN proteins can also be directed to the vacuole for their degradation via the late endosome compartment (PVC/MVB). For this process, ARF-GEF proteins and vacuolar sorting receptors (VSRs) are required. VSRs are type I membrane proteins involved in the sorting and packaging of soluble vacuolar proteins into transport vesicles with the help of various accessory proteins. They are able to recognize the vacuolar proteins thanks to the presence in their sequence of an aminoacid motif, the “sorting signal”. VSRs bind to cargoes at the donor compartment and release them at the TGN (Kang and Hwang, 2014). After that, the VSRs are recycled back to the ER (Seaman *at al.*, 2005; Niemes *et al.*, 2010) by a process mediated by the retromer, a pentameric complex that localizes to early endosomes and sorts transmembrane proteins back to the TGN. The structure of the retromer is composed by a dimer of sorting Nexin 1 (SNX1) and a trimer of vacuolar protein sorting proteins (VPS26, VPS29 and VPS35). The SNX dimer recruits the complex to the endosomes, whereas the VPS trimer mediates the binding to the

ligand (Bonifacino and Hurley, 2008; Seaman, 2005). SNX1 and VPS29 regulate the balance between vacuolar degradation and recycling of PIN proteins. They function either at the PCV/MVB or at the TGN/EE, where they can retain PIN proteins in the recycling pathway (Kleine-Vehn et al., 2008c). Once the PM proteins are at the PVC/MVB, they are targeted to the lumen of their transporters endosomes and directed to the vacuole for degradation. This internalization is mediated by the endosomal sorting complexes required for transport (ESCRT). The activity of ESCRT is the opposite to that of the retromer because it facilitates the movement of proteins from the membrane to the endosomal lumen, whereas the retromer facilitates the movement of proteins from the endosomal membrane back to the TGN.

### *2.5. Root phototropism and auxin transport*

Roots are underground organs that grow into the soil following the gravity vector. Contrary to what is believed, roots are not growing in complete darkness, because sunlight can penetrate the upper layers of the soil for several millimetres (Mandoli et al., 1990). Roots respond to light by growing away from it, a phenomenon called *negative phototropism* and also *root light avoidance*. Plant roots are extremely sensitive to light. However, most studies conducted in the lab use uniformly illuminated roots during the whole growing period of the seedlings. Continuous light generates a burst of reactive oxygen species (ROS) and induces altered salt stress responses in plants (Yokawa et al., 2014, 2011). Despite the study of root phototropism is extremely interesting and in constant evolution, it is important to be aware that exposure of roots to light constitutes a stress factor for the Arabidopsis seedlings grown in the laboratory.

The biological and ecological significance of the negative phototropism might be to enhance the chances of seedlings' survival under dry conditions, and hence it would have an adaptive value for the developing plant (Galen et al., 2007; Kutschera and Briggs, 2012). When light penetrates the soil, its spectral characteristics are altered by the depth. Photons from red and far red light can penetrate deeper than blue light photons; furthermore, vascular tissue can conduct the light to the roots (Mandoli and Briggs, 1984; Sun et al., 2003). Light affects the cell distribution of auxin carriers, leading to changes in auxin fluxes that can favour differential growth during root negative phototropism (Zhang et al., 2014, 2013). Contrary to the auxin gradients generated by positive phototropism, in which auxin accumulates at the shaded side of the hypocotyls after blue light induction, negative phototropism causes auxin accumulation at the illuminated side of the roots in the meristem of epidermal cells (Zhang et al., 2013). This does not agree with the results of Wan et al. (2012) that show auxin accumulation at the shaded side of the roots exposed to the light. Hence, the pattern of auxin accumulation in the negative phototropism still needs to be clearly resolved. However, the existence of an auxin gradient suggests a regulated control of auxin transport in the roots after light stimuli. Indeed, experiments with auxin transport mutants such as *pin2* and *pin3*, show defects in the root phototropic response (Wan et al., 2012; Zhang et al., 2014, 2013).

PIN1 has a relevant role in the root phototropic response (Zhang et al., 2014). In darkness, PIN1 is internalized and accumulates in intracellular compartments, while blue light illumination increases its distribution at the basal (rootward) plasma membrane of the root stele cells, increasing the auxin flow towards the root tip.

Blue light also induces the redistribution of the auxin efflux carrier PIN3 in the columella cells of the roots. This redistribution is required for both gravitropic and phototropic responses. PIN3 proteins show apolar localisation at the membrane of columella cells and rapidly relocalise laterally, following the change of direction of the gravity vector (Friml et al., 2002) or after lateral illumination with blue light (Zhang et al., 2013). After light induction it aligns with the light direction and redirects auxin flow towards the irradiated side of the root.

PIN2 is also necessary for the asymmetric auxin distribution (Wan et al., 2012). In the root tip, under physiological and dark conditions, PIN2 is targeted to the rootward domain of the root cortex cells by a process that is GNOM-dependent (Kleine-Vehn et al., 2008a, 2009, 2008b), whereas PIN2 is targeted to the shootward domain of epidermis cells (Baskin et al., 2010; Suprasanna and Bapat, 2005). In the absence of light, PIN2 on the PM is greatly reduced and its location in part switched from the PM to vacuoles. It has been shown that light enhances PIN2 redistribution to the plasma membrane in epidermis root cells by retromeric components, like sorting Nexin 1 (SNX1) and vacuolar sorting proteins (SVP9) (Kleine-Vehn et al., 2008c), which recover PIN2 from the late/pre vacuolar compartment back to the recycling pathways, increasing the flow of auxin toward the shoot.

Taken together, the rootward polarization of PIN1 in the cells of root stele, the relocalisation of the PIN3 in columella cells exposed to light, and the shootward polarization of PIN2 in epidermis cells result in an asymmetric auxin flow through the root, which leads to the root bending away from the light source (Zhang et al., 2014). While the mechanisms underlying asymmetric auxin distribution in hypocotyl

phototropic response have been extensively investigated, the molecular and cellular mechanisms that underlie root negative phototropism, especially the role of auxin, remain to be deciphered.

In this study we wanted to further characterize the phenotype of CK2mut plants. For this purpose we analyzed the root phototropic response and the auxin distribution in the root of CK2 seedlings lacking CK2 activity.

# 3. Overview Of Salicylic Acid And Its Interplay With Auxin

Plant hormones are small molecules that regulate every aspect of plant life, including development and responses to biotic and abiotic stress. They can act locally, at the sites of their synthesis, or in distant tissues, and they can function independently or in cooperation with other hormones (Depuydt and Hardtke, 2011). In the recent years, the crosstalk between salicylic acid (SA) and auxin has been discovered to play a crucial role in plant defence against both biotic and abiotic stresses (Iglesias et al., 2011), although the mechanisms underlying its regulation is far for being understood.

## *3.1. SA biosynthesis*

Salicylic acid (SA) is a phenolic compound essential for plant immunity. Two main SA biosynthetic pathways are known, the isochorismate pathway that takes place in the chloroplast, and the phenylalanine ammonia-lyase (PAL) pathway in the cytoplasm. Both use chorismate as precursor to synthesize SA. In the chloroplastic-localized pathway, chorismate is converted to SA in two steps, involving isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL) (Dempsey et al., 2011; Vlot et al., 2009). The cytoplasmic-localized pathway starts with L-phenylalanine (derived from isochorismate), which is converted to *trans*-cinnamic acid (*t*-CA) by the enzyme PAL, and then to SA via two possible intermediates, *ortho*-coumaric acid or benzoate. In Arabidopsis, most of the pathogen-induced SA biosynthesis arise from the isochorismate pathway. Arabidopsis genome contains two ICS-encoding genes,

*ICS1* and *ICS2*. SA production as well as pathogen resistance are strongly compromised in mutants lacking a functional *ICS1*, which appears to be responsible for approximately 90% of SA production induced by pathogens (Garcion et al., 2008). The presence of residual SA in the *ics1/ics2* double mutant confirms that the ICS pathway is not the only source of SA in Arabidopsis (Garcion et al., 2008). After its synthesis, SA can be found in plant tissues as a free or as a conjugated form. SA can undergo several biologically relevant modifications, including glycosylation, methylation, and amino acid (AA) conjugation. Most of them, but not all, result in inactive pools of SA. Arabidopsis genome encodes two SA-glucosyltransferase (SAGT) enzymes. One converts SA into the most abundant SA derivative (*O*-*β*-glucoside, SAG), whereas the other converts SA in salicyloyl glucose ester, (SGE) (Vlot et al., 2009). SAG is actively transported from the cytosol into the vacuole, where it is storage as an inactive form that can be converted back to SA by hydrolysis (Dean et al., 2005). The SA storage as SAG might be a mechanism induced by several pathogens to increase plant susceptibility by decreasing free amounts of active SA (Boatwright and Pajerowska-Mukhtar, 2013). However, as mentioned above, not all the conjugated forms of SA are inactive, as for instance SA conjugated with amino acids can activate defence responses (Loake and Grant, 2007). The acyl-adenylate/thioester-forming enzyme (GH3.5) catalyzes the conjugation of amino acids to SA. Loss-of-function mutants of GH3.5 show partially compromised defence responses, thus GH3.5 has been proposed to be a positive regulator of SA signalling (Zhang et al., 2007).

### 3.2. Functions of SA

Salicylic acid has many functions in plants, even though recent researches have focused mainly on its function as an endogenous signal mediating local and systemic plant defence responses against pathogens. Plants are susceptible to attacks by a wide variety of microbial pathogens and insect herbivores. SA is a signalling molecule that induces defence against biotrophic pathogens (that feed and reproduce on live host cells). Pathogens produce elicitors called pathogen/microbe-associated molecular patterns (PAMP/MAMP) to suppress plant defence. Those include peptides, metabolites, cell wall components, enzymes, and toxins. The PAMPs are recognized by membrane-localized pattern-recognition receptors (PRRs), which initiate an active defence response called basal immunity (PTI) that is very similar to the innate immunity system in animals. Successful plant pathogens that are able to escape from this first barrier deploy effectors that contribute to pathogen virulence, resulting in effector-triggered host susceptibility (ETS). During plant–pathogen coevolution, plants developed resistance proteins (PR) that are able to recognize at least some of the pathogen effectors, and activate plant defence. The majority of R proteins are intracellular nucleotide binding-leucine rich repeat (NB-LRR) proteins and they recognize the pathogen effectors directly or indirectly. This second layer of defence, which starts with the recognition of the pathogens effector by a plant R protein, is called effector-triggered immunity (ETI) and induces a rapid reprogramming of gene expression. ETI activation induces a rapid burst of reactive oxygen species (ROS), production and accumulation of SA and of nitric oxide (NO), and expression of *pathogenesis-related* (*PR*) genes. Concomitantly, programmed cell death (PCD) in the local tissue, called hypersensitive response (HR), occurs (Wu et al., 2014).

ETI also induces the production of mobile signals such as MeSA, azelaic acid (AzA) or glycerol-3-phosphate (G3P), triggering the systemic acquired resistance (SAR). SAR consists of a broad-spectrum long-lasting resistance in the whole plant after local exposure to an avirulent pathogen (Fu and Dong, 2013). SAR can also be induced by exogenous application of SA in the absence of pathogen, and confers immune "memory" to plants, protecting them from a second infection (Durrant and Dong, 2004). In contrast to ETI, SAR is not associated with programmed cell death. SAR induces a big transcriptional reprogramming, which is dependent on the transcription cofactor NPR1 and on its associated transcription factors (TFs) of the TGA and WRKY families (better explained in the next paragraph "*SA signalling*"). Strong evidences supporting the role of SA as a defence signal came from the analyses of mutant plants with altered SA levels. Mutants containing elevated SA levels correlated with enhanced resistance to pathogen infection, whereas mutants with reduced SA levels showed enhanced susceptibility to pathogens. Studies performed with transgenic tobacco and Arabidopsis plants expressing the bacterial *nahG* gene, encoding the SA-metabolizing enzyme salicylate hydroxylase, showed that these plants were unable to accumulate high SA levels after pathogen infection, and they displayed susceptibility to both virulent and avirulent pathogens (Delaney et al., 1994). This mutant plant represents an example of defence-related phenotype in SA mutants that has been used in this work. Others SA mutants are reviewed in Vlot et al. (2009). SA also plays an important role in the response to abiotic stresses, including drought, low temperature, and salinity stresses. The effects of SA are dependent on the concentration, the mode of application, and the state of the plants (Rivas-San Vicente and Plasencia, 2011). For

example, both drought tolerance and plant growth are suppressed when high concentrations (2–3 mM) of SA were applied to wheat seedlings, whereas plant growth is enhanced by the application of low concentrations (0.5 mM) of SA (Kang et al., 2012). Generally, low exogenous SA concentrations alleviate the sensitivity to abiotic stresses, whereas high concentrations produce oxidative stress, leading to a decreased tolerance to abiotic stresses (Miura and Tada, 2014). In *A. thaliana*, exogenous SA treatment (100 µM and 1 mM) reduces density and number of trichomes, which are important structures for plants' resistance to herbivores (Traw and Bergelson, 2003).

### *3.3. SA signalling*

After its synthesis in the chloroplast, SA is transported by enhanced disease susceptibility 5 (EDS5) to the cytoplasm. Studies with *eds5* mutant showed that SA accumulated in the chloroplast after stress-induction and inhibited its own biosynthesis, leading to increased susceptibility to pathogens (Serrano et al., 2013). Consequently, EDS5 is necessary for SA accumulation in the cytoplasm after biotic and abiotic stress in Arabidopsis.

NPR1 is considered a master regulator of the SA-signalling pathways in plant defence. It belongs to a small multigene family, with two other members, NPR3 and NPR4 (Fu et al., 2012; Wu et al., 2012). They contain a BTB/POZ (broad-complex, tramtrack), and a bric-à-brac/poxvirus and zinc-finger domains, an ankyrin repeat domain, and a nuclear localisation signal (Seyfferth and Tsuda, 2014). Mutations in *NPR1* lead to almost complete loss of SA-mediated transcriptional reprogramming, and plants present greater susceptibility to pathogens. Moreover, *npr1* mutants are insensitive to

exogenous SA (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). Despite NPR1 was reported to be a *bona fide* SA receptor directly binding SA (Wu et al., 2012), other studies showed that NPR1 does not bind SA (Yan and Dong, 2014). Instead, two homologous of NPR1 (NPR3 and NPR4) have been identified as SA receptors that bind SA (Fu et al., 2012). Another model of SA perception has been proposed considering that NPR1 is degraded in absence of SA via proteosome 26S (Spoel et al., 2009). This model is based on the formation of a gradient of NPR1 protein concentration, which is critical for the SA response. NPR1 accumulation is regulated by SA, through the SA receptors NPR3 and NPR4. In the case of low SA, the SA-receptor NPR4 triggers NPR1 degradation through the 26S proteosome. When SA levels are intermediate, NPR1 protein accumulates. High SA-concentration promotes the interaction of the SA-receptor NPR3 with NPR1, leading to NPR1 degradation. Thus, only intermediate levels of SA achieve NPR1 accumulation activating SA-mediated transcriptional reprogramming (Fu et al., 2012). This is not the only way to regulate NPR1, as it has been reported that NPR1 is also regulated by SA-triggered redox changes (Mou et al., 2003). In the absence of SA, NPR1 is an oligomer localized in the cytosol, formed by cross-linking of several monomers through intermolecular disulphide bonds. Pathogen infection or SA treatment produce changes in the cellular redox potential, reducing the cysteine residues and converting the NPR1 oligomer to the monomeric form (Mou et al., 2003). The NPR1 monomers translocate to the nucleus, where they act as transcription cofactors (Kinkema, 2000). On the other hand, SA triggers phosphorylation of the monomeric form of NPR1 at its N-terminal end, its posterior ubiquitination and proteosome-mediated degradation (Spoel et al., 2009). It has been found that NPR1

regulates plant immunity through a transcription cascade involving multiple transcription factors. NPR1 modulates transcription of SA-responsive genes, as a cofactor, through interactions with TGA transcription factors (Zhang et al., 1999; Zhou et al., 2000). TGA belongs to a subclass of the basic leucine zipper transcription factor family. NPR1 also directly activates the expression of several WRKY transcription factors that exhibit both activator and suppressor activities (Wang et al., 2006). The transcription factors WRKY bind specifically to the w-box promoter elements, which are over represented in SAR-related genes including *ICS1*, *NPR1* and *PR1.* Thus, the presence of multiple w-boxes in the *NPR1* promoter suggests a positive regulation of *NPR1* transcription by WRKY during the activation of plant defence (Fu and Dong, 2013). WRKY factors are also negative regulators of SA biosynthesis, functioning as repressors of the defence responses when the infection disappears (Fu and Dong, 2013). This has been observed with the characterization of two mutants *wrky54 wrky70,* in which ICS1 was over expressed and SA accumulated at higher concentrations than in the WT. SA biosynthesis is repressed by low levels of WRKY54 and WRKY70. Because this phenotype is similar to that of *npr1* and because WRKY70 and WRKY54 are both NPR1 targets, it was hypothesized that they are also involved in shutting down SA biosynthesis after the pathogen attack is over (Wang et al., 2006). Proteosome mediated degradation of NPR1 has important roles. From one side, in absence of pathogen infection (or increased SA concentrations), the NPR1 monomers degradation from the nucleus restrict their activity as transcriptional coactivators, preventing wasteful activation of SAR related genes. From the other side upon infection, the degradation of NPR1 is necessary for the maintenance of an high

rate of SAR related genes expression. Proteolysis is required to sustain a high ratio of transcriptional active over inactive activator, in order to have continuously delivering 'fresh' activator to gene promoters (Collins and Tansey, 2006; Tansey, 2001). Next to this mechanism of SA signalling mediated by NPR1, others SA-dependent, NPR1-independent signalling pathways have been identified (revised in An and Mou, (2011)). These evidences are supported by studies of different Arabidopsis mutants screened for suppressors of *npr1-1*. Among them the *sni1* (suppressor of *npr1-1*, inducible 1) mutant, which was able in double mutants *sni1 npr1* to restore near wild-type levels of *PR1* expression and resistance to pathogens after induction (Li et al., 1999). The *sni1* mutation suggested that wild-type SNI1 might function as a negative regulator of SAR. Another study that supports these evidences concerns the mutants *cpr1, cpr5* and *cpr6,* which exhibit increased expression levels of the *PR* genes and enhanced resistance. When *npr1* was crossed into a *cpr5* or *cpr6* background the constitutive disease resistance was not completely rescued to wild-type levels (Clarke et al., 2000).

### *3.4. Auxin signalling and interplay between auxin and SA in plant defence*

Auxin regulates gene expression through a family of transcription factors called auxin response factors (ARFs). The ARFs bind as homodimers to the auxin-response elements (AuxRE), located upstream of auxin-inducible genes. In the presence of low auxin concentrations, the ARFs are repressed by binding to members of the AUX/IAA protein family of transcriptional repressors (Tiwari et al., 2004). When auxin reaches a certain threshold level inside the cell,

the AUX/IAA repressors become ubiquitinated and are targeted to the 26S proteosome for degradation. Degradation of AUX/IAAs releases the ARFs, resulting in ARF–ARF dimerisation and their binding to DNA to induce gene expression (Santner and Estelle, 2009).

The intracellular auxin receptor is the F-box protein TIR1, which is a subunit of the protein ubiquitin ligase E3, $SCF^{TIR1/AFB}$. Auxin acts as a “molecular glue” to stabilize the interaction between TIR1 and the Aux/IAA proteins, in order to promote their degradation (Tan et al., 2007) reviewed in Calderon-Villalobos et al. (2010). Because the AUX/IAA genes themselves are rapidly induced by auxin, a negative-feedback loop exists, as the newly synthesized AUX/IAA proteins restore repression of the auxin pathway (Gray et al., 2001). Most Aux/IAA proteins have four conserved domains. Domain I contains an ETHYLENE RESPONSE FACTOR-associated amphiphilic repression (EAR) motif that physically interacts with the corepressor TOPLESS (TPL) (Causier et al., 2012). At low auxin levels, the Aux/IAA proteins form multimers with ARFs and recruit TPL to the chromatin through EAR domain, resulting in the repression of auxin-responsive genes. Domain II contains the degron sequence, which interacts directly with the TIR1/AFB protein and auxin. Domain III and Domain IV are responsible for dimerization with other Aux/IAA proteins and heterodimerization with ARF protein (Ulmasov et al., 1997).

Recent studies showed the existence of an antagonistic action between auxin and salicylic acid (SA), which is exploited in plant defence. Many plant pathogens have evolved the ability to produce auxin or to induce auxin biosynthesis in plant host cells to disrupt the normal growth of infected plants (Chen et al., 2007; Robert-Seilaniantz et al., 2007). Plants, on their side, evolved mechanisms to

repress auxin signalling during pathogen infection as a defence strategy by producing elevated amounts of SA, which causes transcriptional repression of genes encoding auxin receptors (protein TIR1 and AFB1) (Wang et al., 2007). The same authors demonstrated that SA stabilizes the auxin repressor AXR3 (belonging to the AUX/IAA protein family), resulting in inhibition of auxin responses. Analysis of the transcriptional changes triggered by the SA analogue benzothiadiazole S-methylester (BTH) in Arabidopsis, showed a general repression of auxin-related genes and the stabilization of Aux/IAA repressor proteins. The reduction of the auxin responses might attenuate the action of the auxin induced by the pathogens and contribute to disease resistance as a part of the SAR response (Wang et al., 2007).

To the contrary, other genes were found upregulated by BTH. Two of them encoded auxin conjugating enzymes, thus BTH might also affect auxin homeostasis by decreasing the amount of free auxin (Woodward and Bartel, 2005). Studies with SA over-accumulating mutants, such as cpr5, cpr6, and snc1, revealed morphologies of reduced apical dominance and retarded growth, which are characteristics of auxin-deficiency mutants (Bowling et al., 1997; Clarke et al., 1998; Wang et al., 2007). It has been also found that the IAA-conjugating enzyme GH3.5, involved in the conversion of free IAA into IAA-Asp (inactive auxin), is transcriptionally induced by SA (Staswick, 2005). Experiments using a transgenic line overexpressing the *GH3.5* gene in Arabidopsis showed increased resistance to infection by *P. syringae* pv. *tomato* (*Pst*) DC3000 and a dwarf phenotype, with increased *PR1* gene expression (Zhang et al., 2008). Moreover, knockout *gh3.5* mutants showed compromised SAR induction with diminished *PR-1* expression in systemic tissues, similar

to the SA-deficient mutant *NahG,* which fail to accumulate SA after pathogen infection and cannot activate SAR (Delaney et al., 1994; Huot et al., 2014; Zhang et al., 2008). Overexpression of the auxin receptor *AFB1* (encoding a F-box protein of the same family as TIR1) in Arabidopsis increased auxin signalling and at the same time produced significant reduction of SA accumulation after pathogen infection and increased susceptibility to the pathogen (Robert-Seilaniantz et al., 2011). The role of auxin during pathogenesis was also tested by studying plants with elevated endogenous auxin levels. Transgenic plants overexpressing the auxin biosynthesis gene *YUCCA1* (YUC1) exhibited higher auxin levels and enhanced susceptibility to *P. syringae* strain DC3000. IAA levels did not interfere with host defences, as effector-triggered immunity was active in *YUC1* overexpressing plants. Elevated auxin levels appear to promote pathogenesis by a mechanism that is independent of the suppression of SA-mediated defences (Mutka et al., 2013).

### *3.5. Protein kinase CK2 and crosstalk between auxin and SA*

Previous studies highlighted the participation of the protein kinase CK2 in the SA-mediated signalling pathways in tobacco. Experimental evidences demonstrated that CK2 participates in the SA-induced phosphorylation of proteins which enhance binding activity of nuclear factors to *as-1*. *As-1* are promoter sequences controlling transcription of early SA-responsive genes (Hidalgo et al., 2001). The same authors showed that CK2 inhibitors hindered the transcriptional activation of early SA-regulated genes in tobacco cell extracts. Moreover, it was also found that CK2 phosphorylates *in vitro* several members of transcription factors (TFs) of the TGA family, which

recognize the *as-1* elements in response to SA (Kang and Klessig, 2005). Recently it was shown that the characteristic phenotype of CK2mut line is an SA-mediated effect (Armengot et al., 2014). Interestingly, it was found that CK2mut roots accumulate high levels of salicylic acid (SA) and that the genes encoding isochorismate synthase (SID2) and phenylalanine ammonia-lyase1 (PAL1) were overexpressed (Armengot et al., 2014), explaining the high SA content found in CK2mut seedlings. *NPR4,* was also found up-regulated in CK2mut seedlings. Thus, these data support the idea that both SA homeostasis and SA signalling are disturbed in CK2mut seedlings (Armengot et al., 2014). Furthermore, it was found that SA activates transcription of CK2-encoding genes and, thus, SA and CK2 appear to be part of an autoregulatory feed-back loop and that the NPR1-mediated pathway is involved in this regulation (Armengot et al., 2014). In this work we wanted to investigate if there is a possible link between the CK2 auxin-related phenotypes and a constitutive stabilization of auxin repressors.

## 4. Overview Of Actin Cytoskeleton

Actin is a highly conserved globular protein that forms cellular scaffold structures, the function of which is involved in maintaining cells shape, tension support, intracellular vesicular transport, cell attachment, adhesion properties and the ability to move. Most animal cells use microtubules for organelles transport (Yi et al., 2004), but plant cells use actin filaments as a primary track for long distance transport (Olyslaegers and Verbelen, 1998). Cytoskeleton has also an important role in cellular responses to biotic and abiotic stimuli, and, for this reason, its ability to rapidly remodel its architecture is important. It has been speculated that the actin binding proteins (ABPs) are required for cytoskeleton remodelling. At least a dozen conserved ABP families are known in plants, and their biochemical properties have been studied. The direct interaction of ABPs with actin filaments influences the behaviour of actin filaments and its function.

Globular actin subunits (G-actin) (~42 kDa) are ATPases that can form polymeric structures called F-actin (7-9 nm), assembled in a double strand string with helical twist (Holmes et al., 1990). The elongation phase occurs at both ends of the filament with the monomers being added to one end at a faster rate than to the other. The two ends are identified as plus “barbed” end, where preferentially G-actin monomers in their ATP-bound state are assembled, and minus “pointed” end, where monomers disassembly upon hydrolysis of ATP into ADP and phosphate. This process, known as F-actin turnover, enables to increase and decrease the length of the polymerised actin filament (Pollard et al., 2000). The equilibrium of polymerisation and depolymerisation in the cell is dependent on the pH, salt concentration and ATP (Culuccio and Tilney, 1983; Wang et

al., 1989). Under physiological conditions and in the absence of ABPs, this equilibrium is shifted towards polymerization (Henty-Ridilla et al., 2013), and this process is known as “treadmilling” (Pollard, 1986). For long time it was thought that the treadmilling mechanism could explain the dynamic equilibrium between polymerisation and depolymerisation of the F-actin filaments, but the finding that the disappearance of actin filaments is not mediated by depolymerisation at their ends, but rather by severing activity, revealed another mechanism that is called “stochastic dynamic” (Staiger et al., 2009). After two decades of biochemical analysis, it has been proposed that the ABPs have a role in the regulation of actin stochastic dynamics. The entire process of F-actin formation can be divided into three overlapping levels of control: filament initiation, stochastic dynamics, and filament organization.

### *4.1. Actin genes in Arabidopsis thaliana*

*Arabidopsis thaliana* contains ten actin genes, relatively small and individually dispersed through the genome. They have been cloned, sequenced, and characterized (McDowell et al., 1996b). The resulting isovariant proteins are specialized to perform many important actin functions in different organs and tissues. Two of these genes are pseudogenes (Kandasamy et al., 2009) and the other eight genes are grouped into two classes, vegetative and reproductive. The vegetative actins are constitutively expressed in all vegetative organs and cell types, in mature pollen and in growing pollen tubes (Meagher et al., 1999b). Furthermore, the vegetative class has two distinct subclasses of actin isovariants: ACT2 and ACT8 (subclass 1) and ACT7 (subclass 2). ACT7 is the most abundant in the root apical meristem and young root tissue, while ACT2 and ACT8 are expressed in late stages of root development (Kandasamy et al., 2009; McDowell et al., 1996a). *act2*

and *act8* mutants show defects in root hair elongation, and complementation studies revealed that ACT2 and ACT8 rescue the root hair defects of *act2* mutants. The *act7* mutant shows sever root growth defects in root epidermal cells with altered root architecture and shorter roots. These results suggest that the isovariants ACT2 and ACT8 are functionally redundant and can mutually substitute each other but are functionally divergent from ACT7 (Kandasamy et al., 2009).

The vegetative actins differ from the reproductive actins by only 4-7% at the amino acid level, even if the two classes of actin-based cytoskeletal systems appear to be functionally distinct in most angiosperms (Kandasamy et al., 2007). Previous studies of ectopic expression of a reproductive actin in vegetative tissues suggested distinct roles for vegetative and reproductive classes of actins in Arabidopsis (Kandasamy et al., 2002). Misexpression of the pollen-specific reproductive actin ACT1, but not the overexpression of the vegetative actin ACT2, in vegetative tissues was extremely toxic and results highly altered organization of the actin cytoskeleton. Since it has been found that also profilins and actin-depolymerising factors (ADFs), the two most highly expressed ABPs in plants, contain subclasses of genes that are differentially expressed in patterns vegetative and reproductive, it was hypothesized that the high level ectopic expression of pollen actin in vegetative tissues affected actin dynamics, perhaps due to the weak interactions among the reproductive actin and the endogenous vegetative ABPs (Kandasamy et al., 2002). Thus, the ABPs preferentially interact with the diverse classes of actin isovariants and regulate actin dynamics to coordinate

different aspects of plant growth and development (Kandasamy et al., 2007).

### 4.2. Actin nucleators

The initial step of actin filament formation, is known as nucleation, in which actin monomers assemble to form dimeric and then trimeric complexes, which are known as nucleation seeds. The *de novo* formation of an actin filament is energetically unfavourable and it is inhibited by proteins that sequester actin monomers, such as profilin (Safer et al., 1990). Profilin (PFN) is an ubiquitous protein with high affinity for cytoplasmic ATP-actin monomers ($K_d$ = 0.1 μM) rather than cytoplasmic ADP-actin monomers ($K_d$ = 0.5 μM). The affinity of profilin for actin filaments is low, because the binding site on the barbed end of actin is hidden by the filament structure. Profilin-ATP-actin complexes elongate the barbed end of actin filaments, but do not nucleate or elongate the pointed end of actin filaments (Pollard et al., 2000). Profilin sequestering of G-actin monomers insures a pool of actin monomers available for F-actin polymerisation and also prevents spontaneous nucleation and F-actin elongation.

The cells require the activity of actin-nucleating factors to overcame the inhibitory effect of profilins (Pollard and Borisy, 2003). These nucleation factors contain actin-binding sites and stabilize spontaneously formed actin nucleation seeds, which are able to generate actin filaments.

#### 4.2.1. The Arp2/3 complex

The Arp2/3 complex was the first relevant actin nucleation factor identified. It consists of seven highly conserved polypeptides. Two subunits, Arp2 and Arp3, are actin-related proteins. The

complex initiates a novel “daughter” filament, attaching itself to the flanks of a “mother” existing filament and initiates a new F-actin branch with an angle of 70° relative to the parent filament and remains associated with the pointed end of the “daughter” filament at the branch junction (Mochida et al., 2002). The biological function of the Arp2/3 complex has been studied in *Drosophila melanogaster.* Homologs of all the Arp2/3 complex subunits are present in plants (Mathur et al., 2003). Three different mutants of orthologs of Arp2/3 subunits have been reported in Arabidopsis: *wurm* is a mutant of the Arp2 subunit, *distorted1* is a mutant of the Arp3 subunit, and *crooked* is a mutant of the ArpC5/p16 subunit (Mathur, 2003; Mathur et al., 2003). All these loss-of-function mutants showed similar defects in development of epidermal pavement cells and of branched trichomes in the leaf. They caused mislocalisation of diffuse cortical F-actin and inhibited lobe extension in pavement cells (Li et al., 2003). Furthermore, treatments with the actin-depolymerisating drug, cytochalasin D, showed defects in trichome shape that have been seen in the “distorted” class of mutants. This suggests that the mutants phenotypes are due to defects in the actin cytoskeleton (Mathur et al., 1999; Szymanski et al., 1999). Also root hair growth under certain conditions was found disturbed in Arp2/3 mutants. They were showing wavy phenotype and sometimes multiple tips (Mathur, 2003). ARP3 was found localized to the apical membrane during root hair tip growth in maize (Van Gestel et al., 2003) and in Arabidopsis mutants. The smallest subunit (ARPC5) of the ARP2/3 complex, frequently form multiple hairs on one trichoblast (Mathur, 2003). This suggests that ARP2/3 is involved in the formation of polar membrane domain, responsible for the fast polar growth of tip

growing plant cells such as pollen tubes and root hairs (Baluška and Volkmann, 2002).

### *4.2.2. Formins*

Formins represent a second group of actin nucleators. They initiate *de novo* actin nucleation and elongation from the fast growing barbed end of the actin filament. In contrast to the ARP2/3 complex, most formins remain associated with the barbed end and consecutively elongate growing filaments (Blanchoin and Staiger, 2010). Formins work as consecutive assembly motors that enhance the rate of growth of the actin filaments. They were first identified in mammals in the early 1980s and they are required for the normal formation of limbs in mice (Kleinebrecht et al., 1982). Formin homology (FH) proteins are proteins with sequence and structural homology to the original mammalian formins. They present two conserved domains FH1 and FH2. FH1 is located at the N-terminal of the FH2 domain and contains one or more poly-L-proline regions where profilin–actin complexes can bind to increase the concentration of actin monomers and favour polymerisation (Pruyne, 2002). The FH2 domain caps the barbed end of the filament, interacts with actin and is also involved in elongation (Otomo et al., 2005). Four formins were identified in Arabidopsis, AtFH1, AtFH4, AtFH5, and AtFH8. Homology searches with the FH2 domain, revealed the presence of 21 Arabidopsis genes that are predicted to contain this domain. Arabidopsis formins can be divided into two distinct clades: type-I and type-II. Most of the type-I formins contains an N-terminal signal peptide followed by a transmembrane domain. Type-I formins are the only ones that have been studied *in vitro*. Overexpression of the actin nucleating domain of AtFH1 in tobacco pollen tubes increases the number of actin cables, suggesting

that it induces actin polymerisation *in vivo* (Cheung and Wu, 2004). At the same time, when individual pollen tubes were examined, those who were transformed with less than 1 µg of the full-length *AtFH1* transgene usually showed retarded growth compared to control tubes (Cheung and Wu, 2004). *In vivo* and *in vitro* experiments in plants overexpressing *AtFH8* showed that more actin monomers were recruited and that they formed more actin filaments, affecting the formation of the initial bulge at the distal end of the root hair cell and the process of tip growth of the root hairs (Yi et al., 2005). Expression of the N-terminus of AtFH4 without the actin-nucleating C-terminus also disrupted root hair growth (Deeks et al., 2005). Overexpression of AtFH1, AtFH4, and AtFH8 showed that formins can affect growth through F-actin formation, but the actual function of most of the plant formins remains unknown.

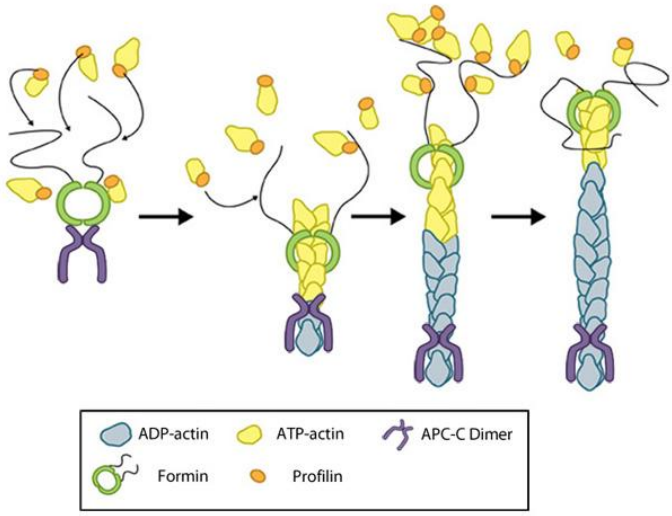



**Figure 5. Formin nucleation of actin filaments.**

The FH2 domains of the formin dimer are shown in green. The FH2 domain binds to actin monomers to initiate filament assembly. The black lines represent the FH1 domains of the formin dimer, which contain sequences that interact with profilin. Profilin binds to both formins and actin monomers to increase the addition of actin monomers to the barbed end of the filament.
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## *4.3. Actin depolymerising factors and actin-bundling proteins*

The actin-depolymerising factors (ADF) are a family of proteins of low molecular weight that regulate actin assembly. ADFs bind to monomers of G-actin and to F-actin filaments, and increase actin dynamics by severing the growing actin filaments. ADFs increase the number of filament ends for new polymerisation (Carlier et al., 1997) and/or increase the turnover of filaments by accelerating the depolymerisation rate at the pointed end (Gungabissoon et al., 1998).

*Arabidopsis thaliana* genome encodes 11 ADF proteins which play important roles in different biological processes. In animals and plants the F-actin disassembly activity of ADFs is regulated by different mechanisms. One of them is phosphorylation. Phosphorylation of ADFs in Ser-6 results in a reduction of their F-actin disassembling activity *in vitro* assays (Smertenko et al., 1998). Plant ADFs phosphorylation is regulated by calmodulin-like protein kinases (CDPKs) that co-localize with F-actin in plant cells (Smertenko et al., 1998). The F-actin disassembling activity of ADF is decreased also by the binding to phosphatidylinositol 4,5-bisphosphate (PIP2) or phosphatidylinositol 4-monophosphate (PIP) (Gungabissoon et al., 1998). Another factor regulating ADFs activity is pH. At pH 6, ADF binds to F-actin inhibiting polymerisation at both filament ends, whereas at pH 8, ADF severs actin filaments (Bernstein et al., 2000).

In order to investigate the ADF functions *in vivo*, transgenic Arabidopsis lines expressing a cDNA encoding an ADF protein under the control of a strong constitutively promoter were created (Dong et al., 2001). Overexpression of ADF1 produced disruption of F-actin cables and resulted in defects of stomatal closure. Also in *Nicotiana tabacum* the overexpression of ADF2 caused alterations in cell cycle by depolymerisation and fragmentation of the actin cortical network (Durst et al., 2013).

Plants have at least three classes of actin bundling proteins: villins, fimbrins, and elongation factor-1α. Villins have been identified in the Arabidopsis genome (Klahre et al., 2000). Immunostaining using antiserum against villins showed that the proteins were localized along the actin cables in lily pollen tubes (Yokota and Shimmen, 1998) and in root hairs (Tominaga et al., 2000). After microinjection of villin antiserum, the thick actin bundles disappeared and the nucleus

migrated toward the apex of growing root hairs (Ketelaar et al., 2002), suggesting that villin plays a central role in actin bundling. Fimbrins are cross-linkers of the actin filaments, and protect them against the profilin-induced depolymerisation (Kovar et al., 2000). Fimbrin contains two highly conserved repeats of actin binding domains (ABD1 and ABD2). Finally, elongation Factor-1α (EF-1α) is a protein with two functions. It binds aminoacyltRNA to the ribosome, but it also binds to actin and bundles, and inhibits the incorporation of monomeric actin at low pH (Gungabissoon et al., 2001). The activity of EF-1α is enhanced by ADF (Gungabissoon et al., 2001).

### *4.4. Actin and microtubule cytoskeleton interactions*

Microtubules (MTs) are rigid cylindrical tubes of approximately 25 nm of diameter. Like the actin filaments, MTs are dynamic structures that undergo continual assembly and disassembly within the cell. They function to maintain cell shape and in a variety of cell movements. MTs are composed of a single type of globular protein, tubulin. Tubulin is a dimer consisting of two closely related 55 kDa polypeptides, α and β-tubulin. A third type of tubulin (γ-tubulin) plays a critical role in initiating the assembly. MTs are polar structures with two distinct ends: a fast-growing plus end and a slow-growing minus end. Visualization of F-actin and MT organization in mutants defective in one of this two cytoskeletal components, suggested that both are physically linked and may share common interactors (Sampathkumar et al., 2011) These components may act to modify the cell shape and the organization of the cytoskeleton during fundamental developmental processes, such as cell division and cell or tissue polarity formation.

### 4.5. Studies on F-actin turnover

Analysis of the F-actin turnover has been facilitated by the improvements in microscopy and in fluorescent protein reporters (Henty-Ridilla et al., 2013). Studies with a chimeric protein, consisting of GFP fused to the actin binding domain of mouse talin (GFP-mTalin), showed a localisation pattern that decorated all the F-actin (Doyle and Botstein, 1996; Li et al., 2014b). However, it was also found that GFP-mTalin caused severe defects in actin organization and cell expansion in Arabidopsis root hairs, and that it inhibited, *in vitro,* the actin depolymerising activity of the endogenous actin binding protein ADF (Ketelaar et al., 2004). To overcome this problem, other constructs, in which the expression of GFP was fused to the actin binding domain of actin binding proteins such as talin, plastin, and fimbrin, had been generated. These constructs have been largely used to visualize the actin cytoskeleton in living cells. One in particular has been used in this work, GFP-FABD2. This construct has been generated by Voigt et al. (2005) (see experimental procedures), and it offers the opportunity to study the actin cytoskeleton *in vivo* in all cell types throughout the plant body. Alternative tools to study F-actin are several drugs that either inhibit or increase actin polymerisations. One of them is jasplakinolide, used in this work.

### 4.6. Auxin and actin cytoskeleton

Polar auxin transport and actin cytoskeleton are tightly interconnected. Auxin modulates actin expression and its organization, and auxin transporter's polarity and their flexibility is mediated by continuous recycling vesicles along the actin filaments (Kleine-Vehn and Friml, 2008).

Several authors have shown that the right position and function of auxin efflux carriers (PINs), involved in polar auxin transport in plant cells, depends on an intact actin cytosketon (Kleine-Vehn et al., 2006; Muday, 2000).Tissues in expansion show higher ACT7 expression levels and higher auxin concentration, suggesting a role for auxin in increasing actin expression (McDowell et al., 1996a). Furthermore, it is known that high IAA concentration leads to inhibition of cytoplasmic streaming and vice versa, low IAA concentration increases cytoplasmic streaming (Sweeney and Thimann, 1938). Actin cytoskeleton is involved not only in recycling/exocytosis processes, but also in PINs endocytosis/internalization processes, and thus chemical treatments with actin drugs and plant hormones, as well as genetic mutations, interfere with PIN trafficking. For example pre-treatments with BFA (and inhibitor of vesicle trafficking) followed by treatments with cytochalasin D or latrunculin B, which depolymerise the actin filaments, inhibited intracellular PIN1 accumulation as well as its re-localisation to the plasma membrane when BFA was washed out (Geldner et al., 2001). This suggests that the cycling between PM and endosomal compartments of auxin efflux carriers occurs in an actin dependent manner, despite conflicting opinions that treatments with low concentration of latrunculin B did not alter PIN polarization but caused PIN2 accumulation in bodies of unknown identity in epidermal cells (Rahman et al., 2007). Furthermore, in the same study, it was also observed a reduction of root elongation of about 50% on the third day of the treatment with latrunculin B, suggesting that actin might play a direct role in root growth.

Other studies showed that PIN3 internalizes in smaller compartments without any regular positioning when actin cytoskeleton was disrupted by latrunculin B or cytochalasin D treatment, suggesting

an actin-dependent cycling of PIN3 in root caps (Friml et al., 2002). In addition, recently authors have found that a mutant allele of *ACT2* was responsible for lower bundling of F-actin and delocalisation of PIN2 in Arabidopsis roots (Lanza et al., 2012), highlighting the importance of intact actin organization for the right positioning of PIN proteins for the polar auxin transport.

Previous studies in our lab showed that depletion of CK2 activity in Arabidopsis seedlings resulted in phenotypic traits linked to alterations in auxin-dependent processes (Marquès-Bueno et al., 2011b), and that CK2 is involved in the regulation of auxin-signalling pathways and in auxin transport (Marquès-Bueno et al., 2011a). Here, we wanted to study the effect of CK2 activity depletion on F-actin turnover and on actin spatial organization in root cells, focusing our attention on the root transition zone and the root apex, which are zones of particular interest, especially for PAT. Therefore, we performed *in vivo* visualization of actin filaments using a stably transformed *Arabidopsis thaliana* line expressing the *GFP-FABD2* construct (Voigt et al., 2005). We also used the GFP-MAP4 Arabidopsis line, in order to visualize the microtubules (Marc et al., 1927).

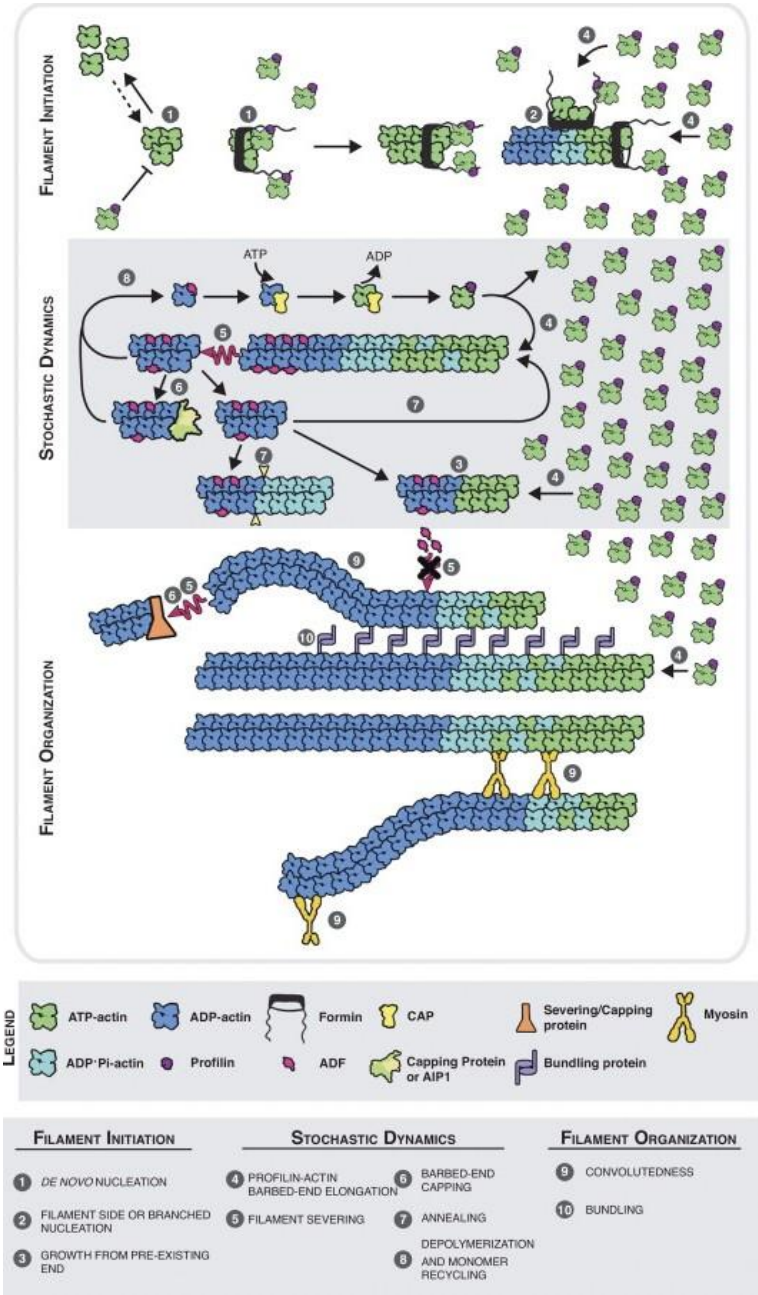
Filament Initiation
Stochastic Dynamics
Filament Organization
ATP
ADP
Legend
ATP-actin
ADP-actin
Formin
CAP
Severing/Capping protein
Myosin
ADP·Pi-actin
Profilin
ADF
Capping Protein or AIP1
Bundling protein
Filament Initiation
1 De novo nucleation
2 Filament side or branched nucleation
3 Growth from pre-existing end
Stochastic Dynamics
4 Profilin-actin barbed-end elongation
5 Filament severing
6 Barbed-end capping
7 Annealing
8 Depolymerization and monomer recycling
Filament Organization
9 Convolutedness
10 Bundling

**Figure 6. Model of actin stochastic dynamics**

This cartoon shows the assembly, disassembly and organization of actin filament arrays in plant cells. The gray box displays the steps of filament assembly and turnover by stochastic dynamics. The numbering of steps is intended to mirror the live-cell observations of single filament turnover. Red arrows denote filament severing events, which are a major feature of stochastic dynamic turnover. Adapted from Henty-Ridilla et al. (2013).

# Objectives

This thesis is focused on the following objectives:

1. Analysis of phototropism and study of auxin distribution profile in the root apex after blue lateral stimuli. Study of PIN2-GFP recycling in dark and light conditions in CK2mut seedlings.

2. Study of the stability of the auxin repressor AXR3 in seedlings depleted of CK2 activity and in mutants unable to accumulate SA.

3. Analysis of F-actin turnover and of microtubules organization in seedlings depleted of CK2 activity. Bioinformatics analyses for the identification of actin binding proteins as possible substrates of CK2.

# Experimental procedures

## 1. Plant Material And Growth Conditions

Wild-type (WT) *Arabidopsis thaliana* ecotype used in this study was Columbia (Col-0) and the Arabidopsis mutants were generated in the same genetic background. For plant growth, seeds were first surface sterilized and cold-treated (4°C) for 2 days in darkness. Subsequently, seedlings were grown in vertical Murashige and Skoog (MS) plates (Duchefala Biochemie BV, http://www.duchefala.com/) supplemented with 2 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) (pH 5.7), 0.25 to 0.5% (w/v) sucrose and 1 to 1.2% (w/v) agar and grown at 21°C to 22°C on a 16-hour light, 8-hour dark cycle (140 µE $m^{-2}$ $sec^{-1}$). Generation of CK2mut seedlings have been described previously (Moreno-Romero and Martínez, 2008) and VENUS DII line was kindly obtained by Géraldine Brunoud (Brunoud et al., 2012). PIN2::PIN2-GFP plants were crossed with the CK2mut line to obtain CK2mut x PIN2::PIN2-GFP homozygous plants (F3), which were selected by hygromicin and basta resistance. The transgenic Arabidopsis seedlings containing the HS::AXR3.3NT-GUS construct, were kindly provided by Mark Estelle (Salk Institute, LaJolla, CA, USA). The mutant line AXR3.3NT-GUS was sexually crossed with the NahG mutant line and plants in heterozygosis (F1) were selected by kanamicin resistance and GUS essay. The Arabidopsis transgenic

seeds NahG (Delaney et al., 1994) were obtained from P.Tornero (IBMCP-Valencia, Spain). *Arabidopsis thaliana* expressing *gfp-map4* was previously described by Marc et al. (1927), and contain a microtubule reporter gene generated by fusing the microtubule binding domain of the mammalian microtubule-associated protein 4 (MAP4) with the green fluorescent protein (GFP) under the control of the constitutive 35S promoter. GFP-FABD2 line was kindly provided by Dr. Voigt (Voigt et al., 2005). An actin reporter based on fusions between the carboxy terminal region, 325–687 bp, harboring the second actin binding domain of the *A. Thaliana* fimbrin1 (AtFIM1) and the N-terminal domain of the green florescent protein (GFP). CK2mut x GFP-FABD2 mutant line was generated by sexually crossing the CK2mut line and GFP-FABD2 line. Then, F3 generation of homozygosis lines were selected by hygromycin and *basta* resistance.

## *2. Elimination of the CK2 activity in Arabidopsis plants*

Expression of the *CK2mut* transgene in CK2mut plants was induced by incubation with 1 μM dexamethasone (Dex) (Sigma, www.sigmaaldrich.com) dissolved in ethanol (30 mM stock solution) during 48 h or with 5 μM Dex during 24 h. Inhibition of CK2 activity has been achieved by treating Arabidopsis WT seedlings with 10 μM 4,5,6,7-tetrabromobenzotriazol (TBB) (Calbiochem, www.emdmillipore.com) dissolved in DMSO (20 mM stock solution) during 16 h. For the analysis of F-actin turnover, GFP-FABD2 seedlings were treated with 100 μM TBB for 1 h or with 50 μM TBB for 2 h. For the study of microtubules organization GFP-MAP4 seedlings were treated with 10 μM TBB for 2 h or with 10 μM TBB for 3 h.

### 3. Confocal microscopy

Confocal images were obtained with an Olympus FluoView™ FV1000 confocal microscope (www.olympusamerica.com), equipped with diode (405 nm), argon-ion (458, 488 and 514 nm) and helium–neon (543 nm) lasers and 4.0 Fluoview software for image analysis. GFP was excited at 488 nm and the emission signals were collected at 500-600 nm. Auxin distribution in VENUS DII line was visualized by exciting the yellow fluorescent protein (YFP) at 488 nm and collecting the emission signal at 510 nm. FM4-64 was excited at 543 nm and the emission was collected at 581nm. Z-stack images of the roots at different intervals were acquired. DAPI fluorescence was detected with an excitation wavelength of 405 nm and the emission was collected at 581 nm. For image processing IMAGEJ and GIMPS programs were used (www.imagej.net) (www.gimp.org).

### 4. Phototropism and blue light experiments

Three-days-old CK2mut seedlings were grown in darkness on vertical plates. Incubation with 1 µM Dex to induce the *CK2mut* gene was performed 24 hours prior to exposure to blue light and continued until the end of the experiment. At the same time, control seedlings were transferred to MS medium solid plate containing 1 µM ethanol 24 hours before blue light treatment. The blue light treatments were carried out with an array of light emitting diodes (LED). The irradiance was measured with a photometer (HD2302.0, Delta Ohm) equipped with a probe (LP471RAD, Delta Ohm). All root samples were irradiated at 20 to 25 cm distance from the light source, and two black panels were positioned at both sides of the slides in order to avoid lateral light reflections. The seedlings were exposed to lateral blue light (10 µmol $m^{-2}$ $sec^{-1}$) during 48 hours. Pictures were taken with a

confocal microscope and the angles of roots curvature were measured with the IMAGEJ software (www.imagej.net). More than 100 roots were measured for both mutant and control seedlings.

### *5. Auxin distribution in VENUS DII roots*

Five-days-old VENUS DII seedlings were used. The seedlings were treated with TBB at a final concentration of 10 µM for 1 h. Later, they were placed between a microscope slide and a cover slip, kept in a vertical position and treated with 0.5 mg/ml of FM4-64 fluorescent dye (SynaptoRed$^{TM}$C2), (Sigma), at 6°C during 10 minutes to stain the outer leaflet of plasma membranes. Afterwards, seedlings were exposed to unilateral blue-light illumination for 40 minutes. Auxin distribution was measured using laser-scanning confocal microscope. To get information about the asymmetric auxin distribution, the ratio of fluorescence intensity between the shaded and the irradiated side of the VENUS DII-YFP roots was measured and averaged using IMAGEJ software. As DII-VENUS construct contains an *in frame* nuclear localisation signal (NLS), the fluorescence was localized in the nuclei.

### *6. PIN2-GFP distribution in dark and light conditions*

Three-days-old seedlings CK2mut x PIN2::PIN2-GFP grown either in dark or in light conditions, were incubated with Dex for 24 hours prior to the confocal analysis. Control seedlings were incubated respectively with ethanol. Subsequently, seedlings were well positioned on vertical microscope slides and treated with FM4-64. After that, the seedlings were exposed to unilateral blue light and images were acquired by confocal microscopy.

## 7. Endocytic vesicle recycling of PIN2-GFP

Four-days-old CK2mut x PIN2::PIN2-GFP seedlings were grown in MS solid medium on vertical plates in darkness and subsequently incubated with Dex for 24 hours prior to the treatment with inhibitors. Seedlings were then placed between a microscope slide and a cover slip with MS solution. After that, they were stained with 0.5 mg/ml of FM4-64. Then, seedlings were incubated with 30 μM brefeldin A (BFA) (Sigma-Aldrich Co, www.sigmaaldrich.com) at room temperature for 60 minutes in darkness, in order to block exocytosis and endocytic vesicle recycling. Confocal images of the roots at 2 μm intervals were acquired by confocal microscopy. To study the effect of BFA under blue light conditions, seedlings were first treated with 0.5 mg/ml of FM4-64 as above and, at the same time, were exposed to blue light for 40 minutes.

## 8. Heat induction and GUS assays

Seven-days-old seedlings were grown in MS plates and then transferred to liquid MS medium before the treatments. HS::AXR3.3-GUS and HS::AXR3.3NT-GUS x NahG seedlings were heat shocked (HS) for 2h at 37°C and then left for one additional hour at room temperature with shaking. Treatments with TBB (25 μM in DMSO) and MG132 (50 μM in DMSO) (Sigma) were carried out for two hours, starting one hour after launching HS. Treatment with 2,4-D (5 μM in ethanol) was carried out for one hour, starting at the end of HS. Treatment with 10 μM 1-naphthaleneaceticacid (NAA) (Sigma) dissolved in ethanol has been used for the fluorimetric assay during 80 min after the HS. All these treatments were performed in liquid MS medium for 3 h and appropriate controls with the corresponding solvents were always carried out. GUS staining for Arabidopsis roots

was performed as in Stomp (1992); seedlings were mounted with 50% glycerol (v/v) and observed with a Leica DMRB microscope (Leica, http://www.leica.com). Images were taken with a Leica DFC500 digital camera. Fluorimetric assays of GUS activity for HS::AXR3.3-GUS seedlings were carried out with 4-methylumbelliferyl-β-D-glucuronide (MUG) (Sigma) as a GUS substrate, following the procedure described in Gray et al. (2001). Plants were sampled at 20 min intervals and stored in liquid nitrogen until GUS activity was measured. Protein extracts were obtained in GUS buffer (50mMKPO4, pH 7.0,0.1% sarkosyl, 0.1% Triton X-100,10mM β-mercaptoethanol,10mM EDTA). Equal volumes of protein extracts and of MUG solution (2 mM in GUS buffer) were incubated for 16 h in darkness. The reaction was stopped with 900 µl of 0.2 M$Na_2CO_3$ and fluorescence was measured in a spectrophotometer Cary Eclipse (Varian) with λ-excitation at 365nm and λ-emission at 450 nm. Extracts were prepared from 10 seedlings and data were normalized against total protein content determined by Bradford assays. Three biological replicates for each sample were performed. The intensity of GUS staining in the HS::AXR3.3NT-GUS x NahG seedlings was quantified by using IMAGE J software, as described by Abd-El-Haliem (2012). After TBB treatment the seedlings were observed with a Leica DM 750 microscope (Leica, http://www.leica.com) and images were taken with a Leica ICC50 HD digital camera.

### *9. Whole mount immunofluorescence labelling*

Root tips of Arabidopsis were fixed for 60 minutes using a fixative solution (1.5% paraformaldehyde + 0.5% glutaraldehyde in 0,5x microtubule stabilizing buffer), under vacuum to ensure proper tissue penetration. The microtubule stabilizing buffer (MTSB) contained 50

mM Pipes, 5 mM $MgSO_4$, 5 Mm EGTA at pH 6.9. The fixative solution was removed and then seedlings were washed once with 0,5X MTSB for 10 minutes and twice with phosphate-buffered saline (PBS) for 10 minutes each. PBS contains 140 mM NaCl, 2.7 mM KCl, 6.5 mM Na2HPO4 and 1.5 mM KH2PO4, pH 7.3. Then seedlings were treated three times with sodium borohydride 1 mg/ml NaBH4 in PBS for 10 minutes each. Afterwards, samples were briefly rinsed in PBS, three times during 5 minutes, and then incubated with 2% driselase + 2% cellulose + 1% pectolyase in PBS, at 37°C for 30 min. Then, cells were permeabilised by incubation with 10 mM glycine, four times for 5 min each, and then with 2% Nonidet P40 + 10% DMSO in PBS for 1h. Seedlings were then washed with PBS for 10 min and subsequently blocked with 2% BSA in PBS. After incubation with rabbit anti-maize actin primary polyclonal antibodies (1:200) at 4°C overnight, seedlings were washed six times with PBS for 10 min each and then incubated with anti-rabbit IgG antibody conjugated to Alexa Fluor® 488, during 1.5 h at 37°C and 1.5 h at room temperature. Seedlings were then washed six times with PBS for 10 min each and nuclei were stained with 0.5 µM DAPI in PBS. Samples were washed twice with PBS for 5 min before confocal imaging.

### *10. F-actin turnover*

Six-days-old GFP-FABD2 seedlings were submerged in one of the following MS liquid solutions: 100 µM TBB for 60 minutes; 2 µM jasplakinolide for 60 min; 100 µM TBB and 2 µM jasplakinolide for 60 minutes; 50 µM TBB for 120 minutes and 2 µM) jasplakinolide for 60 minutes added just at the end of the first 60 minutes of TBB treatment. All experiments where performed at room temperature in darkness and in shaking plates. Jasplakinolide, purchased from

Enzolifesciences, (www.enzolifesciences.com) is a cyclic peptide isolated from marine sponges (Spector et al., 1999), known as a potent inducer of actin polymerisation and/or stabilization. Stock solutions were prepared in dimethyl sulfoxide (DMSO) at final concentration of 1 mM.

### *11. Bioinformatics tools*

Prediction of putative CK2 phosphorylation residues were identified using UniProt (www.uniprot.org) and NetPhos 3.1 Server (www.cbs.dtu.dk/services/NetPhos) using a threshold of 0.5 (Blom et al., 2004).

# Results

## 1. Analysis Of Phototropic Responses In CK2-Defective Plants

### *1.1. Inhibition of protein kinase CK2 affects Arabidopsis phototropic responses*

Previous work in the laboratory of Dra. Martínez showed that depletion of CK2 activity by using the dexamethasone-induced CK2mut line resulted in phenotypic changes linked to alterations in auxin-dependent processes. Among them, loss of hypocotyls phototropic curvature in response to directional white light was reported (Marquès-Bueno et al., 2011b). In order to better characterize the phototropic phenotype of the CK2mut line, root and hypocotyl phototropic responses were examined in this work. For the analysis of the root phototropic response, illumination with unilateral blue-light (BL) at 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ was carried out. The response was quantified by measuring the deviation angle, α, of root tips from the vertical (see the scheme at Figure 7a). The results are shown in the form of a histogram, representing the deviation angle and its frequency, both in CK2mut seedlings and their respective controls (Figure 7c). It can be seen that control roots exhibited a negative phototropic response, growing away from the light source with an average deviation angle of -20°. Moreover, loss of CK2 activity

strongly influenced the root phototropic response, as the roots of CK2mut seedlings showed an average deviation angle of -40°. These results evidence an enhanced phototropic negative response in CK2mut seedlings. However, the histogram (Figure 7c) also shows a no significant minority of roots with positive phototropic response, which might be due to experimental errors and sample variability.

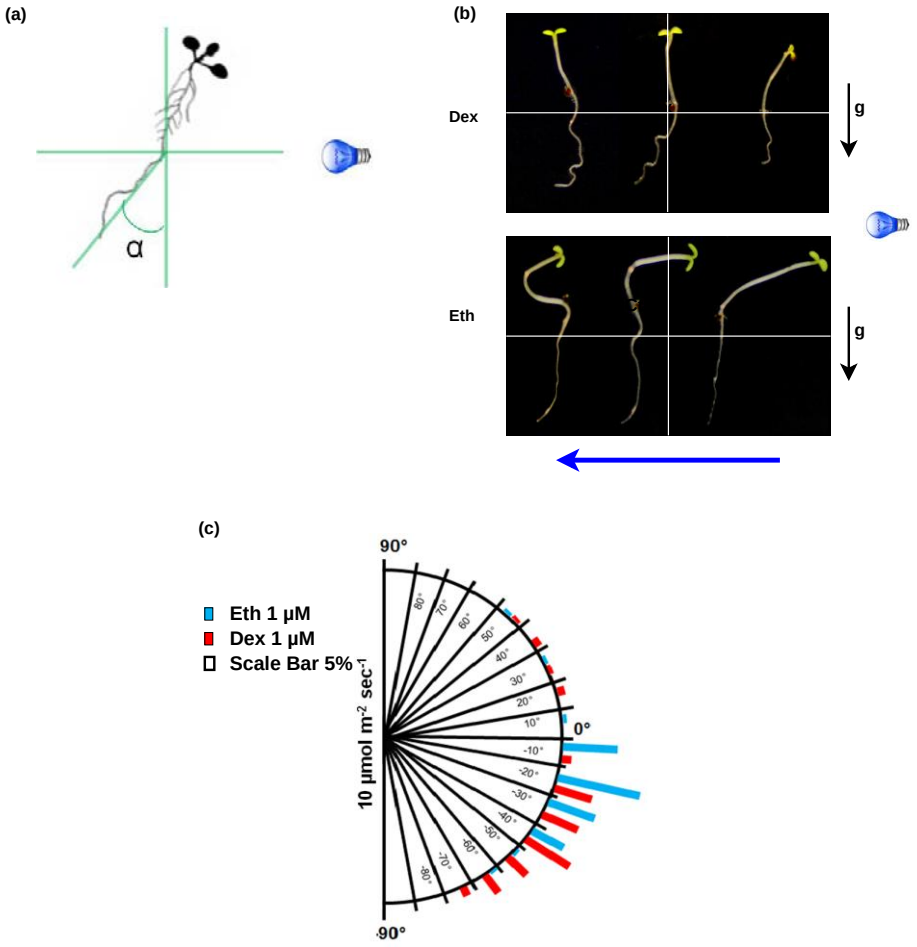

**Figure 7. Enhanced negative root phototropic response of CK2mut seedlings.**

***(a)*** *Schematic representation of the measured α angle used to quantify the curvature. The α angle represents the deviation of the root tip from the vertical.* ***(b)*** *Pictures from a typical experiment, taken from three-days-old WT and CK2mut seedlings grown in vertical plates in darkness and incubated respectively with 1 μM Dex or Ethanol (control) for 24h prior to blue-light exposure. The bending phenotype was analyzed 48h after illumination with blue-light (10 μmol·m$^{-2}$·s$^{-1}$).* ***(c)*** *Histogram shows the results of the root bending angles α in both Dex and Eth treated seedlings. Roots of Eth treated seedlings bend away from the light source with an average deviation angle of -20°. Roots of Dex treated seedlings bend away from the light source with an average deviation angle of -40°. The number of individuals examined respectively for Dex treatment was n=97 and for Eth treatment was n=109 and it is represented as the percentage of all plant analyzed.*

We also analysed the bending curvature of CK2mut hypocotyls under unilateral blue light (Figure 8a). The results of the deviation angle, α, of cotyledons from the vertical (Figure 8b), together with the deviations frequencies, are represented in the histogram below (Figure 8c).

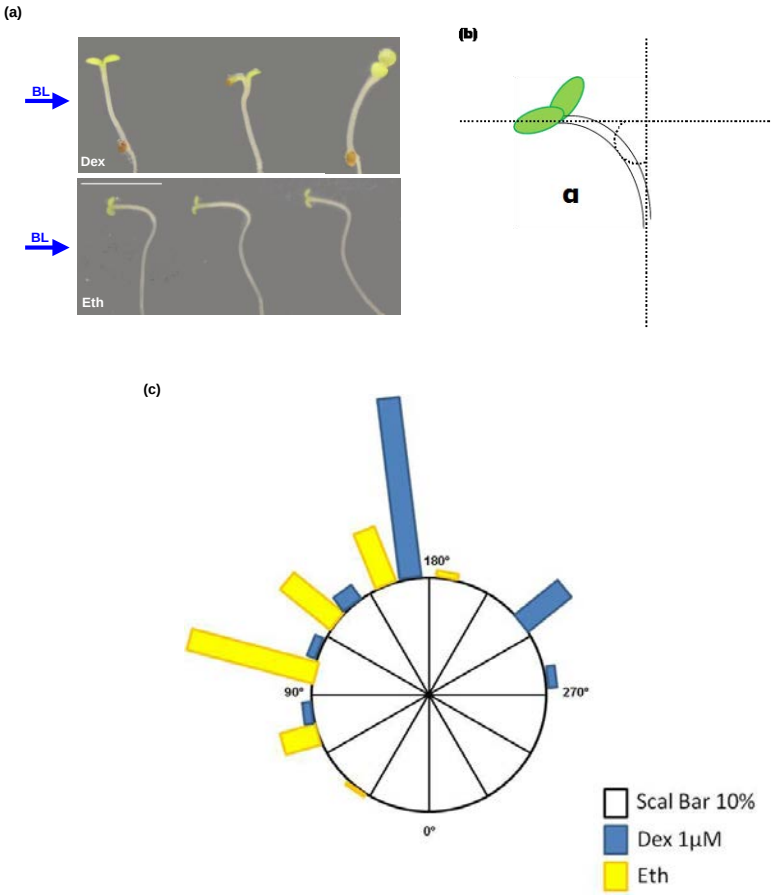


**Figure 8. Hypocotyl phototropic response of CK2mut seedlings.**

***(a)*** *Analysis of hypocotyl phototropic curvature in CK2 defective seedlings. Pictures were taken from three-days-old WT and CK2mut seedlings grown in vertical plates in darkness and incubated respectively with 1 µM Dex for 24h prior to blue-light exposure. Control seedlings were treated with Ethanol. The bending phenotype was analyzed 48h after the application of blue-light (10 µmol·m$^{-2}$·s$^{-1}$).* ***(b)*** *Schematic representation of the measured angle used to quantify the hypocotyls curvature. 180° represents no bending.* ***(c)*** *Histograms of hypocotyl bending angles. The number of individuals examined for both treatments was n=100 and it is represented as the percentage of all plants analyzed.*

Our results show average angles of 100° and 170° for control and CK2mut seedlings respectively, indicating that the hypocotyls of CK2mut seedlings have null capacity to bend towards the light source.

These results corroborate previous data obtained by illumination with white light, in the laboratory of Dra. Martínez (Marquès-Bueno et al., 2011a).

In summary, our data strongly suggest a functional implication of protein kinase CK2 in the negative and the positive phototropic responses exhibited by Arabidopsis roots and shoots, respectively.

### *1.2. CK2mut plants show impaired auxin redistribution*

In order to visualize the dynamic changes of auxin cellular distribution, a novel Aux/IAA-based auxin signalling sensor (DII-VENUS) was used in this work. This auxin sensor was constructed by *in frame* fusion of the fast maturing form of the yellow fluorescent protein (VENUS) to the Aux/IAAs auxin-interaction domain (DII), and the chimeric protein was expressed under a constitutive promoter (Brunoud et al., 2012). The distribution of the yellow fluorescent protein (YFP) in root apices of DII-VENUS line was analysed by laser scanning confocal microscopy, giving us an indirect evidence of auxin distribution (Figure 9a,b). We also analysed YFP fluorescence in DII-VENUS line treated with TBB, which is a strong inhibitor of CK2 activity (Sarno et al., 2001), in order to study the influence of CK2 inhibition in auxin distribution (Figure 9c,d).

Our results show that the YFP signal was uniformly distributed along the root tip in DII-VENUS control seedlings grown in darkness (Figure 9a). We then investigated the effect of blue-light irradiation on auxin distribution. Interestingly, 40 minutes after blue-light irradiation, the amount of fluorescence strongly decreased at the irradiated side, indicating an increase of auxin concentration in this part of the root (Figure 9b). This asymmetric distribution of auxin induced by unilateral

light is in agreement with the results previously reported by Zhang et al. (2014, 2013). TBB-treated DII-VENUS seedlings grown in darkness showed a decrease of fluorescence in the upper part of the root (the transition zone), indicating accumulation of auxin in this region (Figure 9c). Similar results were previously obtained in the laboratory of Dra. Martinez, using a DR5::GFP line (Marquès-Bueno et al., 2011a). Moreover, by illumination with unilateral blue light, the fluorescence signal became very weak and widespread, and its asymmetry was completely lost (Figure 9d). Thus, normal auxin redistribution through the root triggered by the BL stimulus was impaired after CK2 inhibition. This was a striking and unexpected result, since we have shown that CK2 inhibition results in a phenotype of enhanced negative root phototropism.

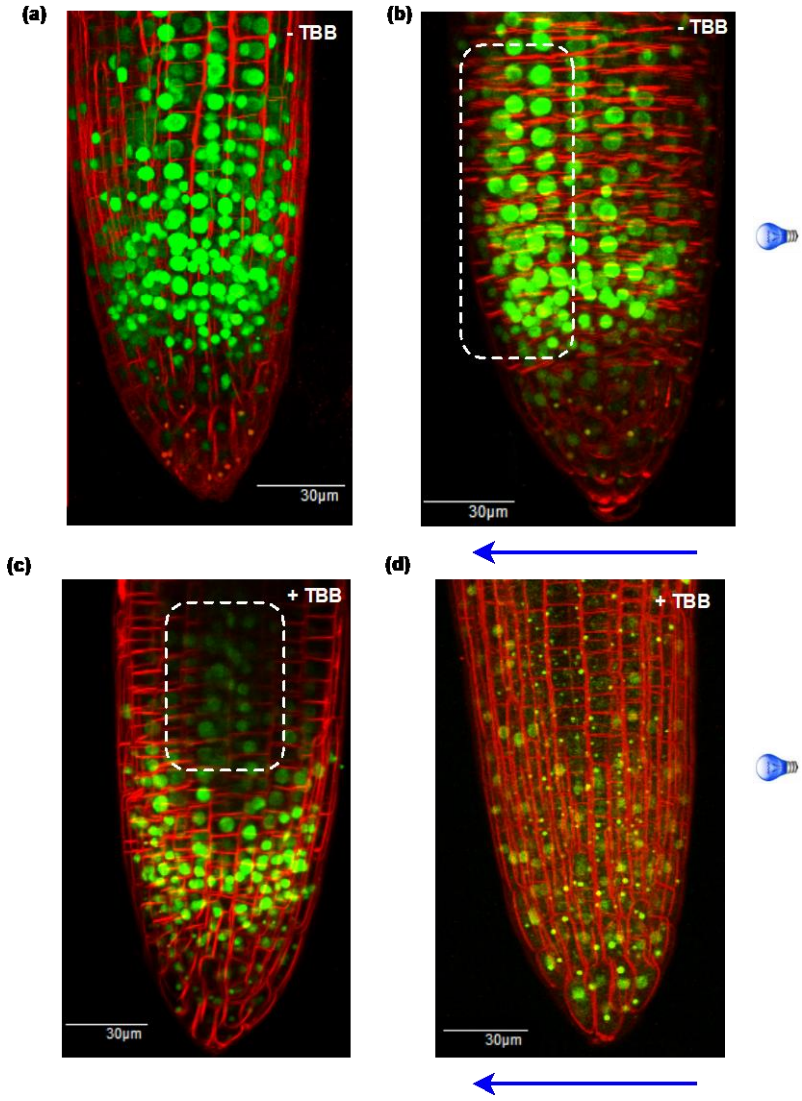


**Figure 9. Auxin distribution in the root apex and its relocation after BL induction.**

*The images correspond to Z-stacks of five-days-old DII-VENUS roots. Fluorescence intensity of nuclei (green signal) and of FM4-64 staining (red signal) in DII-VENUS roots grown in darkness* ***(a)*** *and then exposed to BL for 40 minutes* ***(b)****. The same experiment was carried out after root incubation with 10 µM TBB for 1 hour* ***(c)*** *and* ***(d)****, n ≥ 27, for each condition.*

In order to quantify these results, we calculated the ratio of fluorescence intensity between the right (illuminated) and the left (shaded) side of the roots after 20 minutes of blue-light irradiation (Figure 10). The part of the root analysed in all conditions was the area from the transition zone until the root tip. The results confirmed a higher asymmetric auxin distribution in control roots after blue light stimuli, whereas more homogeneous values through the whole root tip have been found in dark grown control roots . The statistical analysis of the TBB-treated roots was not satisfactory, likely due to the loss of fluorescent signal after lateral blue light irradiation.

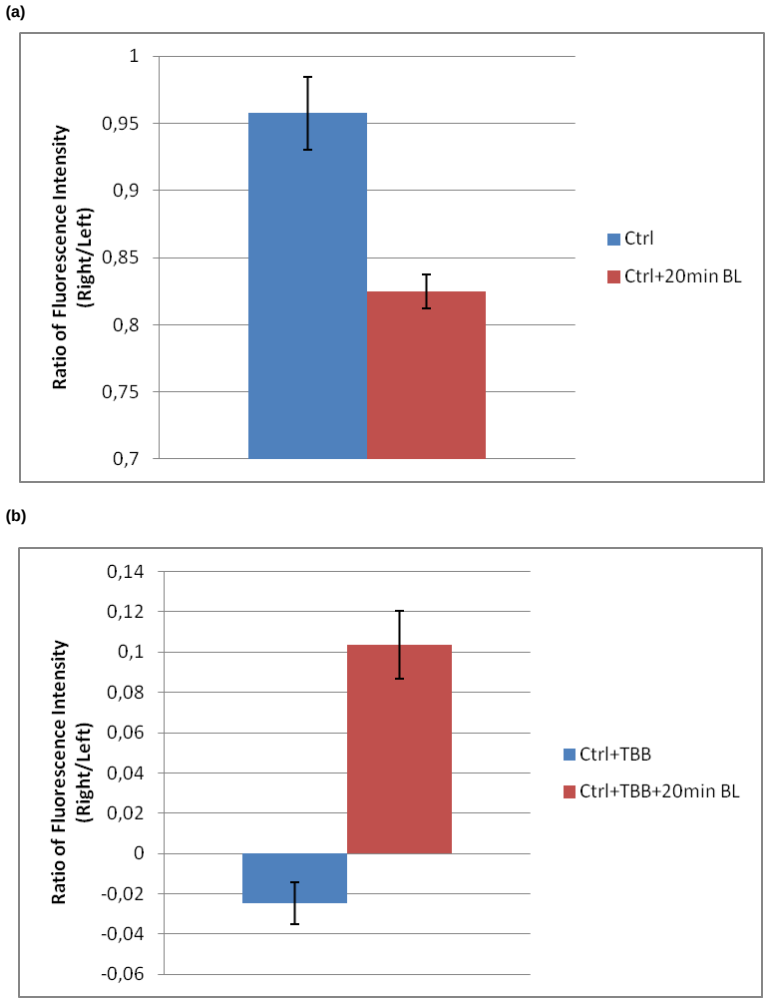


**Figure 10. Quantification of auxin redistribution after BL induction.**

***(a)*** *Ratio of DII-VENUS fluorescence intensities between right (illuminated) and left (shaded) side in control seedlings. Fluorescence was measured in the root epidermal cells of root apex (n=3)* ***(b)*** *Ratio of DII-VENUS fluorescence intensities in TBB-treated seedlings, measured as in panel (a).*

### 1.3. PIN2 localisation in CK2mut roots in dark and light conditions

The relevant role of PIN2 in the phototropic responses, led us to investigate whether its localisation and trafficking was affected in the CK2mut line. To do that, we sexually crossed the CK2mut line with the PIN2::PIN2-GFP line and we isolated the homozygous seeds (F3 generation). We then analyzed PIN2-GFP localisation in the root apex under dark and blue-light conditions, and in the presence and absence of dexamethasone (Dex), which is the inducer of the transgene. Figure 11a shows that PIN2-GFP is correctly localized at the basal membrane of the cortical cells and the apical membrane of the epidermal cells in light-grown, control plants (-Dex) (see the white arrows, Figure 11a). Moreover, when PIN2-GFP seedlings were grown in darkness, a great amount of PIN2 was found in the vacuole-like compartments (VLCs) (Figure 11b and c). Dex-treated CK2mut x PIN2::PIN2-GFP roots exhibited similar PIN2-GFP localisation in seedlings grown either in light or darkness (Figure 11d and e, respectively). In both cases, PIN2 was found polarly localized in the plasma membrane, suggesting that dark-triggered PIN2 internalization was impaired by inhibition of CK2 activity. Our results related to the control seedlings are in agreement with those reported by other authors (Kleine-Vehn et al., 2008c; Laxmi et al., 2008; Wan et al., 2012). It has been previously described that the light/dark-associated states of PIN2 localisation are reversible (Wan et al., 2012). A hypothesis to explain the above results is that CK2mut roots are always in the “light status”, regardless of the conditions they were grown under (light or darkness). We can conclude that CK2mut roots exhibit impaired vesicular trafficking that regulates PIN2

internalization. Furthermore, this putative constitutive “light status” might also account for the enhanced negative phototropic response of CK2mut roots.

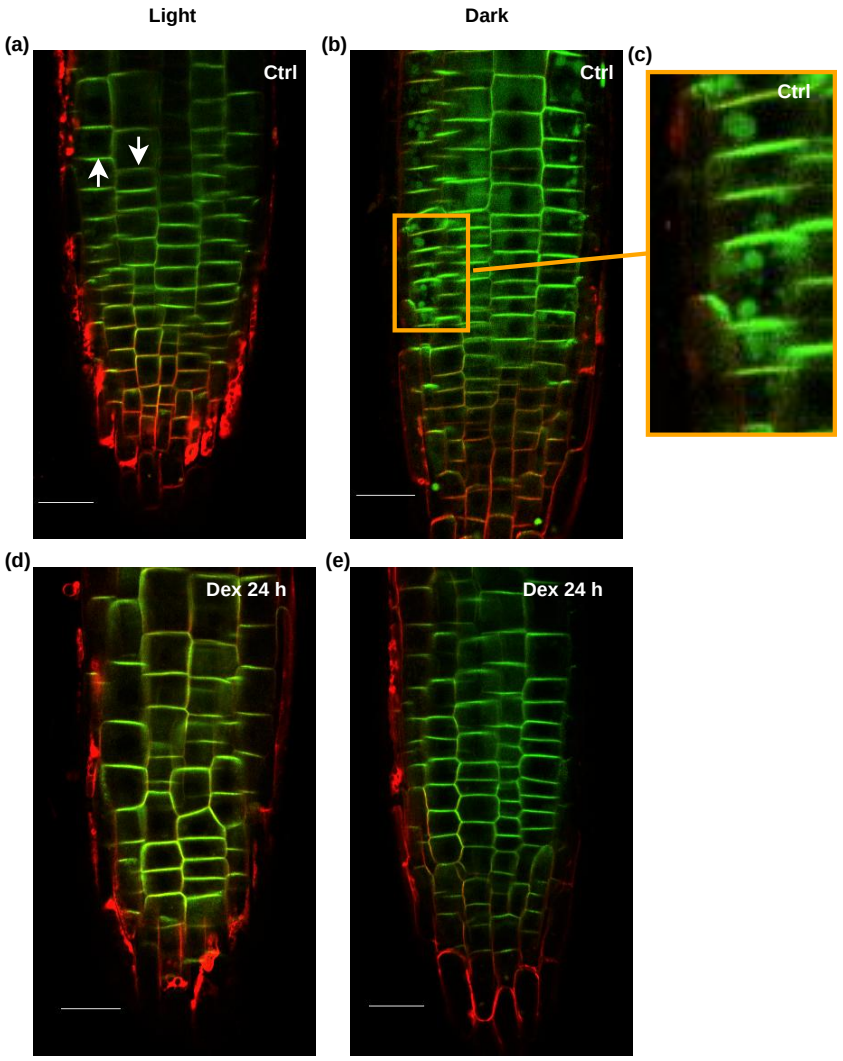

**Figure 11. PIN2 localisation in control and CK2mut lines under light and dark conditions.**

*Images were selected from three independent experiments and at least three seedlings for each condition were analyzed. Three-days-old dark-grown CK2mut x PIN2::PIN2-GFP roots were transferred to MS medium solid plates containing 5 µM Dex or Ethanol (ctrl) for 24 h and stained with FM4-64. Bars = 20 µm.*

***(a, b)** Localisation of PIN2-GFP in CK2mut x PIN2::PIN2-GFP roots treated with Eth and grown respectively in light **(a)** and dark **(b)** conditions. **(c)** Magnification of epidermal cells of the transition zone of the root. It shows PIN2-GFP accumulation in vacuole-like compartments. **(d, e)** Localisation of PIN2-GFP in CK2mut x PIN2::PIN2-GFP roots treated with Dex either in light-grown condition **(d)** and dark-grown condition **(e)**.*

### *1.4. PIN2 recycling in CK2mut seedlings*

In order to study the role of CK2 in the regulation of vesicular trafficking of PIN2, we studied PIN2-GFP relocation after BFA treatments in CK2mut line, both under dark and light conditions.

Four-days-old CK2mut x PIN2::PIN2-GFP seedlings grown in MS solid medium on vertical plates in darkness were incubated with 5 µM Dex during 24 hours prior to BFA treatments. Seedlings were then stained with 8.2 µM of FM4-64 at 6°C for 10 minutes to trace internalizations and the endosomal aggregates produced by BFA. Afterwards, seedlings were incubated with 30 µM BFA at room temperature for 60 minutes in darkness, and observed with a confocal microscope. As shown in Figure 12a dark-grown CK2mut x PIN2-GFP control roots treated with BFA show visible PIN2-GFP internalizations (green dye) that colocalize with small vesicles stained by FM4-64 (red dye), indicative of BFA bodies (yellow arrows). We also observed internalizations of PIN2-GFP that do not colocalize with the FM4-64 vesicles, and show the appearance of vacuole-like compartments (VLCs) (white arrows). These VLCs have been previously identified by other authors (Kleine-Vehn et al., 2008c; Laxmi et al., 2008). By contrast, CK2mut x PIN2-GFP control roots treated with BFA under blue lateral light conditions (10 µmol m-2 sec-1) showed PIN2

internalization only in the BFA bodies (Figure 12b). We then performed the same experiments in Dex-treated CK2mut x PIN2-GFP line. Figure 12c and Figure 12d show that PIN2 is found internalized in large BFA bodies, with a similar pattern both under light and dark conditions. These results show that in the absence of CK2 activity PIN2 proteins fail to accumulate in the VLCs, suggesting that CK2 is required in the retromeric pathway but not in the recycling pathway to the plasma membrane. It was previously hypothesized that NPH3 acts as a switch, changing the dynamic equilibrium of PIN2 between the VLCs and the plasma membrane trafficking pathways (Wan et al., 2012). Since it has been found that proteins of the NPH3 family contain consensus phosphorylation sites for CK2, we can speculate that CK2 might control PIN2 trafficking between PVCs and plasma membrane pathways by controlling the activity of NPH3 in light and dark transitions.

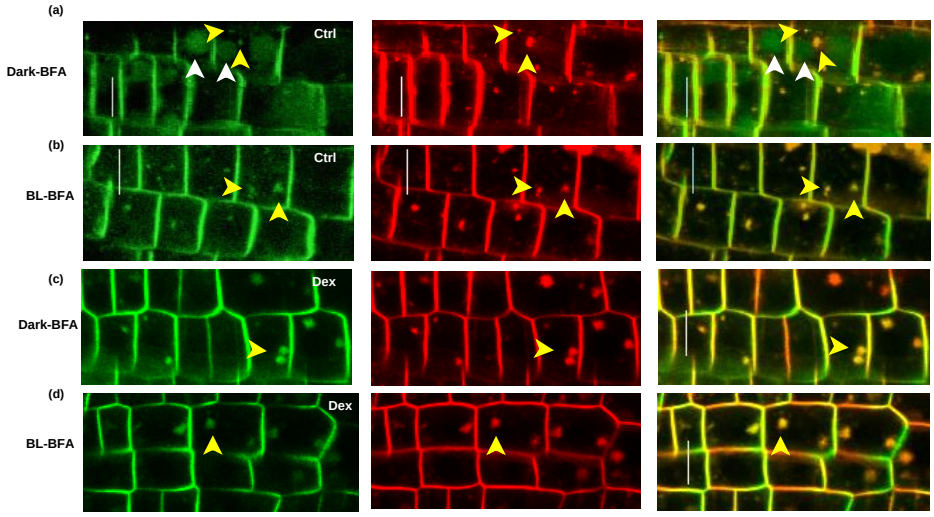

**Figure 12. BFA-sensitive recycling of PIN2 in CK2mut seedlings.**

*Confocal sections of five-days-old dark-grown CK2mut x PIN2::PIN2-GFP roots. Three-days-old dark-grown CK2mut x PIN2::PIN2-GFP roots were transferred to MS medium solid plates containing 5 µM Dex or Ethanol during 24 h in darkness. Then, roots were transferred to microscope slides and treated with 8.2 µM FM4-64 (red dye) during 10 minutes, washed, and incubated with 30 µM BFA during 60 minutes in darkness* ***(a, c)*** *or during 40 minutes under blue light lateral exposure (10 µmol* $m^{-2}$ $sec^{-1}$ *)* ***(b, d)****. White arrows point to VLCs and yellow arrows point to BFA-induced compartments. Images were selected from four independent experiments and at least three seedlings for each point were analyzed. (Scal Bars = 10 µm).*

# 2. Role Of CK2 In The Crosstalk Between Salicylic Acid And Auxin signalling pathways

## *2.1. CK2 inhibition enhances the stability of the auxin repressor AXR3*

In order to study whether the auxin-signalling repression in CK2-defective Arabidopsis seedlings was a consequence of the stabilization of the auxin repressor AXR3, we used HS::AXR3NT-GUS Arabidopsis seedlings (Gray et al., 2001) treated with TBB.

Figure 13a shows that GUS activity was detected in HS::AXR3NT-GUS roots 2 h after HS treatment (control) and disappeared when the HS treatment was followed by incubation with 2,4-D. Moreover, as expected, the 2,4-D-induced AXR3 degradation was prevented in the presence of the proteosome inhibitor MG132. These results confirmed that AXR3-GUS chimeric protein was post-translationally regulated in a similar way than wild-type AXR3, i.e. by auxin-induced degradation via the proteosome. Figure 13a also shows that incubation with TBB increased AXR3 stability. Furthermore, exogenous 2,4-D added to TBB-treated plants was unable to target AXR3-GUS for degradation, as GUS activity did not decrease. Moreover, simultaneous incubation with MG132 and TBB potentiated the MG132 effect, suggesting that TBB and MG132 are acting on different steps of the protein degradation pathway (Figure 13a).

We quantified the effects of TBB and auxin on AXR3 stability by using a fluorimetric assay. AXR3NT-GUS activity was measured at 20-min intervals after heat shock using 4-methylumbelliferyl-β-D-glucuronide (MUG) as a GUS substrate (Figure 13b). Whereas AXR3NT-GUS levels decreased rapidly in control plants and NAA-

treated plants, GUS activity remained high in TBB-treated roots (blue line, ANOVA>0.05), with only a slight but not significant decrease at 80 min after HS (to 68% of the initial GUS activity value). Moreover, treatment with exogenous NAA was unable to reverse the TBB effect. We conclude that protein kinase CK2 functions as a regulator of AXR3 stability, which is in agreement with our previous hypothesis that CK2 activity was required in the early steps of auxin-driven gene transcription.

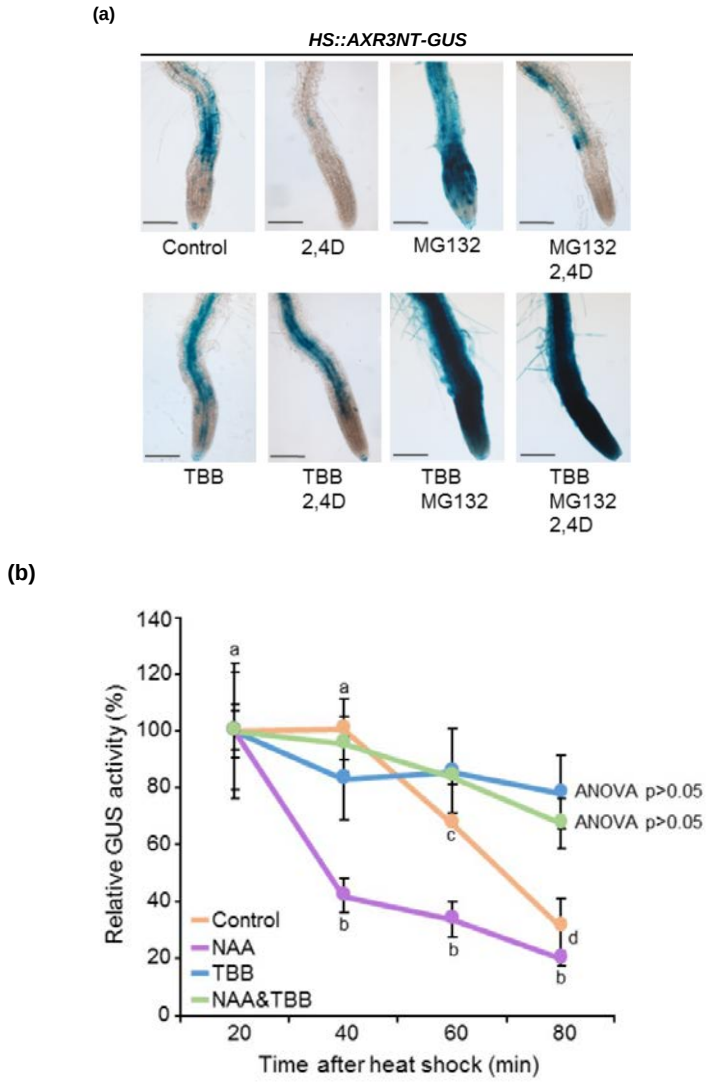


**Figure 13. CK2 activity regulates the stability of AXR3, an auxin transcriptional repressor.**

***(a)*** *GUS activity staining. Seven-day-old HS::AXR3.3-GUS seedlings were heat shocked for 2 h (control) and treated with 2,4-D, MG132, TBB in different combinations, as indicated. n= 10-20.****(b)*** *Fluorimetric assay of GUS activity. Relative activity is expressed as percentage of the 20-min GUS activity levels. Error bars indicate standard deviations. Same letters above the bars indicate not significant differences from each other (ANOVA P≤0.05). Abbreviations: 2,4-D, 2,4 dichlorophenoxyacetic acid; TBB, 4,5,6,7-tetrabromo benzotriazol.*

### *2.2. AXR3 over-stability in CK2-defective plants is SA-dependent*

It has been reported that SA stabilizes the auxin repressor AXR3, resulting in inhibition of auxin responses (Wang et al., 2007). As CK2mut roots accumulate high levels of salicylic acid (Armengot et al., 2014) we wanted to know if the AXR3 stabilization found in CK2 depleted seedlings was mediated by their high endogenous SA levels. We generated a transgenic line by sexual crossing of HS::AXR3NT-GUS Arabidopsis plants with NahG plants. NahG plants are SA-defective due to the overexpression of a bacterial salicylate hydroxylase (Delaney et al., 1994). The subsequent experiments were performed with the F1 generation and HS::AXR3.3-GUS roots were used as controls. Both, HS::AXR3.3-GUS x NahG and HS::AXR3.3-GUS were subjected to 1h of heat shock and then treated with TBB for 2 h before GUS activity detection. As shown in Figure 14a and Figure 14b, TBB treatments produced enhanced GUS signal in HS::AXR3.3-GUS roots as compared to the HS::AXR3.3-GUS x NahG roots. This demonstrates that the AXR3 stabilization, obtained by CK2 inactivation (with TBB treatment), is likely due to their high salicylic acid content. Quantification of the results was carried out with IMAGEJ software and are shown in Figure 15. A significant decrease of fluorescence intensity (up to 3 times) in HS::AXR3.3-GUS x NahG as compared to HS::AXR3.3-GUS roots was measured.

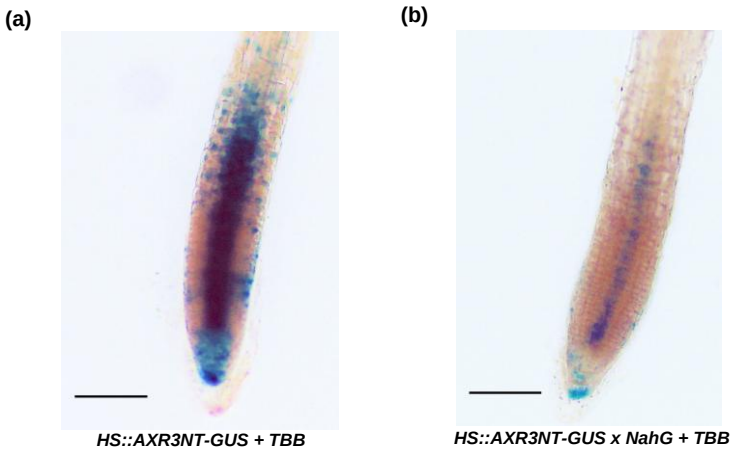


**Figure 14. Salicylic acid stabilizes AXR3 under condition of CK2 depletion.**

*GUS activity staining. Seven-day-old HS::AXR3.3-GUS **(a)** and HS::AXR3.3-GUS x NahG seedlings **(b)** were heat shocked for 2 h (control) and treated with TBB at the indicated concentration. n= 20-30 for each condition. Bars = 0.10 mm*

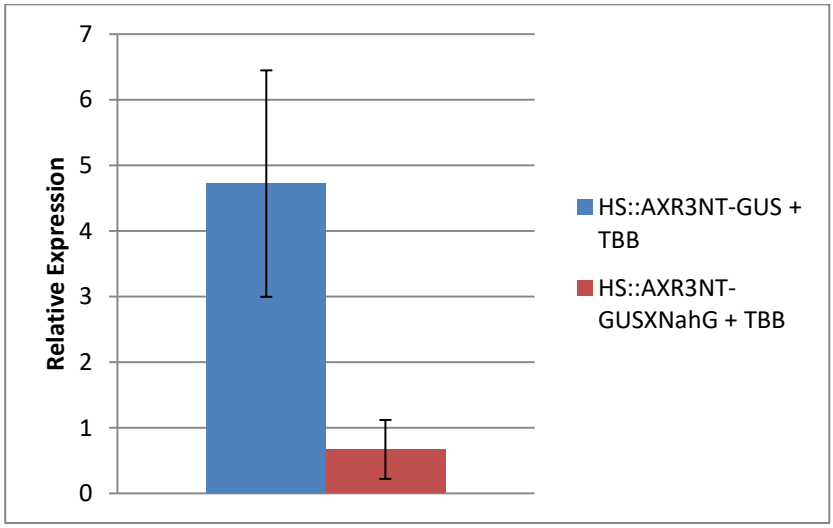


**Figure 15. Salicylic acid regulates the stability of AXR3 in CK2mut seedlings.**

*Quantification analysis of relative GUS expression in seven-day-old HS::AXR3.3-GUS and HS::AXR3.3-GUS x NahG seedlings after TBB treatment, using IMAGEJ software, with n=19 for both.*

# 3. Analysis Of Actin Cytoskeleton In CK2mut Seedlings

## *3.1. Root cells cytoskeleton is altered in CK2-depleted seedlings*

The actin cytoskeleton plays important roles in many cellular processes, among them maintaining cell shape and transport and positioning of organelles. In order to study whether the growth phenotype of the CK2-depleted line was associated to impairment of cytoskeleton organization, we performed *in vivo* visualization of F-actin using the actin reporter GFP-FABD2 line (Voigt et al., 2005) treated with TBB. The pictures corresponding to the GFP fluorescence were taken from the transition zone of the root apex.

A dramatic redistribution of F-actin networks in cells of GFP-FABD2 seedlings treated with TBB treatment is showed in Figure 16a. This effect can be seen both in cortex and epidermal cells, as compared to the controls (Figure 16b). In seedlings depleted of CK2 activity, F-actin bundles appeared to be collapsed in the nucleus and the nucleus position was shifted towards the cell periphery close to the plasma membrane in the transition zone cells. Furthermore, the shape of vacuoles and of whole cells appeared heavily affected, indicating that the depletion of CK2 activity strongly affects actin polymerisation in root cells.

To confirm these data, we then performed actin immunolocalisation experiments on WT roots in the presence and absence of TBB (Figure 17). The results obtained confirmed that F-actin structure was lost after inhibition of CK2 activity with TBB.

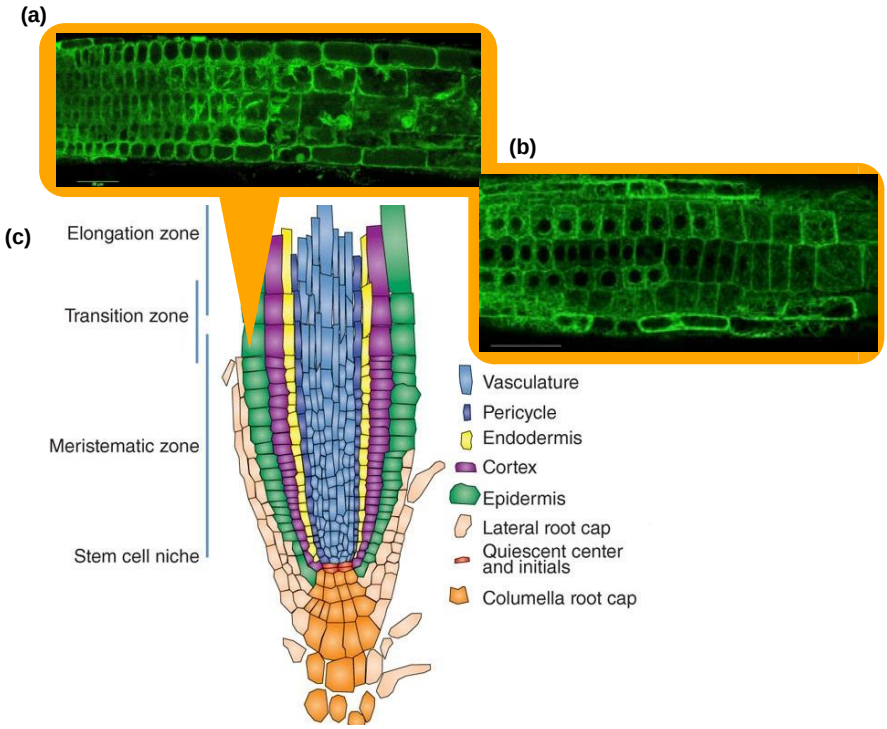


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**Figure 16. Architecture of actin cytoskeleton in the cortical and epidermal cells of CK2mut roots.**

***(a, b)*** *In vivo visualization of F-actin in GFP-FABD2 seedlings. Single confocal sections of the transition zone taken from 6-days-old seedlings after 16 h of TBB treatment (10 μM).* ***(c)*** *Schematic representation of the root apex* (Jaillais and Chory, 2010) *(Scal bars: 30 μm).*

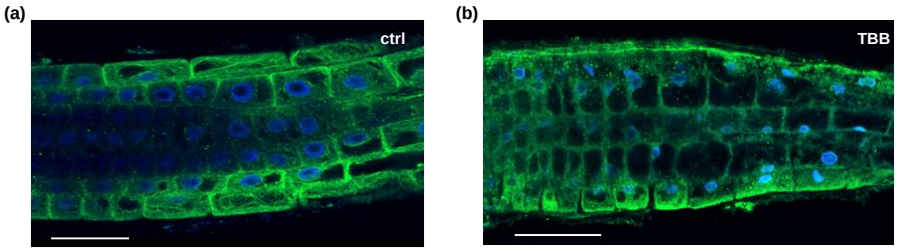



**Figure 17. Whole-mount immunolocalisation of actin.**

*Confocal sections of immunostained roots with anti-actin antibodies and with DAPI (blue fluorescence) to detect nuclei in 6-days-old WT Arabidopsis seedlings **(a)** and in 6-days-old WT Arabidopsis seedlings after 16 h of 10 µM TBB treatment **(b)**. Scal bars: 30 µm.*

## *3.2. In vivo visualization of F-actin in CK2mut x GFP-FABD2 seedlings*

To further characterize the role of CK2 in actin architecture, GFP-FABD2 seedlings were sexually crossed with the loss-of-function CK2 mutant (CK2mut line), and homozygous lines for both transgenes (F3 generation) were isolated. Transient inductions of the *CK2mut* transgene with dexamethasone (Dex) were performed during 24 hours, and then seedlings were observed by confocal microscopy. As shown in Figure 18a (white arrow), F-actin bundles appear thicker and the GFP-FABD2 signal stronger in root endodermis cells of Dex-treated CK2mut x GFP-FABD2 seedlings.

Transition zone exhibits impaired F-actin networks after Dex induction (Figure 18c). Magnified pictures of single cells from the transition zone (Figure 18e and f) show that the shape of the nucleus as well as its central position were lost in Dex-treated CK2mut x GFP-

FAB2 seedlings (Figure 18e), as compared to the controls (Figure 18f). Moreover, actin filaments going from nucleus to plasma membrane are thicker (red arrows) and several F-actin spots and F-actin bundles are evident (white arrows), resulting in a confused, patchy network of actin. In contrast, we observe a very well organized F-actin framework in the control line, with a distribution all around the nuclear envelope. It has been previously reported that auxin alters the appearance of vacuoles, increasing the amount of SNARE proteins around them (Löfke et al., 2015). Actin cytoskeleton in plants shows proximity to vacuoles and it controls its volume in an auxin-dependant manner. Furthermore, exogenous auxin applications induce vacuolar constrictions (Scheuring et al., 2016). A comparable scenario is visible in Dex-treated CK2mut x GFP-FAB2 seedlings (Figure 18e) where we can identify black holes around the nuclei that represent vacuole's constrictions.

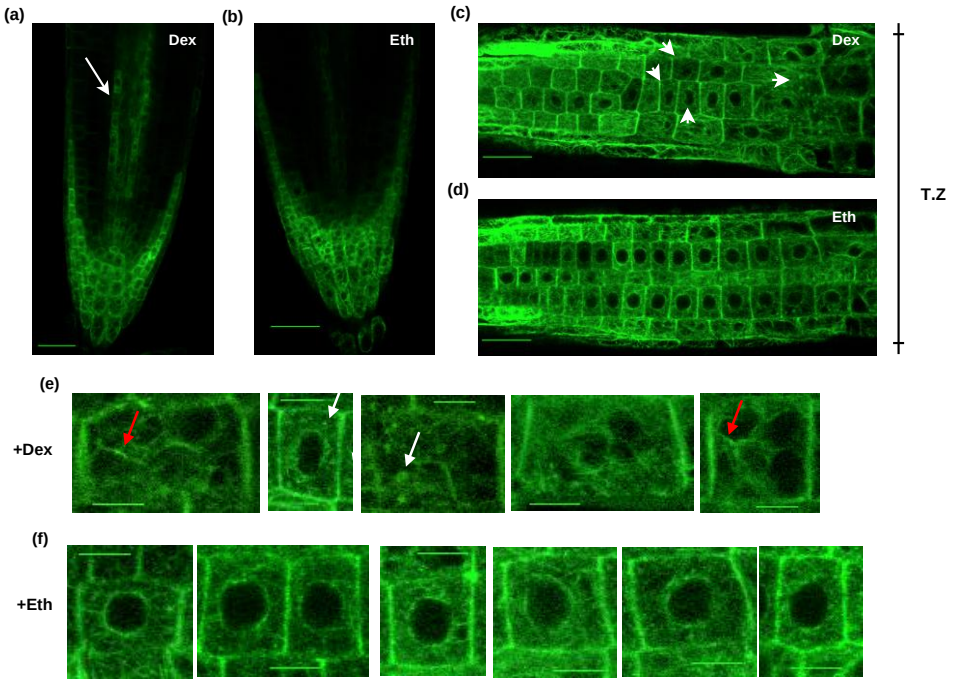



**Figure 18. *In vivo* imaging of *CK2mut x GFP-FABD2* seedlings**

*Confocal sections of six-days-old CK2mut x GFP-FABD2 Arabidopsis seedlings. Five days-old seedlings were transferred to MS medium solid plates containing 5 µM Dex. White arrow indicate thick F-actin bundles and strong GFP signal of root endodermis cells* ***(a)*** *or ethanol* ***(b)*** *for 24 h. Overview of transition zone of five days-old seedlings transferred to MS medium solid plates containing 5 µM Dex* ***(c)*** *or Ethanol* ***(d)*** *for 24 h. (Scal bars: 30 µm). Single cell magnifications from transition zone of five days-old seedlings transferred to MS medium solid plates containing 5 µM Dex* ***(e)*** *or Ethanol* ***(f)*** *Red arrows indicate thick actin bundles extended from the nucleous to the PM. White arrows point to F-actin spots (Scal bars: 10 µm).*

## *3.3. Analysis of F-actin turnover*

Cytoskeleton dynamics play an important role in cell morphogenesis, organelle trafficking and endocytosis. F-actin bundles are constantly remodelled and respond rapidly to cellular stresses (Li et al., 2015). F-actin undergoes a rapid turnover by which the

assembly of new actin subunits at the barbed ends is connected with the disassembly of the actin subunits at the pointed end. This determines the length of the final actin filament.

In order to study the F-actin turnover in CK2 mutant seedlings, the drug jasplakinolide (Bubb et al., 2000) was used. Jasplakinolide induces spontaneous nucleation of G-actin monomers and polymerisation and stabilization of F-actin filaments, and inhibits filament disassembly. GFP-FABD2 seedlings were treated both with TBB to eliminate CK2 activity and with jasplakinolide to promote actin polymerisation, and single optical sections were collected by confocal microscopy from the root cap, the transition zone and the elongation zone.

As shown in Figure 19a, treatments with TBB promote actin depolymerisation in the root cap, which is partially prevented by the jasplakinolide treatment. The diffuse and patchy pattern of GFP-FABD2 signal in optical sections of the root cap, at middle plane of the columella, is typical of the GFP-FABD2 line itself, as it was already found by Voigt et al. (2005).

In the transition zone, cells treated with TBB lost the rounded form of the nucleus and exhibited thicker F-actin bundles (white arrows) (Figure 19b) that accumulate in the plasma membrane. Along the plasma membrane, several individual spots are visible (red arrows), probably generated by the overlapping of F-actin bundles, generated by the loss of their tidy parallel orientation. The cell walls between adjacent cells (also called cross-walls) are known to be actin-enriched domain (Baluška et al., 2003), but in seedlings in which CK2 function was depleted by TBB, this actin polarity is lost. This is showed by the decreased F-actin bundles signal in the cross-walls (yellow asterisks) in Figure 19b. It has been reported the existence of putative actin

filament organizing centers in plant cells (AFOCs), that consist of distinct domains localized at the nuclear envelope and at the plasma membrane and act as support for actin filament assembly (Staiger et al., 2000). In contrast to the TBB treatment, jasplakinolide leads to stabilization of F-actin bundles around the nuclear envelope, where these centers (AFOCs) are localized and where it is possible to find more actin dynamicity (Figure 19b blue arrows, Jasplakinolide). In fact in cells of the transition zone, actin filament (AF) bundles develop to participate in the onset of rapid cell elongation. These AF bundles are initiated at the nuclear peripheries and are anchored to the cell walls. This AF dynamicity has been shown to be essential for the cell elongation and for root hair formation (Baluška et al., 2003).

The simultaneous treatment with jasplakinolide and TBB showed that the strong actin accumulation around the nucleus was not present, although disorganized and patchy actin filaments were still evident and the shape of the nucleus and its central position were not lost (Figure 19b Jaspl + TBB). To the contrary, pre-treatments with TBB prior to addition of jasplakinolide, revealed a strong depolymerisation of actin (white arrows), indicating that the effects of TBB cannot be reversed by jasplakinolide treatment (Figure 19b 2 h Jaspl + 1 h TBB). Similar results were found by analysing the cells of the elongation zone. These results suggest that CK2 acts as a regulator of F-actin filament assembly, modulating the F-actin turnover and the cell cytoskeleton organization.

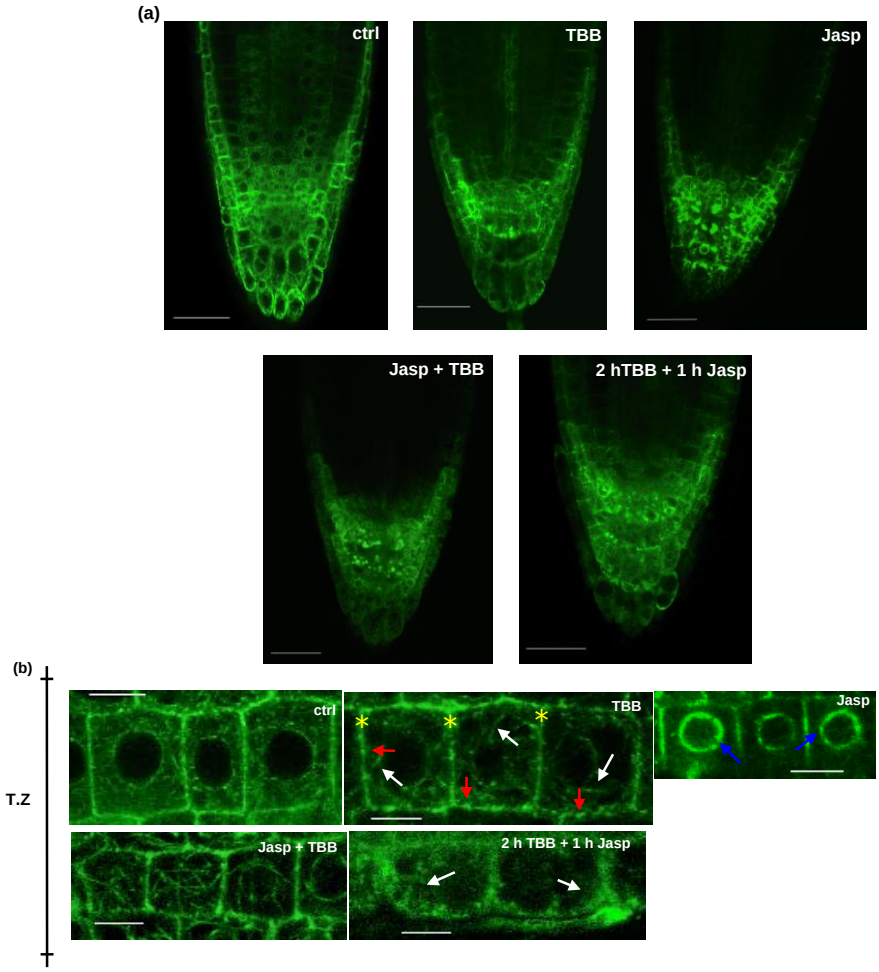
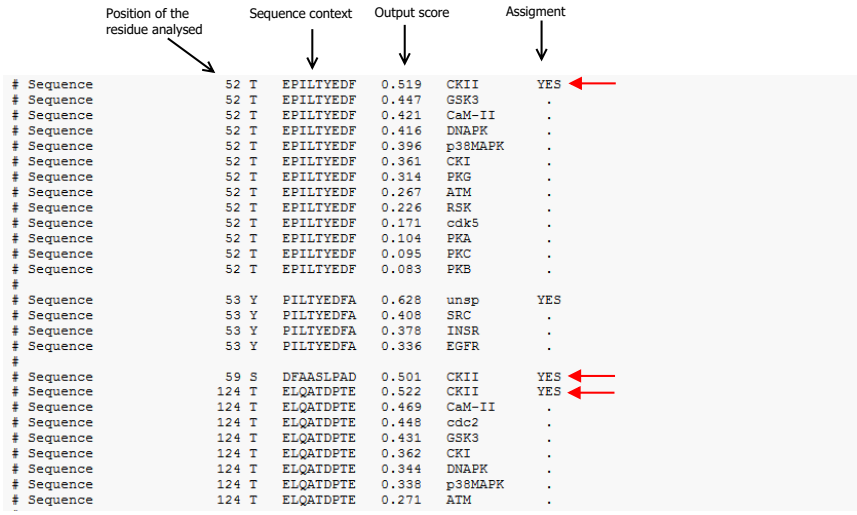



**Figure 19. F-actin dynamics in *GFP-FABD2* seedlings without CK2 activity.**

*Confocal sections of six-days-old GFP-FABD2 Arabidopsis seedlings. Six-days-old seedlings were submerged in MS liquid solution containing 100 µM of TBB for 60 minutes (**TBB**); 2 µM of Jasplakinolide for 60 min (**Jasp**); 100 µM of TBB and 2 µM of Jasplakinolide for 60 minutes (**Jasp+TBB**); 50 µM of TBB for 120 minutes and 2 µM of Jasplakinolide for 60 minutes added just at the end of the first 60 minutes of TBB treatment (**2 h TBB + 1 h Jasp**). (Scal bars: 30 µm) **(a)**. Higher magnifications of single cells from transition zone of the same GFP-FABD2 seedlings in (a). White arrows in (TBB) indicate thick F-actin bundle, red arrows point to spots in PM and yellow asterisks indicate the decreased F-actin bundles signal in the cross-wall. Blue arrows in (Jasp) point to the stabilized F-actin bundles around the nuclear envelope. White arrows in (2 h TBB + 1 h Jasp) indicate strong actin depolymerisation. (Scal bars: 10 µm) **(b)**.*

### *3.4. ADF4 as a possible CK2 substrate*

An important role in the stochastic dynamic of actin turnover is played by severing component proteins, like ADF or villin. The actin depolymerising factor (ADF) family is considered to be a central regulator of actin filament turnover in eukaryotes (Van Troys et al., 2008). It was found that protein casein kinase 1- like protein 2 of *Arabidopsis thaliana* (CKL2), which is a member of casein kinase 1 protein family localized to the cytoplasm and nucleus, physically interacts with and phosphorylates actin depolymerizing factor 4 (ADF4), and inhibits its activity in F-actin filament disassembly (Zhao et al., 2016). In order to identify whether ADF4 could be a potential substrate for Arabidopsis protein kinase CK2, the ADF4 amino acid sequence was analyzed using NetPhos 3.1 software. As shown in Figure 20, three predicted sites of potential phosphorylation sites have been identified, S-59, Thr-52, and Thr-124. From the results obtained, the confidence for these phosphorylation sites is not very high, thus further studies are needed to confirm which of these sites are phosphorilated by CK2 protein. These results suggest that CK2 could interact with ADF4, as a potential substrate and therefore be involved in the regulation of actin filament organization.



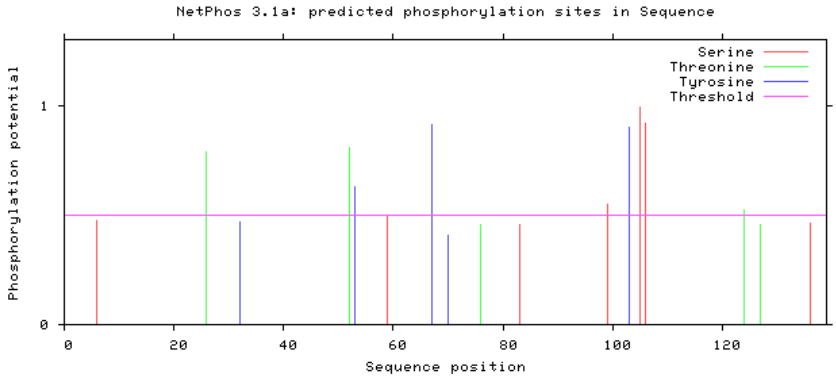




**Figure 20. Predicted phosphorylation sites for ADF4.**

*Representation of prediction of putative CK2 phosphorylation sites (Ser and Thr residues) red and green columns. Score output is a value in the range [0.000-1.000]. The threshold correspond to (0.500) and indicates that the confidence for this site being a true phosphorylation site is low, (the higher the score, the higher the confidence of the prediction).*

## *3.5. Impairments in microtubule organization*

Microtubules play a relevant role in the deposition of cellulose microfibrils into the cell wall and in guiding movement of cellulose

synthase (CESA) enzyme complex in the plasma membrane (Paredez et al., 2006).

To better characterize the role of CK2 in the cytoskeleton structure we also decided to study the microtubule organization in plants depleted of CK2 activity. *In vivo* visualization of microtubules was performed using GFP-MAP4 (Marc et al., 1927). In Figure 21a, control cells show well ordered, parallel array of epidermis microtubules Dramatic changes in the microtubule organization of root cap cells were visible only after 2 h of TBB treatment (Figure 21 b) and they were more pronounced after 3h (Figure 21c). After TBB treatments, most of the epidermis microtubules are destroyed and appear broken down within the cells. An overall fluorescent background and a high number of brightly fluorescent spots within these cells are displayed. Taken together, our results show that inactivation of CK2 activity produces significant changes in F-actin and in microtubules of root cells.

It has been shown that CK2 associates with the microtubules fraction in purified rat's brain, suggesting that CK2 modulate cytoskeletal assembly and dynamics through the phosphorylation of microtubule proteins, stabilizing and promoting the assembly of microtubules within neuronal process (Serrano et al., 1989). Therefore, we propose that CK2 could have a prominent role in the regulation of cytoskeleton dynamics, F-actin and microtubules assembly in cells, but further studies are necessary to better understand its role in the microtubule organization.

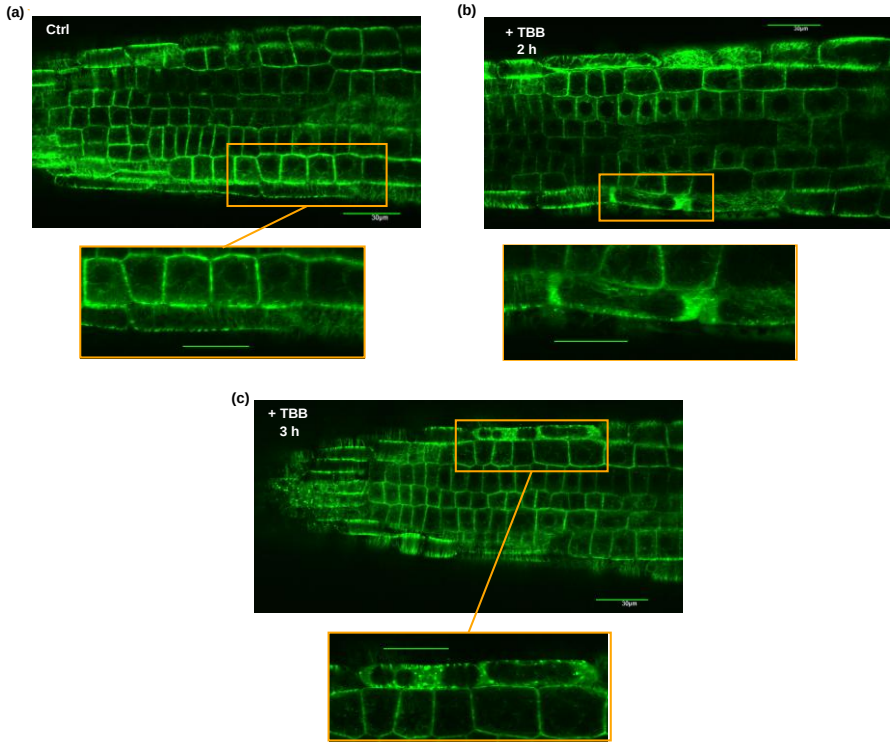



**Figure 21. Microtubule organization in GFP-MAP4 root cap cells.**

*In vivo visualization of microtubules in six-days-old GFP-MAP4 seedlings. Single confocal sections of the transition zone and the early elongation zone* ***(a)****. Six-days-old GFP-MAP4 seedlings after 2 h of 10 µM TBB treatment and magnification* ***(b)*** *Six-days-old GFP-MAP4 seedlings after 3 h of 10 µM TBB treatment and magnification* ***(c)****. (Scal bars: 30 µm).*

# Discussion

## 1. Analysis Of Root Phototropism In CK2-Defective Plants

Plants respond to differences in light quality, intensity and direction. Plant roots grow underground and mostly in darkness and they are very sensitive to light. They behave by bending away from light sources (a response that is called negative phototropism), in order to avoid photo damage and to facilitate water and nutrient absorption (Esmon et al., 2005; Monshausen and Gilroy, 2009).

In this work we studied the phototropic response of CK2mut seedlings, both in hypocotyls and roots. We measured and collected the values of the deviation angles, α, of the CK2mut line after 48h of irradiation with unilateral blue light. Our results revealed an enhanced root phototropic response in CK2mut line and a loss of positive phototropic response of the hypocotyls. The latter data confirmed previous observations obtained by other members of the laboratory (Marquès-Bueno et al., 2011a). Together these results indicate that CK2 activity is involved in the modulation of both the positive and the negative phototrophic responses.

The classical Cholodny-Went theory (Went, 1974) asserts that tropic responses are due to asymmetric distribution of the growth

regulator auxin. For long time, the synthetic transcriptional reporter DR5rev:GFP has been used to monitor intracellular auxin accumulation. DR5rev:GFP is a construct containing a synthetic auxin response element (DR5rev) coupled to the reporter gene GFP (Petersson et al., 2009). The auxin-responsive promoter responds to changes in IAA concentrations and, consequently, the GFP green signal increases or decreases in correspondence to the auxin levels.

Several studies that used the auxin response reporter DR5-GFP have demonstrated that an increase of auxin at the shaded side is essential for hypocotyl phototropism. Thus, application of unilateral light induces auxin accumulation at the shaded side of the hypocotyls, inducing cell expansion and causing plant bending towards the light source (Fankhauser and Christie, 2015; Liscum et al., 2014). In contrast, in the negative phototropic response unilateral blue light induces a higher auxin concentration in the illuminated side of roots exposed to light (Naundorf, 1940; Zhang et al., 2014, 2013). Despite this, until nowadays little is known about the negative phototropic mechanism and there are different conflicting theories.

Thus, we wanted to deepen this topic by studying auxin redistribution through CK2mut roots after their illumination with unilateral blue light. We used a novel auxin sensor, VENUS-DII-YFP, (Brunoud et al., 2012), which give more accurate results for the dynamic changes of auxin concentration. We found decreased YFP fluorescent signal at the illuminated side of the root, which corresponds to increased auxin accumulation. This result corroborates previous studies by other authors (Zhang et al., 2014, 2013) and suggests that auxin accumulation at the irradiated side of the root promotes growth and causes roots bending away from the light (Zhang et al., 2013). We then investigated the auxin distribution in

VENUS-DII-YFP line after CK2 depletion by TBB treatments, in dark and in light conditions. In dark conditions, our data show a clear decrease of fluorescence in the upper part of the root, which corresponds to the transition zone, indicating the presence of higher auxin concentrations in this part of the root. This result confirms previous findings obtained in our laboratory using DR5rev::GFP (Marquès-Bueno et al., 2011a). In light conditions (unilateral blue light), we found a dramatic decrease of the fluorescence signal through the whole root, suggesting impaired auxin distribution. This result gives us further confirmation of the impaired auxin transport in CK2 depleted seedlings, already highlighted in our laboratory (Marquès-Bueno et al., 2011a). Previous studies in the laboratory of Dra. Martínez showed that four out of five genes encoding PM-resident members of the PIN family were significantly misexpressed in CK2-defective plants. Among them, PIN4 and PIN7 were highly overexpressed and they appeared less polarized at the PM and partially internalized in endosome-like particles. Also genes encoding regulators of PIN proteins, like PID, were overexpressed in CK2mut roots (Marquès-Bueno et al., 2011a). Thus, since CK2mut is impaired in auxin transport, it is reasonable to presume that asymmetric auxin distribution, triggered by the light stimuli, will be also affected. However, our data do not show a clear auxin gradient in CK2 depleted seedlings after blue light irradiation and unfortunately they cannot explain the higher negative phototropism of the CK2mut roots. Interestingly, a wavy phenotype mutant, *wavy growth 2* (*wav2*), characterized by Mochizuki et al. (2005), was found to share some of the characteristics of the CK2mut line. The roots of both mutants exhibit a wavy phenotype and bend with larger curvature than the wild-type roots during the gravitropic and phototropic responses.

These three phenotypical characteristics, which occur together in *wav2* and CK2mut mutants, might have a common cause that has not yet been identified.

Then, we wanted to study the intracellular distribution of PIN2 in CK2mut roots, both in dark and in light conditions. In agreement with other authors (Kleine-Vehn et al., 2008c; Laxmi et al., 2008; Wan et al., 2012) we found that in control, dark-grown roots, PIN2-GFP was partially internalized in vacuole compartments ("dark status"), whereas in control, light-grown roots, PIN2-GFP was polar localized at the PM ("light status"). Interestingly, when CK2 activity was depleted by Dex-treatments, the shift between the light and the dark states was impaired and PIN2-GFP was localized at the PM in both cases, suggesting a constitutive "light status" in the CK2mut line. The light and dark states of PIN2 localisation are reversible and it has been hypothesized that NPH3 has an important role in this process (Wan et al., 2012). It has been hypothesized that NPH3 acts as a switch, changing PIN2 targeting by directing it to either the prevacuolar pathway or to the membrane recycling pathway.

Considering our data, we can hypothesize two possible mechanisms by which CK2 activity can regulate PIN2 distribution in the cell: 1) by regulating NPH3 activity, which has been hypothesized to work as a switch determining the equilibrium of PIN2 between PVC-targeting trafficking and membrane-targeting recycling; 2) by regulation of the retromeric complex activity, through the phosphorylation of protein substrates that are part of this complex.

The first hypothesis is based on previous discoveries made by members of our laboratory showing that various members of the

NPH3 family are CK2-interacting proteins (results not published). Moreover, the NPH3 protein sequences contain numerous acidic-based motifs that are predicted CK2 phosphorylation sites and, according to Arabidopsis phosphoproteome databases, several members of the NPH3-containing protein family are predicted to be phosphorylated (Benschop et al., 2007; Marquès-Bueno et al., 2011b). A possible function of CK2 in the regulation of the activity of the protein NPH3 was postulated (Marquès-Bueno et al., 2011b). It is reasonable to think that CK2 might regulate PIN2 steady-state localisation through NPH3. The absence of PIN2-GFP at the vacuole and its increased abundance at the plasma membrane of CK2mut roots in darkness, which represents the physiologic roots growth conditions, might explain the increased auxin accumulation at the transition zone of the roots. Since the light represents a stress condition for the roots and since many other auxin efflux carriers are involved in the light responses, the auxin distribution in CK2mut roots under light condition needs further studies.

The second hypothesis is based on the observation that mutants of the retromeric vacuolar protein sorting 29 (VPS29) show similar phenotypic traits as those of CK2mut seedlings. Both exhibit inhibition of secondary roots formation, reduced primary root length, impaired tropisms, altered auxin distribution and enlarged PIN-containing endosomes (Jaillais et al., 2007). Furthermore, the absence of PIN2 in PVCs in CK2mut seedlings favours the idea of CK2 as a regulator of the retromer recycling pathway.

## 1.1. New insights on negative phototropism

Despite the poor knowledge on root negative phototropism, it is already known that positive phototropism and negative phototropism share some of the components of the signalling pathway, as for example RPT2, NPH3, and PKS1, all of them working downstream of Phot1. However, more studies are necessary to completely understand the similarities and the differences in the signalling pathways of both responses. Our findings show that auxin redistribution in hypocotyls and roots is different under blue-light stimulus, which is in agreement with the results of other authors (Zhang et al., 2014, 2013). Moreover, auxin efflux carriers show different polar localisation in hypocotyls and roots under blue lateral light conditions. For example, PIN1 localisation in hypocotyl reduces auxin fluxes towards the root under light stimuli (Christie et al., 2011) whereas, in roots, relocalisation of PIN1 at the basal membrane in cells of the root stele after blue light stimuli increases auxin accumulation in the root tip, which results in asymmetric auxin distribution and negative phototropic response of the root (Zhang et al., 2014). To confirm this important role of PIN1, analysis in loss-of-function *pin1* mutants has been conducted, showing no response to unilateral blue light illumination (Zhang et al., 2014). Furthermore, it has been found that PIN1 recycling from endosomes is mediated by a BFA sensitive, GNOM-dependent trafficking pathway (Geldner et al., 2001; Muday et al., 2003) and by the activity of PID/PP2A (Ding et al., 2011). We already described in the introduction (see root phototropism and auxin transport) that also PIN3 shows different polar distribution in hypocotyls (Ding et al., 2011) and in roots (Zhang et al., 2013). On the basis of this evidence, it is possible to hypothesize that the mechanism of phototropic regulation is different in hypocotyls and

roots and that independent pathways regulate the two processes. This might explain the coexistence of loss of hypocotyl phototropic response with enhanced root negative phototropic response in CK2mut seedlings.

## 2. Role Of CK2 In The Crosstalk Between Salicylic Acid And Auxin

It was previously shown that Arabidopsis plants in which CK2 activity was inhibited exhibited shorter primary roots and impaired lateral root formation (Marquès-Bueno et al., 2011a). Moreover, CK2mut roots or roots from plants treated with TBB accumulated high levels of salicylic acid (SA) (Armengot et al., 2014). Based on studies with Arabidopsis mutants and with plants treated with exogenous SA, it was proposed that the peculiar root phenotype of CK2-defective plants was an SA-mediated effect (Armengot et al., 2014). The auxin signalling pathway is controlled by a family of nuclear proteins called AUX/IAA that represses the transcription of auxin-regulated genes. AUX/IAA proteins dimerise with auxin response factors (ARFs) preventing their binding to auxin-responsive promoter elements and thus they repress early auxin responses. The AUX/IAA repressors are short-living proteins and their stability is regulated by auxin, which promotes their ubiquitination and degradation via the proteosome (Gray et al., 2001). In order to verify if the auxin signalling pathway in CK2 depleted seedlings was repressed through the stabilization of the auxin repressors, an Arabidopsis line containing AXR3 (an AUX/IAA repressor) fused to GUS was used. Histochemical GUS staining has been performed on control HS::AXR3NT-GUS line and on the same line after TBB treatment. The results showed a higher GUS activity in TBB-treated seedlings, suggesting that the loss of CK2 activity was responsible for the AXR3 stabilization. Moreover, GUS activity did not decrease in TBB-treated HS::AXR3NT-GUS incubated with 2.4-D, indicating that CK2 activity is necessary for the IAA-induced AXR3

degradation. In addition, simultaneous incubation with MG132 and TBB potentiated the MG132 effect, suggesting that TBB and MG132 are acting on different steps of the degradation pathway. The above results were confirmed by quantification analysis.

This suggests that CK2 controls the stabilization of the auxin repressors and that CK2 has an important role as a positive regulator of auxin signalling pathways, indeed its activity is required for the degradation of the auxin repressors. Our next objective was to investigate how CK2 activity controls the stability of auxin transcriptional repressor AXR3. We hypothesized that this could be the consequence of two different mechanisms: 1) by direct post translational modifications on the repressor AXR, and 2) indirectly, through controlling salicylic acid (SA) intracellular levels.

SA has an important role in plant defence against biotrophic and hemi-biotrophic pathogens, inducing the immune system (Fu and Dong, 2013). SA is perceived by the plants through nonexpressor of pathogenesis related protein 1 (NPR1) and its paralogues NPR3 and NPR4 (Fu et al., 2012; Wu et al., 2012). At low SA levels, NPR1 is unable to induce the defence genes since it is targeted through its binding to NPR4 for degradation by the proteosome. As SA concentration increases after infection, SA binds to NPR4 disrupting its interaction with NPR1, and free NPR1 can play its role in the defence gene activation (Fu et al., 2012). At very high concentrations, SA binds to NPR3 and promote its interaction with NPR1, controlling NPR1 turnover (Moreau et al., 2012). Previous studies showed that treating Arabidopsis seedlings with analogues of SA resulted in the repression of genes involved in auxin signalling and auxin biosynthesis and that genes encoding enzymes that bind free IAA were upregulated (Wang et al., 2007). It was also found that SA

represses the expression of the TIR1/ABF F-box receptor complex. The F-box protein TIR1 is a component of an E3 SCF ubiquitin-ligase complex that serves as the auxin receptor and directs the ubiquitin-dependent degradation of repressors of auxin signalling proteins. This leads to the stabilization of auxin repressor proteins of the AUX/IAA family and represses auxin signalling and its responses (Wang et al., 2007). We have already largely discussed about the important role of auxin in plant cellular developmental programmes and how the disruption of auxin signalling influences negatively the normal plant development (Grones and Friml, 2015). Transgenic overexpression of the Arabidopsis gene *GH3.5* (which encodes an enzyme that conjugates amino acids to IAA) showed increased resistance to infection with *P. syringae* and dwarf phenotype (Zhang et al., 2008). Furthermore, *gh3.5* knockout mutants are susceptible to infections and are similar to the SA-deficient mutant line (Huot et al., 2014). However, in the absence of infection, auxin mediated suppression of SA responses. Reciprocally, SA-mediated defences are attenuated by auxin. It has been previously reported from members of our laboratory that the inhibition of CK2 activity in Arabidopsis had important effects on auxin signalling, in particular in PAT (Marquès-Bueno et al., 2011a). It was found that CK2-loss-of-function has enhanced SA levels and that WT plants incubated with exogenous SA and Arabidopsis *cpr* mutants (which contain high SA level) show the same root phenotype of the CK2mut seedlings. Furthermore, SA defective mutants or SA signalling mutants with a pharmacological partial depletion of CK2 activity (*sid2, NahG, npr1*) showed a less severe root phenotype (Armengot et al., 2014). Here, we wanted to know if the same SA was also responsible for the increase of the auxin repressor (AXR3) stabilization. For this purpose, we used the SA deficient

Arabidopsis mutant (NahG) that overexpresses a bacterial salicylate hydroxylase that catalyzes the conversion of SA to catechol (Delaney et al., 1994) and is not able to accumulate SA. Our results showed decreased GUS signal in HS::AXR3NT-GUS x NahG line after TBB treatment, suggesting that salicylic acid accumulation in the root is required for the auxin AXR3 repressor stabilization. We can finally link the down-regulation of auxin signalling pathways in CK2 defective plants with their high SA content.

Our results corroborate that auxin and SA pathways are tightly connected to each other and act antagonistically. Several components of the SA signalling pathway, such as NPR1 (coactivator of the immunity response) are targeted for degradation via the proteosome in SA dependent manner. After pathogen infection and the increase of SA level in plants, the NPR1 in its monomeric form migrates to the nucleus where it works as a coactivator for plant resistance. From our results, it is evident that the CK2-defective line treated with the proteosome inhibitor (MG132) showed a higher stability of the auxin repressor compared to the control line after MG132 treatment. In this circumstance, we might also hypothesize that a proteosome inhibition by MG132 not only inhibits auxin signalling by blocking degradation of auxin repressors but that might may also activate the SA signalling pathway by increasing the NPR1 half-life. However, it is known, as already mentioned, that NPR1 activity depends on SA accumulation (Moreau et al., 2012). Thus, we might also suppose a more general role for CK2 as a regulator of protein degradation via 26S-proteosome.

In conclusion, here we show that under conditions of CK2 inhibition, the auxin signalling pathway is repressed by the enhanced

stability of the AXR3 auxin repressor. This stabilization is due to the high accumulation of endogenous SA levels. However, we cannot discard the hypothesis that CK2 might also function as a regulator of protein degradation by the proteosome, and for this further studies need to be done.

## 3. Functional Involvement of CK2 Activity In Actin Cytoskeleton Structure

Previous analysis of the phenotype of a loss-of-function mutant of protein kinase CK2 revealed a strong connection between CK2 activity and auxin polar transport (PAT). Actin cytoskeleton is functionally interconnected with PAT, since it is required for delivering the auxin transporters vesicles to their final destination. For this reason, we focused our study on the investigation of the role of CK2 in actin cytoskeleton.

*In vivo* confocal imaging enabled us to characterize the actin architecture of CK2 depleted seedlings. Our analyses clearly show that the structure of the actin network is strongly affected in seedlings after inhibition of CK2 activity. Several data from TBB-treated roots support this conclusion: 1) actin distribution in both cortical and epidermal cells of the transition zone is totally lost; 2) F-actin bundles are collapsed around the nucleus; 3) The central nucleus position is lost and nuclei are shifted toward the cellular corners; 4) the shape of the cells is heavily deformed. Furthermore, histochemical immunolabelling assays using antibodies against actin show a strong actin depolymerisation after TBB treatment, in agreement with our previous results. Importantly, the CK2 mutant line also exhibits irregularities in the distribution of actin bundles after treatments with Dex to induce the transgene. Taken together, these results strongly suggest that CK2 activity is required for the correct actin polymerisation and distribution. However, how CK2 regulates actin polymerisation is not yet known, but there is an interesting clue that suggests that CK2 might regulate ADF4, since its amino acid

sequence contains predicted CK2 phosphorylation sites. Interestingly, previous studies conducted in our laboratory (Marquès-Bueno et al., 2011a), also corroborated in this work (see results *1.2 CK2mut plants show impaired auxin redistribution*), showed increased auxin accumulation in the upper part of the root cap of CK2mut seedlings, at the level of the transition zone. It has been previously reported that auxin induces actin cytoskeleton reorganization, inhibiting the cytoplasmic streaming at high concentration (Ayling et al., 1994; Li et al., 2014a). Thus, on the basis of these considerations, we can speculate that a higher auxin distribution of CK2mut line enhances the actin unbundling in the transition zone of the root.

Turnover of actin filaments is important for cellular morphogenesis and cell expansion (Higaki et al., 2010; Smith and Oppenheimer, 2005). Live-cell imaging techniques have allowed us to study actin turnover in Arabidopsis seedlings, using jasplakinolide as a potent inducer of actin polymerisation (Staiger et al., 2009) and TBB to inhibit CK2 activity. Here, we show that the lack of CK2 activity strongly affects actin filaments polymerisation and their polar distribution. We also show that by simultaneous treatment with TBB and jasplakinolide, actin filaments become stabilized all around the nuclear envelope, maintaining the roundish (normal) nuclear shape and its central position. Previous authors have already reported the existence of putative actin filament organizing centers (AFOCs) in plant cells (Staiger et al., 2000). Actin filament bundles are anchored by one end laterally to the nuclear surfaces and by the other end to myosin VIII in cross-walls (cell walls between adjacent cells), supporting actin filaments assembly and giving them specific polarity (Staiger et al., 2000). Our results suggest that protein kinase CK2 regulates the assembly of the actin filaments at the nuclear membrane. How CK2

activity can modulate this process is an interesting question that remains to be answered. The rapid F-actin turnover and the actin filaments rearrangements are regulated by the action of actin binding proteins (ABPs) (Wang et al., 2015; Wasteneys and Galway, 2003). Among them, ADF proteins promote severing and depolymerisation of the actin filaments (Andrianantoandro and Pollard, 2006). The Arabidopsis genome encodes 11 ADF proteins. Knock out mutants for Arabidopsis ADF4 showed longer hypocotyls and more bundled cytoskeletal arrays than the wild-type. Actin bundling is an array of linear actin filaments cross-linked by actin bundling proteins, and its assembly and dynamics are not as well understood. It has been found that Arabidopsis *ADF1* antisense plant show excessively bundled arrays in hypocotyls (Dong et al., 2001), and that inducible RNA interference lines for *ADF2* resulted in dense cytoskeleton arrays with evident bundles (Clément et al., 2009). The actin filaments bundling is an indirect effect of ADF4. It has been hypothesized that in *adf4* mutants the reduced severing activity of ADF4, increased the F-actin length and lifetimes. Since these cells showed increasing in bundling but also less dense cytoskeletal arrays, it has been proposed that there was no polymer available and there was more time for individual actin filament to make contacts with adjacent filaments (Henty-Ridilla et al., 2014). This proves that ADF4 with its severing activity modulates the stochastic turnover behaviour (Henty et al., 2011). Interestingly, CK2mut line showed shorter hypocotyls compared with WT, indicating a change in actin architecture and suggesting the existence of an higher severing activity of ADF4 in CK2mutant.

There are also recent evidences that inhibition of the ADF4 activity regulates actin dynamics during the innate immune signalling, since the ADF4 activity is negatively regulated during plant immune

response (Henty-Ridilla et al., 2014). An interesting recent study shows that the Arabidopsis casein Kinase 1-like protein 2 (CKL2) regulates actin filament stability and its reorganization during stomatal closure by physically interacting and phosphorylating ADF4 (Zhao et al., 2016). In our study we found some putative predicted phosphorylation sites for CK2 protein in ADF4. Based on our results we can speculate that CK2 might be involved in the regulation of ADF4 activity, probably in the AFOCs around the nuclear envelope where the actin filaments are stabilized. Because inhibition of CK2 leads to strong disruption of actin filament network and of microtubules, which affect both cellular shape and plant development, we propose that CK2 modulates actin assembly. Further studies are crucial to better clarify this process and for the understanding of the role of CK2 in cytoskeleton organization.

# Conclusions

1. CK2-defective seedlings showed enhanced negative phototropism in roots and loss of positive phototropism in hypocotyls, concluding that CK2 activity is required for normal phototropisms.
2. Unilateral blue light illumination triggered auxin redistribution in Arabidopsis roots, and we detected increased auxin levels at the illuminated side of the roots. This is the opposite to what has been reported for positive shoot phototropism, suggesting that independent pathways might regulate these two responses.
3. Depletion of CK2 activity resulted in impaired auxin distribution in response to blue-light stimulus, and a dramatic increase of auxin through the root was detected.
4. CK2 activity is required for the regulation of the dynamic equilibrium of PIN2 localisation between the plasma membrane and the vacuole, which is normally regulated by light and is a part of the cellular response linked to phototropisms. Inhibition of CK2 activity resulted in the stabilization of the AXR3 protein, an auxin repressor of the AUX/IAA family. This stabilization was overcome in transgenic plants unable to accumulate salicylic acid. These results reveal that the auxin-related phenotypes of CK2-defective plants are likely due to the

repression of auxin-signalling, caused by the overstability of auxin repressors, and mediated by the high salicylic acid levels of those plants.

5. CK2-depleted seedlings showed strong disorganization of the actin network. The main affected characteristics were collapse of F-actin bundles, loss of the central nucleus position within the cell, and deformation of cell's shape.
6. Studies of actin turnover in Arabidopsis seedlings confirmed that the lack of CK2 activity strongly affects polymerisation of the actin filaments and their polar distribution. Putative predicted phosphorylation sites specific of CK2 activity were found in ADF4, suggesting that CK2 might be involved in the regulation of ADF4 activity, probably at the AFOCs around the nuclear envelope. Interestingly, CK2mut line show shorter hypocotyls as compared to wild-type plants, which might be caused by defects of cell expansion due to changes of actin architecture in the whole seedling.

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