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TITLE: Functional interplay between protein kinase CK2 and salicylic acid sustains *PIN* transcriptional expression and root development

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SUMMARY (243 words)

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

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INTRODUCTION (1232 words)

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

CK2 subunits are encoded by multigene families, which in plants include more members than in mammals and yeast. For instance, the *Arabidopsis thaliana* genome contains four genes encoding the CK2α subunit (one of them with predicted chloroplast localization), and four genes encoding the CK2β subunit (Salinas *et al.*, 2006). Small gene families have been also reported in other plant species (Riera *et al.*, 2001; Espunya *et al.*, 2005; Salinas *et al.*, 2006). Antisense expression of a CK2α-encoding gene revealed some negative effects on light-regulated responses (Lee, et al., 1999), and generation of an Arabidopsis CK2α1α2α3 triple mutant resulted in late flowering, reduced

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hypocotyl growth, smaller cotyledon size, reduced number of lateral roots, and ABA-signalling defects (Mulekar *et al.*, 2012). A stronger impact on plant development was obtained by construction of a CK2 dominant negative mutant. This mutant was generated by conditional overexpression of a CK2α-inactive subunit in Arabidopsis (Moreno-Romero *et al.*, 2008). Long-term induction of the transgene was lethal, confirming that CK2 activity is essential for cell survival. Short-time induction, however, resulted in phenotypes similar to those exhibited by auxin-defective mutants, affecting cell expansion, gravitropism, phototropism, and lateral root formation. The authors demonstrated that auxin transport was partially impaired in this mutant and that most of *PIN*-formed (PIN) genes, encoding auxin-efflux transporters, were misregulated (Marques-Bueno *et al.*, 2011a; Marques-Bueno *et al.*, 2011b). Moreover, some of the PIN transporters showed a tendency to be found internalized in endosome-like particles.

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

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regulates clathrin-mediated trafficking and auxin signaling has been recently proposed (Wang *et al.*, 2013).

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

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

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rapid turnover of NPR1 in the nucleus is essential to promote gene transcription; this is achieved by phosphorylation and ubiquitination, which targets NPR1 to the proteosome (Spoel *et al.*, 2009). Recent results suggest that two adaptor proteins, NPR3 and NPR4, which are SA receptors with different affinities, also bind NPR1 and might be involved in the regulation of NPR1 stability (Zhang *et al.*, 2006; Fu and Dong, 2013). NPR1 also directly activates the expression of the plant-specific WRKY family of transcription factors, which might act as either transcriptional activators or repressors (Wang *et al.*, 2006). NPR1 expression is itself under the regulation of the WRKY factors (Yu *et al.*, 2001). WRKY factors bind to DNA sequences called W-boxes that have been implicated in plant defence responses to pathogens. Moreover, the gene promoter of isochorismate synthase, an enzyme involved in SA biosynthesis, is enriched with W boxes (Wildermuth *et al.*, 2001). In a transcriptional profiling study, Maleck *et al.* (2000) discovered that W boxes are overrepresented in a cluster of genes sharing the induction pattern of *PR-1*, suggesting a role for WRKY factors in the systemic acquired resistance (SAR).

On the other hand, the transcriptional responses to auxin are driven by the well-characterized Auxin Response Factors (ARFs) that bind the Auxin Response Elements (AuxREs). However, promoters of the auxin-regulated genes in Arabidopsis and rice show a high occurrence of b-ZIP and MYB-responsive elements located close to AREs (Berendzen *et al.*, 2012). This observation led to propose that these two families of TFs might act as modulators of the auxin-elicited transcriptional responses. The b-ZIP family of TFs is composed of 162 elements in Arabidopsis, which have been classified in different subfamilies

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according to their structural features, and the Arabidopsis MYB family is composed of 198 genes. Both families of TFs control responses to light, biotic and abiotic stress and plant development, among other biological processes (Jakoby *et al.*, 2002; Bailey *et al.*, 2003; Yanhui *et al.*, 2006).

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

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RESULTS (2861 words)

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

The CK2α-encoding sequence was amplified by PCR using *NtCKA3* cDNA as a template (GenBank/EMBL bank accession no. AJ438263). For immunodetection purposes, a *c*-myc-encoding epitope was introduced at the N-terminal end of the coding sequence. The construct was cloned into a binary vector and used to transform Arabidopsis plants by means of *Agrobacterium tumefaciens.* Several independent transgenic lines were isolated and brought to homozygosis using hygromycin as a selection factor. Expression of the CK2α transgene was confirmed by RT-PCR using specific primers (one of them corresponding to the *c*-myc-encoding region and the other to the CKA3-encoding sequence). We also performed quantitative RT-PCR reactions to amplify separately the transcripts of *AtCK2αA* and *AtCK2αB* genes (the two CK2α-encoding genes predominantly expressed in Arabidopsis, Moreno-Romero *et al.*, 2011) and of *CKA3*, in order to compare the total *CK2α* transcript levels in WT and transgenic plants. The results are shown in Figure 1b. As *CKA3* is only expressed in CK2^{OE} transgenic plants and *CK2αA* and *CK2αB* are similarly expressed in WT and transgenic plants we conclude that the total amount of transcripts encoding the CK2α subunit is higher in CK2^{OE} transgenic plants. Moreover, accumulation of the CKA3 transgenic protein was detected using a *c*-myc antibody (Figure 1c) and measurement of CK2 activity in whole-cell extracts incubated with radiolabelled ATP and with a CK2-specific peptide

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(see Experimental procedures) revealed CK2 activity increments ranging from 5 to 36 % in CK2^{OE} lines, compared to wild-type plants (Figure 1d).

We then to investigated whether CK2α overexpression (CK2^{OE}) produced significant phenotypic changes in Arabidopsis roots. Figure 2a shows that the growth rate of primary root was slightly but significantly enhanced in the different CK2^{OE} lines (Figure 2a). We also found that those lines exhibited an increment in the number of lateral roots (LRs) (Figure 2b-c, and Figure S1a).To further investigate the effect of CK2α overexpression in LR formation we performed a detailed study in one of the lines showing the highest increment in CK2 activity (line 3). We distinguished between lateral root primordia (LRP) and emerged roots (according to the classification in Peret *et al.*, 2009). Figure 2b shows that CK2^{OE} plants had more emerged lateral roots but less LRPs than wild-type (WT) plants. These results suggest that lateral roots arise earlier in CK2^{OE} plants. To check this hypothesis, we quantified the number of LRPs in younger seedlings (5-day-old); we found an average of 4 LRP and 8 LRP in WT and CK2^{OE} seedlings, respectively (Figure 2c). Moreover, lateral root density (number of lateral roots per mm) was higher in CK2^{OE} seedlings (Figure S1b). Meristem size, however, was similar in CK2^{OE} and WT seedlings (Figure S1c), as well as auxin distribution, as measured by the expression of *DR5::GFP* reporter in CK2^{OE} x *DR5::GFP* seedlings (F3 generation) (Figure S1d). On the other hand, CK2^{OE} seedlings showed normal gravitropic response (not distinguisble from that of WT plants) (Figure S1e). Additional pictures of CK2^{OE} root phenotypes are shown in Figure S2.

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

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

We have previously shown that CK2-defective plants (CK2mut plants) were impaired in auxin polar transport, although the content of indole-3-acetic acid (IAA) was unchanged in CK2mut seedlings (Marques-Bueno *et al.*, 2011a). We now determined IAA and SA content in excised roots of WT, CK2mut and CK2^{OE} lines. Our results show similar IAA levels in CK2^{OE}, CK2mut (+/-Dex) and WT roots (Figure 3a). However, CK2mut roots exhibited a spectacular increase in salicylic acid (SA) content, as compared to WT and CK2^{OE} roots. This high SA content was only detected after induction with dexamethasone, indicating that it was a consequence of *CK2mut* transgene expression (Figure 3a). It has been recently reported that auxin and SA counteract during the adaptative response to stress (Wang *et al.*, 2007; Iglesias *et al.*, 2011), and thus we wondered whether the previously described IAA-related phenotypes of CK2mut plants were a consequence of their elevated SA levels. To check this hypothesis, Arabidopsis WT plants were incubated with SA. Figure 3b shows that exogenous SA triggers inhibition of both root length and lateral root

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formation, phenocopying the morphological characteristics of CK2mut roots (Marques-Bueno *et al.*, 2011a). We then crossed CK2mut plants with the SA-deficient *sid2* mutant. *sid2* is a loss-of function mutant of the isochorismate synthase 1 (ICS1) (Wildermuth *et al.*, 2001), an enzyme of the shikimate pathway, involved in SA biosynthesis in plants. We isolated homozygous lines for both mutations (CK2mut x *sid2* double mutant, F3 generation), and we obtained a partial but significant recovery of the WT root phenotype (Figure 3c). The recovery of the root length was small but statistically significant. Moreover, the recovery of the number of lateral roots was clearly visible and statistically significant in CK2mut x sid2 double mutant. Quantification of SA in the CK2mut x *sid2* double mutant revealed that DEX-mediated induction of *CK2mut* transgene still increased SA levels in the *sid2* background (Figure 3a), which agrees with the partial but not complete recovery of the WT root phenotypes.

To corroborate the idea that the CK2mut root phenotypes are a direct consequence of the elevated SA levels in the mutant, we used additional mutants affected in either SA content or SA-signalling. The *CONSTITUTIVE EXPRESSER OF PR1* mutants *(cpr1, cpr5 and cpr6* mutants*)* show high levels of SA (Clarke *et al.*, 2000). We measured the primary root length and the number of lateral roots in *cpr1, cpr5 and cpr6* and we found that they exhibited shorter roots and fewer lateral roots, with the only exception of the number of lateral roots in *cpr1*, which was similar to that in WT plants (Figure 4a). We also used two Arabidopsis mutants that are impaired in SA-mediated effects, the SA-defective *NahG* mutant (expressing bacterial SA hydroxylase, Delaney *et al.*, 1994), and the *npr1-1* mutant *(NONEXPRESSER OF PATHOGENESIS-*

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RELATED PROTEIN1), which is impaired in SA-signalling (Durrant and Dong, 2004)**.** *NahG* and *npr1-1* mutants were incubated with 4,5,6,7-tetrabromobenzotriazole (TBB), a strong inhibitor of CK2 (Shugar, 1994) that has been previously used by us in combination with the CK2 mutant (Moreno-Romero *et al.*, 2008; Marques-Bueno *et al.*, 2011a; Moreno-Romero *et al.*, 2012). We first measured IAA and SA levels in WT plants incubated with TBB. Figure 4b (left panel) shows that IAA levels were unchanged, whereas SA levels increased significantly, as it happened in Dex-treated CK2mut plants. Moreover, incubation of the SA-defective *NahG* plants with TBB produced a slight increase of SA, but to a much less extent than in WT plants (Figure 4b, right panel). The effect of TBB on the number of lateral roots is shown in Figure 4c. TBB-treated WT plants show a significant decrease of lateral roots (number of LRs in TBB-treated plants versus that in control plants: 0.48), whereas TBB-treated *NahG* or *npr1-1* mutants show a higher ratio of LRs in TBB-treated versus control plants (0.74 and 0.71, respectively). The experiments were performed with a high number of individuals (≥40) and the statistical analyses show significant differences between genotypes.

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

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Interplay between CK2 activity and SA-triggered transcriptional responses

We have previously reported that the basic machinery for polar auxin transport (PIN protein family and protein kinase PINOID) was misregulated in CK2mut plants (Marques-Bueno *et al.*, 2011a). To study the contribution of SA, if any, to this misregulation, we performed a time-course study of *PIN* and *PID* expression in Arabidosis WT plants incubated with 0.25 mM salicylic acid (Figure 5a). Transcript levels were measured in roots by quantitative RT-PCR. Our results show that exogenous SA down-regulates *PIN1*, *PIN4* and *PIN7* and up-regulates *PID*, and that those effects remained for as long as 48H. *PIN2* and *PIN3* showed a bimodal response to SA, with transient up-regulation at the beginning of the treatment (Figure 5a). Moreover, a time-course study of *PIN/PID* expression in Dex-treated CK2mut roots revealed that *PIN2, PIN4* and *PIN7* were up-regulated in CK2mut plants, in spite of the elevated SA content of this mutant. On the other hand, *PIN1* and *PID* expression showed similar responses in CK2mut or WT + SA plants (down-regulation for *PIN1* and up-regulation for *PID*) (Figure 5b).

To further study the influence of SA content and CK2 activity on *PIN* and *PID* expression, we used the SA-defective *sid2* mutant and the CK2mutx*sid2* double mutant. In an independent experiment, *PIN* and *PID* transcript levels were measured in all the conditions and lines shown in Table I. The results are shown as fold changes of gene expression (in Dex-treated versus untreated roots for CK2mut, *sid2* x CK2mut and *sid2*; versus WT roots for WT+SA and CK2^{OE}). Dex inductions and SA treatments were carried out for 48 H in these experiments. Interestingly, our data show that *PIN1* and *PIN3* fold-changes

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were similar in CK2mut roots and SA-treated plants, and showed a tendency to increase in the CK2mut x *sid2* double mutant (which contains less amounts of SA) (statistical analyses of these data, using the Students' *t*-test can be seen in Table S1). These results strongly suggest that SA is sufficient to repress *PIN1* and *PIN3* expression, and that *PIN1* and *PIN3* down-regulation in CK2mut roots is a consequence of the high SA content in this mutant. To the contrary, *PIN4* and *PIN7,* which were also repressed by exogenous SA, appeared strongly up-regulated in CK2mut roots, revealing a CK2-dependent mechanism underlying their response to SA. Concordantly, *PIN4* and *PIN7* transcript levels were similar in CK2mut and CK2mut x *sid2* mutants.

On the other hand, *PID* transcript levels were much higher in CK2mut roots than in SA-treated WT roots, and they did not decrease in the CK2mut x *sid2* double mutant. Thus, although SA is sufficient to increase *PID* transcription, additional mechanisms, involving CK2 activity, might come into play in order to explain the high *PID* transcript levels found in CK2mut roots.

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

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CK2-encoding genes are transcriptionally regulated by SA in Arabidopsis

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

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

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

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

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

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

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idea that both SA homeostasis and signalling are disturbed in CK2mut seedlings.

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

In silico promoter analysis of auxin-responsive genes

It is well known that the transcriptional responses to auxin are primarily mediated by *cis*-regulatory Auxin Response Elements (AREs), which are recognized by *trans*-factors called Auxin Response Factors (ARFs) (Ulmasov *et al.*, 1999). Although ARE sequences are sufficient to confer auxin responsiveness, they are often found as composite elements in the natural promoter context (Ulmasov *et al.*, 1995) or in association to other *cis*-elements recognized by different families of transcription factors (Berendzen *et al.*, 2012). In concordance with these findings, putative MYB related elements (MREs) or bZIP response elements (ZREs) have been found to modulate transcriptional auxin responses (Heinekamp *et al.*, 2004; Shin *et al.*, 2007). MREs and ZREs are also involved in responses to biotic and abiotic stresses. On the other hand, the WRKY plant-specific family of transcription factors has a prominent role in the SA-triggered responses, particularly through NPR1, and in SA biosynthesis

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through the shikimate pathway (Wildermuth *et al.*, 2001; Yu *et al.*, 2001; Wang *et al.*, 2006).

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

It has been postulated, that those bipartite and tripartite organizations of *cis*-elements might have functional significance in the modulation of gene transcriptional responses (Berendzen *et al.*, 2012). In the case of auxin–mediated responses, bZIP- and MYB-related binding sites are potential AuxRE-coupling elements in auxin-mediated transcription. The promoters of the *PIN* and *PID* genes show significant differences among them, both in abundance

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and distribution of auxin- and SA-responsive elements. Although experimental analysis is needed to test the functionality of the *cis*-elements, those differences might account for the differential qualitative and quantitative responses to SA measured for *PIN* and *PID* genes in this work.

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DISCUSSION (785 words)

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

Very little is known about the signalling pathways in which plant CK2 participates. We previously showed that CK2 loss-of-function mutants (CK2mut plants) have shorter roots and are impaired in lateral root formation. In this work we show that those are salicylic acid-mediated effects. Several data support our conclusion: 1) CK2 loss-of-function mutants have enhanced levels of SA; in particular, roots of CK2mut seedlings show increments of 3.7-fold as compared to the WT levels; 2) both, Arabidopsis WT plants incubated with exogenous SA, and Arabidopsis *cpr* mutants (which contain high constitutive SA levels), show the same root phenotypes as CK2mut seedlings; and 3) partial depletion of CK2 activity (using genetic of pharmacological tools) in SA-defective and SA-signalling mutants (*sid2*, *NahG, npr1*) resulted in less severe root phenotypes. Moreover, these findings reveal the existence of a negative regulatory point in the SA biosynthesis that is bypassed in CK2-defective seedlings. In addition, we demonstrate that CK2-encoding genes are transcriptionally up-regulated by SA, and that the NPR1-mediated pathway is involved in this regulation. Taken

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together, these data strongly suggest the existence of an autoregulatory feed-back loop between CK2 and SA. Analysis of transcript profiles in CK2mut seedlings revealed up-regulation of *SID2* and *PAL1* genes, involved in two alternative SA-biosynthesis pathways, as well as of many transcription factors belonging to families involved in the transcriptional regulation of SA-responsive genes (Qin *et al.*, 1994; Maleck *et al.*, 2000; Krawczyk *et al.*, 2002). It has been previously reported that CK2 mediates post-translational regulation of transcription factors belonging to the TGA family, which recognize the SA-responsive *as-1* element (Kang and Klessig, 2005). These findings support the idea that CK2 activity is required for both SA signalling and homeostasis.

SA levels can also influence auxin polar transport in plants (Du *et al.*, 2013). Stimulation of SA biosynthesis and the subsequent SA accumulation, as it occurs during biotic stress, inhibits auxin polar transport, and this appears to be a plant defence mechanism to hinder pathogens from growth and reproduction (Wang *et al.*, 2007). As CK2mut seedlings accumulate such high levels of SA (which was concomitant with constitutive up-regulation of *PR-1*), and show up-regulation of *PIN2*, *PIN4* and *PIN7*, as well as of *PID* (Marques-Bueno *et al.*, 2011a; Moreno-Romero *et al.*, 2012), we decided to investigate the role, if any, of SA in the transcriptional regulation of *PIN* and *PID* genes. Surprisingly, Arabidopsis WT plants incubated with SA showed significant down-regulation of all the members of the *PIN* gene family, with the exception of *PIN2*. SA-mediated repression of *PIN7* was previously reported by other authors (Wang *et al.*, 2007), and now this SA-mediated effect can be extended to most of the *PIN* family members, suggesting that this might be the mechanism underlying

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inhibition of auxin transport by SA. Moreover, these results also show that the high SA content in CK2mut roots is not able to mediate repression of *PIN4* and *PIN7* genes, and, thus, that this particular SA-signalling pathway is impaired in CK2-defective plants. We propose that the SA-mediated transcriptional regulation of *PIN4* and *PIN7* is CK2-dependent. CK2 activity might be required for transcriptional regulation and/or post-translational modification of particular TFs involved in *PIN* transcriptional control. Analysis of the 5'-regulatory sequences in *PIN* promoters showed the presence of many copies of *cis*-acting elements that mediate both SA- and auxin-responses, in particular of W-boxes.

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

In conclusion, this work presents evidence that CK2 mediates SA responses by affecting both SA biosynthesis and SA-signalling pathway. Overexpression of CK2 does not have a significant impact on SA-mediated effects, indicating that an excess of CK2 activity does not produce an imbalance in the CK2/SA mutual influence. To the contrary, loss-of-function of CK2 mutants presents defects in SA accumulation and in SA-mediated root phenotypes. Very importantly, loss-of-function of CK2 activity also impairs the SA-signalling pathway that links SA and auxin transport, a mechanism exploited by pathogens to bypass plant defences. A working model summarizing all these data is shown in Figure 6.

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EXPERIMENTAL PROCEDURES (895 words)

Plant material

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

Plant treatments and phenotypes

Expression of *CK2mut* transgene was induced with 1 µM Dexamethasone (DEX) for the indicated times, and controls with DEX solvent (ethanol) were performed in all cases. Salicylic acid (SA) was dissolved in ethanol and treatments were performed at 0.25 mM for the indicated times. Treatments with 10 µM 4,5,6,7-tetrabromobenzotriazol (TBB) dissolved in DMSO were performed on 5-day-old WT plantlets for 16 h. Lengths of primary roots were measured in seedlings grown vertically in Petri dishes and analyzed using the IMAGEJ software (http://rsb.info.nih.gov/ij). The number of lateral roots was

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determined using a Leica DMRB optical microscope and the results are represented as frequency distributions in histograms (frequency denotes the number of plants containing the indicated number of emerged lateral roots or of lateral root primordia) or as mean values ± SD. For the root gravitropic assay, 5-day-old seedlings grown on vertically-oriented plates were reoriented by 90º, left to grow for 24h, and reoriented again by 90º. Plants were scanned with a Bio-Rad GS-700 Imaging Densitometer (Bio-Rad Laboratories, http://www.biorad.com/). Statistical analyses of data were performed either in EXCEL (Microsoft, http://www.microsoft.com) or the R program (http://www.R-project.org/), using the Student's two-tailed t-test for independent samples ($p \leq 0.05$).

Generation of transgenic CK2^{OE} plants

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

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obtained by sexual crossing, and *DR5::GFP* detection was performed by confocal microscopy, as in (Marques-Bueno *et al.*, 2011a).

RT-PCR analysis

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

Protein extracts, western blots, enzymatic activities and hormone analysis

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Proteins were extracted from frozen root tissue homogenized in cold protein extraction buffer (50 mM Tris–HCl, pH 7.5, 100 mM KCl, 10% glycerol, 0,1% Triton x-100, 2mM DTT, 5mM EDTA, 0,5% (w/v) polyvinylpolypyrrolidone). For immunoblots, proteins were electrophoresed on 10% SDS-PAGE gels, transferred to immobilon-P membranes (Millipore, http://www.millipore.com), and then incubated with 1:1000 anti-*c*-myc antibodies (GenScript, http://www.genscript.com/). The immunocomplexes were revealed using the Lumi-Light Western Blotting Substrate system (Roche, http://www.roche.com/). Loading of equal quantities of proteins was controlled by Bradford's analysis using BSA as a standard, and by Ponceau-staining of the membranes. Indole-3-acetic acid and salicylic acid (SA) were determined as described in (Muller and Munne-Bosch, 2011). CK2 enzymatic assays in crude extracts were performed as described by Espunya et al. (1999) using 50 µM of the specific peptide RRRADDSDDDDD (Jena Bioscience GmbH, http://ww.jenabioscience.com) and $[\gamma^{32}P]$-ATP (1000-2000 c.p.m. $pmol^{-1}$). Proteins were extracted with Tris-HCl pH 7.5 50 mM, NaCl 50 mM, MgCl2 10 mM, PMSF 1 mM, beta-glicerophosphate 25 mM, NaF 20 mM, sodium orthovanadate 0.2 mM, and protease Inhibitor cocktail 1/1000 (Sigma). One enzymatic unit was defined as the amount of enzyme that incorporates 1 pmol of ^{32}P into the substrate per minute at 30ºC. All the assays were made in triplicate.

In silico analysis of promoters

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

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ACKNOWLEDGEMENTS (109 words)

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SHORT LEGENDS FOR SUPPORTING INFORMATION (83 words)

The following materials are available in the online version of this article:

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

Figure S2. Root development of CK2α-overexpressing plants.

Figure S3. *Cis*-element organizations in gene promoters of *PINs*, *AUX1* and *PID*.

Table S1. *p*-values of the statistical analysis of Table I and Table II.

Table S2. SA-regulated genes with differential expression in CK2mut seedlings.

Table S3. Ocurrence list of *cis*-acting elements in *PIN*, *PID* and *AUX1* gene promoters.

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

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

Table II. Regulation of CK2-encoding gene expression by salicylic acid (SA). Transcript levels of CK2-encoding genes were measured in WT plants incubated with 0.25 mM SA for 48 H (WT +SA) and in SA-biosynthetic and SA-signalling mutants. Values were obtained by quantitative RT-PCR in 7-day-old roots and normalized to those of *EF-1-α* gene. Mean values of three biological replicates are shown as fold-changes of transcript levels versus those in WT roots, with standard deviations always ≤30%. Statistical analyses to assess differences in gene expression between the different lines and conditions were carried out for each gene, using One-way ANOVA ($p≤0.05$). The expression changes were statistically significant for all genes. Pairs of conditions were also compared by the Student's *t*-test and the *p*-values are shown in Table S1.

Abbreviations: CK2αA and *CK2αB*: Arabidopsis CK2α-encoding genes. *CK2β1-4*: Arabidopsis CK2β-encoding genes. *npr1-1 (NONEXPRESSER OF PATHOGENESIS-RELATED PROTEIN1)* (Durrant and Dong, 2004)*:* Arabidopsis mutant impaired in SA-signalling; *cpr1* and *cpr6 (CONSTITUTIVE EXPRESSER OF PR1)* (Clarke *et al.*, 2000)*:* Arabidopsis mutants with constitutive high levels of SA; *NahG (SA HYDROXYLASE)* (Delaney *et al.*, 1994): SA-defective Arabidopsis mutant .

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

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FIGURE LEGENDS (954 words)

Figure 1. Molecular characterization of Arabidopsis transgenic lines overexpressing CK2α subunit.

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

Abbreviations: WT, wild-type Arabidopsis plants; CK2^{OE}, CK2α-overexpressing plants; a.u., arbitrary units.

Figure 2. Root phenotypes of CK2α-overexpressing plants.

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(a) Quantification of root lengths (primary roots) in WT and CK2^{OE} seedlings. Results shown for WT and for four independent transgenic lines are the mean values ±SD (n=10-25); the experiment was repeated two times with similar results, and only the data from one of them is shown. (*) Asterisks denote statistically significant differences between WT and CK2^{OE} lines at the indicated times. (b) Number of lateral roots in 10-day-old seedlings (CK2^{OE}3). The histograms show frequency distributions of the number of emerged lateral roots (top) or of root primordia (middle), according to the classification in Peret *et al*. (2009). The frequency denotes the number of plants containing the indicated number of emerged lateral roots or of lateral root primordia. Mean values ±SD (n≥40) are shown at the bottom panel; three independent experiments were performed. The insets show pictures of lateral roots at the indicated stages. (c) Number of lateral roots in 5-day-old seedlings (CK2^{OE}3). Data are represented as in (b), but note that only root primordia are seen at this developmental stage (top). Mean values ±SD (n≥25) are shown at the bottom panel; three independent replicates were performed. *Abbreviations:* WT, wild-type Arabidopsis plants; CK2^{OE}, CK2α-overexpressing plants; SD, standard deviations. Statistical analyses were performed using Student's *t*-test at p≤0.05, and statistical significances are marked with asterisks (*).

Figure 3. Influence of salicylic acid on root phenotypes.

(a) Quantification of indole-acetic acid (IAA) and salicylic acid (SA) in 10-day-old roots of different Arabidopsis lines. CK2mut and CK2mut x *sid2* lines were

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incubated with either dexamethasone (+DEX) or ethanol (-DEX) for the last 72 h before hormone determinations. Values shown are the mean (±SE) of 10 biological replicates. (b) Root phenotypes of Arabidopsis wild-type seedlings incubated with 0.25 mM SA for 48 h. Mean values (±SD) are shown (n≥20). (c) Root phenotypes of CK2mut x *sid2* double mutant (± DEX, as in a). The CK2mut line (± DEX) was used as a control. Mean values (±SD) are shown (n≥20). Statistical analyses were performed using Student's *t*-test at p≤0.05, and significant differences were marked with asterik (*).The experiments in b) and c) were carried out three times with similar results. *Abbreviations:* FW, fresh weight; CK2^{OE}, CK2-overexpressing line; SE, standard errors; SD, standard deviations.

Figure 4. Salicylic acid mutants and inhibition of CK2 activity with 4,5,6,7-tetrabromobenzotriazol (TBB).

(a) Primary root length and number of lateral roots in *CONSTITUTIVE EXPRESSER OF PR1 (cpr)* mutants. Experiments were performed with 10-day-old seedlings of *cpr1*, *cpr5* and *cpr6.* Data shown are the mean values ± SD (n ≥ 10). (b) Effects of TBB on hormone levels. Quantification of indole-acetic acid (IAA) in WT Arabidopsis roots (±TBB) (left panel), and of salicylic acid (SA) in WT and *NahG* roots (± TBB) (right panel). Hormones were quantified in 10-day-old roots after 16 h of TBB treatments (10 µM). Data shown are the mean values (±SE) of 10 biological replicates. (c) Quantification of the number of lateral roots in TBB-treated plants. Five-day-old plants were incubated with 10

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μM TBB for 16 h and then transferred to plates without TBB. The number of lateral roots was counted 5 days after removing theTBB. *Abbreviations:* Wild-type plants (WT), SA HYDROXYLASE mutant (*NahG*), and *npr1-1* *(NONEXPRESSER OF PATHOGENESIS-RELATED PROTEIN1)* mutant. Statistical analysis was performed using Student's *t*-test at p≤ 0.05. Asterisk (*) indicates statistically significant differences in comparison to the corresponding control plants.

Figure 5. Influence of salicylic acid on *PIN* and *PID* expression.

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

Figure 6. Proposed model for the interplay between CK2, salicylic acid and *PIN* transcription.

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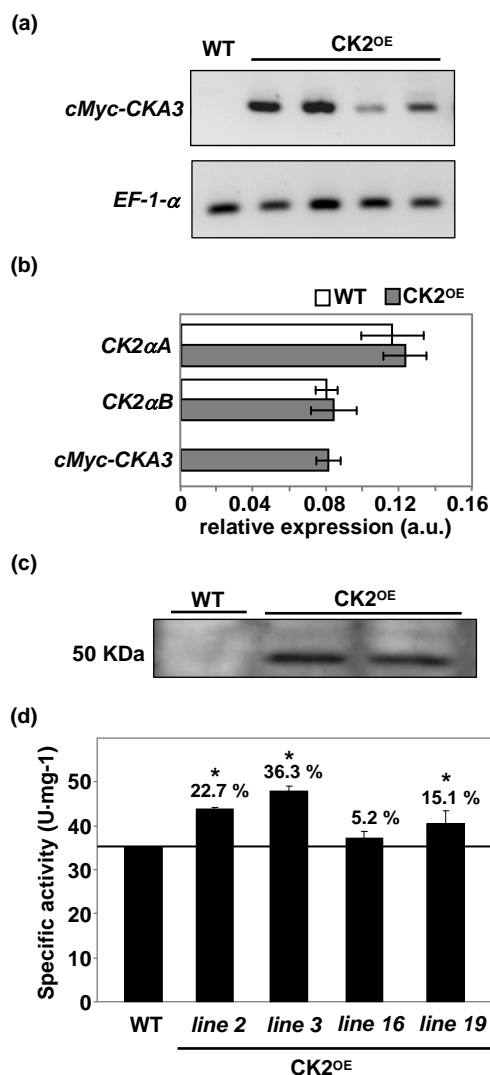
Symbols: **CK2** *Inactive CK2*

Figure 1. Molecular characterization of Arabidopsis transgenic lines overexpressing CK2α subunit.
(a) Transgenic Arabidopsis lines, previously selected by Hy^R (F3 generation), were analyzed by RT-PCR, using specific primers to amplify the CK2α transgene. Amplified *EF-1-α* transcript levels were used as loading control. (b) Quantification of CK2α-encoding gene expression in $CK2^{OE}$ roots. Transcript levels of endogenous Arabidopsis CK2α-encoding genes (*CK2αA* and *CK2αB*) and of CK2α transgene (cMyc-*CKA3*) were measured separately by quantitative RT-PCR. Values are the means of three biological replicates (±SD) and are shown as relative expression versus that of the constitutive *actin2* gene (*at3g18780*). (c) Western blot, using an anti-*c*-myc antibody. Only two of the several analyzed lines are shown. (d) Overall CK2 activity in wild-type and $CK2^{OE}$ transgenic lines. The data shown are the mean of three replicates (±SD), and two independent experiments were performed. The activity percentage for each $CK2^{OE}$ line (relative to wt) is shown above each bar. (*) Asterisks denote statistically significant differences using Student's *t*-test at p≤0.05.
Abbreviations: WT, wild-type Arabidopsis plants; $CK2^{OE}$, CK2α-overexpressing plants; a.u., arbitrary units.
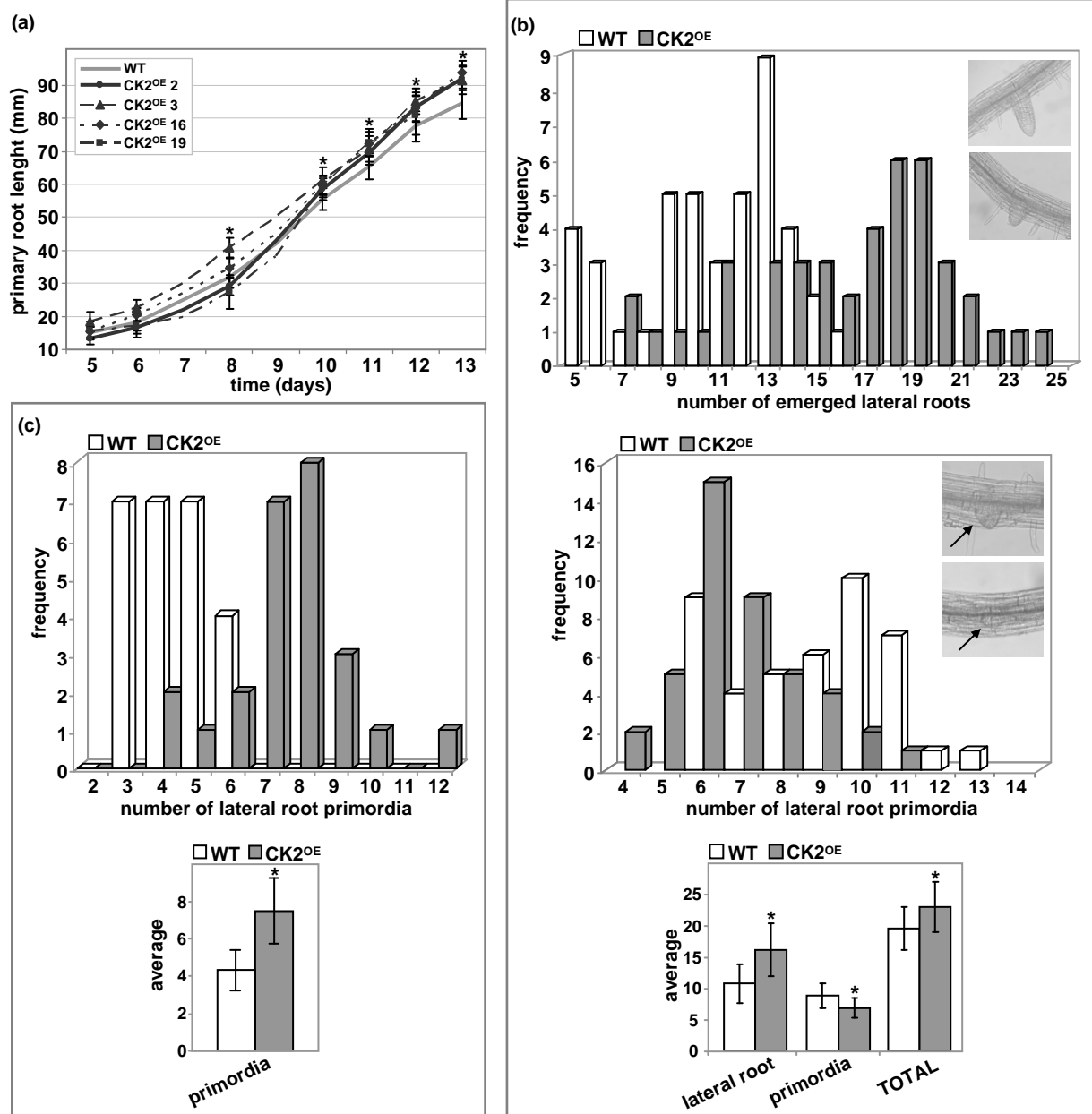
Figure 2. Root phenotypes of CK2α-overexpressing plants.

(a) Quantification of root lengths (primary roots) in WT and CK2[OE] seedlings. Results shown for WT and for four independent transgenic lines are the mean values ±SD (n=10-25); the experiment was repeated two times with similar results, and only the data from one of them is shown. (*) Asterisks denote statistically significant differences between WT and CK2[OE] lines at the indicated times. (b) Number of lateral roots in 10-day-old seedlings (CK2[OE]3). The histograms show frequency distributions of the number of emerged lateral roots (top) or of root primordia (middle), according to the classification in Peret *et al.* (2009). The frequency denotes the number of plants containing the indicated number of emerged lateral roots or of lateral root primordia. Mean values ±SD (n≥40) are shown at the bottom panel; three independent experiments were performed. The insets show pictures of lateral roots at the indicated stages. (c) Number of lateral roots in 5-day-old seedlings (CK2[OE]3). Data are represented as in (b), but note that only root primordia are seen at this developmental stage (top). Mean values ±SD (n≥25) are shown at the bottom panel; three independent replicates were performed. *Abbreviations:* WT, wild-type Arabidopsis plants; CK2[OE], CK2α-overexpressing plants; SD, standard deviations. Statistical analyses were performed using Student's *t*-test at p≤0.05, and statistical significances are marked with asterisks (*).
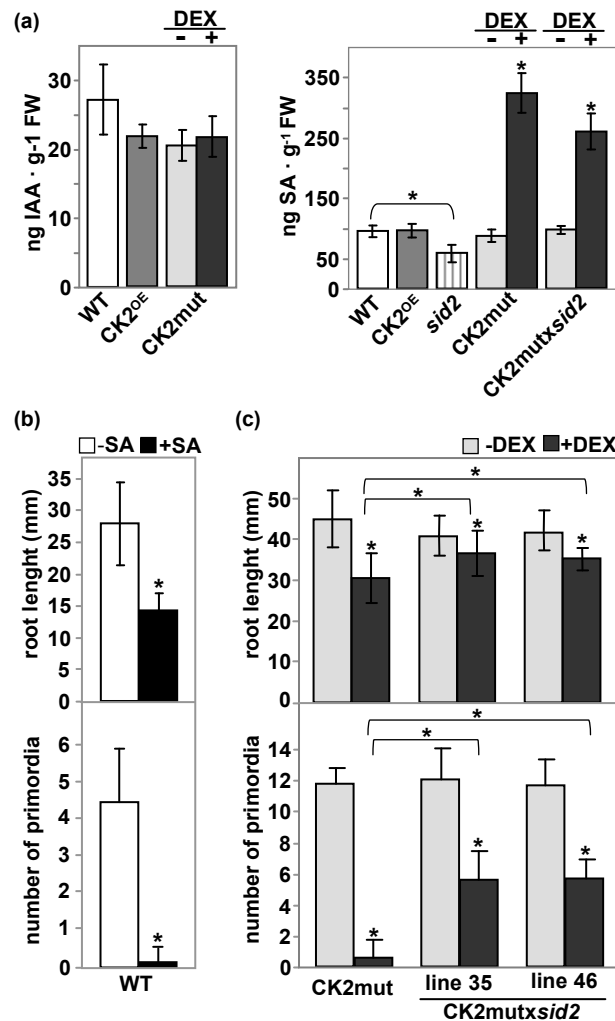
Figure 3. Influence of salicylic acid on root phenotypes.
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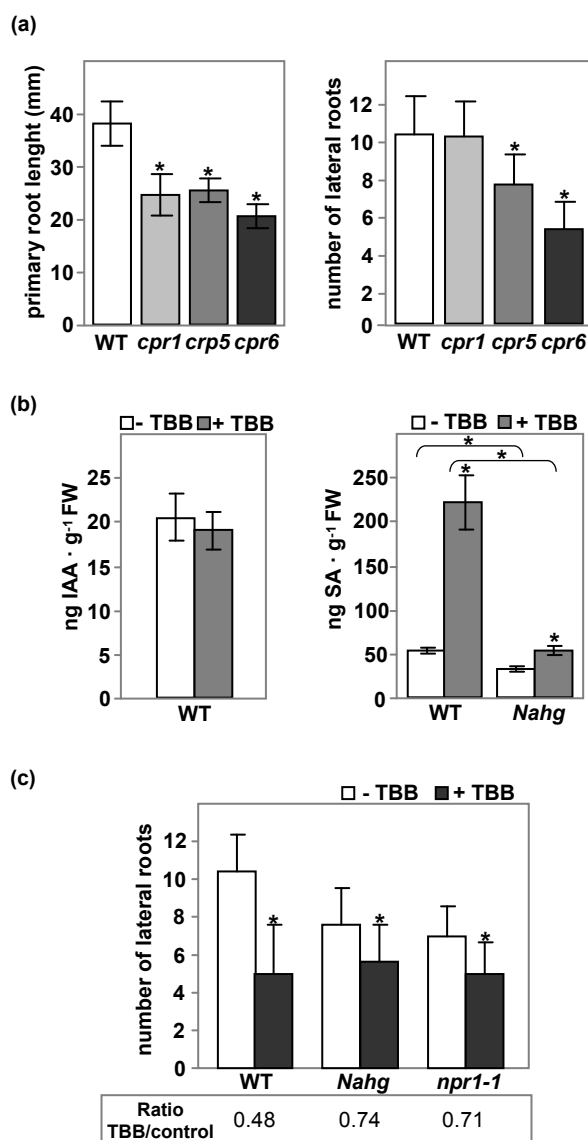| Ratio TBB/control	0.48	0.74	0.71

Figure 4. Salicylic acid mutants and inhibition of CK2 activity with 4,5,6,7-tetrabromobenzotriazol (TBB).
(a) Primary root length and number of lateral roots in *CONSTITUTIVE EXPRESSER OF PR1 (cpr)* mutants. Experiments were performed with 10-day-old seedlings of *cpr1*, *cpr5* and *cpr6*. Data shown are the mean values ± SD (n ≥ 10). (b) Effects of TBB on hormone levels. Quantification of indole-acetic acid (IAA) in WT Arabidopsis roots (±TBB) (left panel), and of salicylic acid (SA) in WT and *NahG* roots (± TBB) (right panel). Hormones were quantified in 10-day-old roots after 16 h of TBB treatments (10 µM). Data shown are the mean values (±SE) of 10 biological replicates. (c) Quantification of the number of lateral roots in TBB-treated plants. Five-day-old plants were incubated with 10 µM TBB for 16 h and then transferred to plates without TBB. The number of lateral roots was counted 5 days after removing theTBB. *Abbreviations:* Wild-type plants (WT), SA HYDROXYLASE mutant (*NahG*), and *npr1-1 (NONEXPRESSER OF PATHOGENESIS-RELATED PROTEIN1)* mutant. Statistical analysis was performed using Student's *t*-test at p≤ 0.05. Asterisk (*) indicates statistically significant differences in comparison to the corresponding control plants.
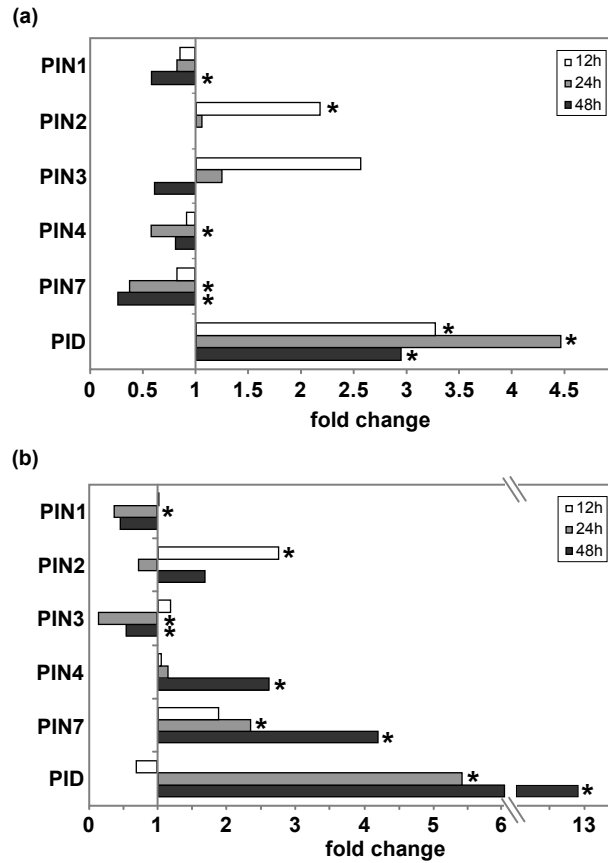
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Figure 5. Influence of salicylic acid on *PIN* and *PID* expression. Fold changes of *PIN* and *PID* transcript levels in Arabidopsis WT plants incubated with 0.25 mM salicylic acid (SA) (a) or in CK2mut plants treated with Dex (b) for the indicated times. Transcript levels were measured by quantitative RT-PCR in roots and normalized to those of *EF-1-α* gene. Mean values of three biological replicates were obtained, with standard deviations always ≤30%. The data are represented as fold changes in SA-treated or Dex-treated plants versus their respective controls. Asterisks (*) indicate statistical significant differences of treated plants versus untreated plants, using the Student's *t*-test ($p \leq 0.05$). Statistical significance was assigned to a fold-change value of 2.
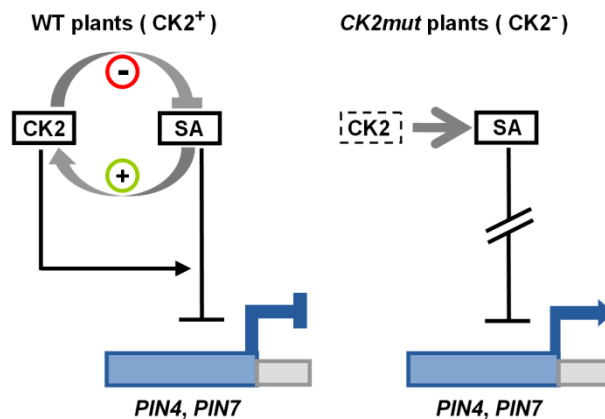
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Figure 6. Proposed model for the interplay between CK2, salicylic acid and *PIN* transcription.

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

Symbols: **CK2**   *Inactive CK2*

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(d)

DR5rev::GFP plants

DR5rev::GFP x CK2^{OE} plants
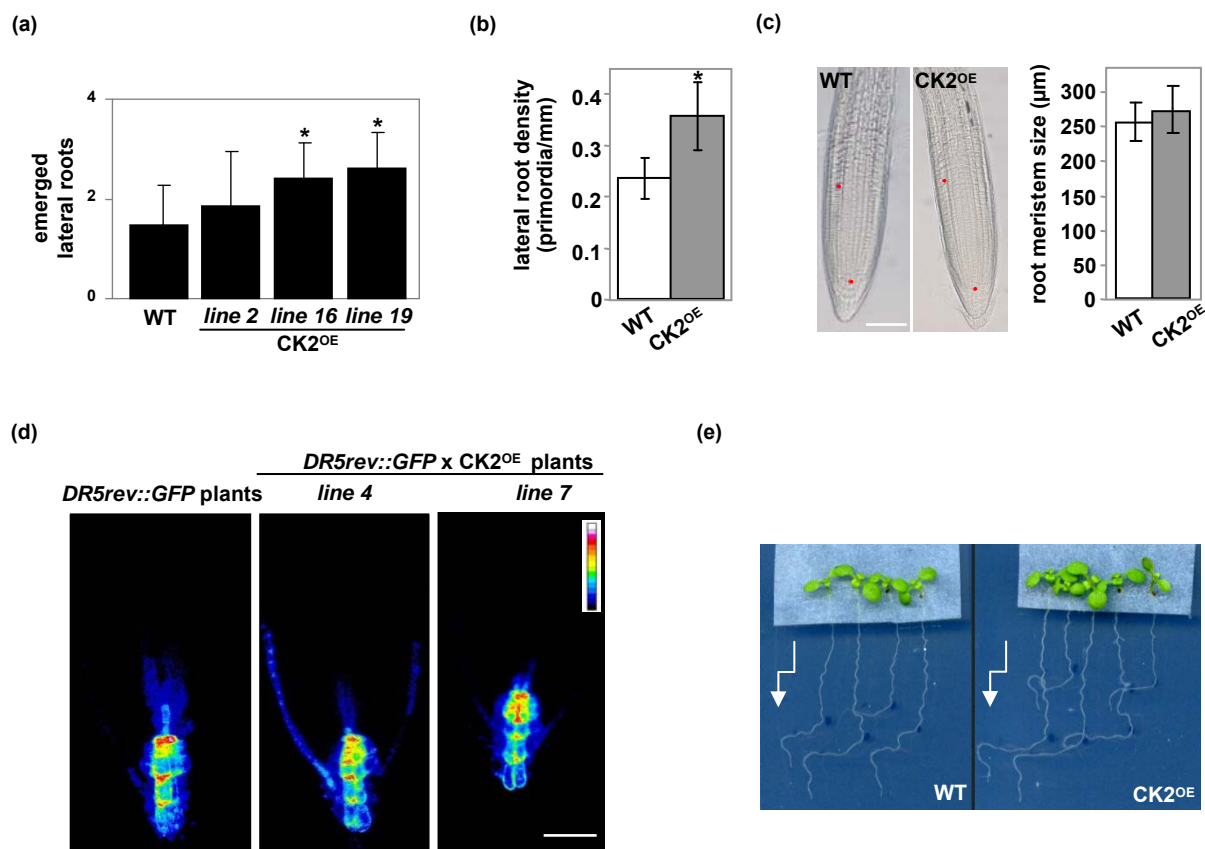
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(e)

Figure S1. Lateral root density, meristem size, auxin distribution, and gravitropic response in CK2^{OE} roots.
(a) Number of emerged lateral roots in six-day-old Arabidopsis seedlings (WT and different CK2^{OE} lines). Mean values (±SD) are represented (n=10-25). (b) Lateral root densities, measured as number of root primordia per mm, in 5-day-old CK2^{OE}3 seedlings. Mean values (±SD) are shown (n≥25). The experiment was carried out three times with similar results. (c) Root meristem sizes of 5-day-old CK2^{OE}3 seedlings. Red dots in the pictures mark the meristem boundaries. Scale bar: 100 µm. (d) Expression of *DR5::GFP* reporter in CK2^{OE}3 roots, recorded by confocal microscopy as in Marques-Bueno *et al.* (2011a). Scale bar: 50 µm. (e) Root gravitropic response in CK2^{OE}3 seedlings. The changes in the gravitropic vector (carried out twice) are indicated by the connecting arrow.
Statistical analyses were performed using Student's *t*-test at p≤0.05 and significant differences are marked by asterisk (*). WT: wild-type; CK2^{OE}, CK2-overexpressing plants.

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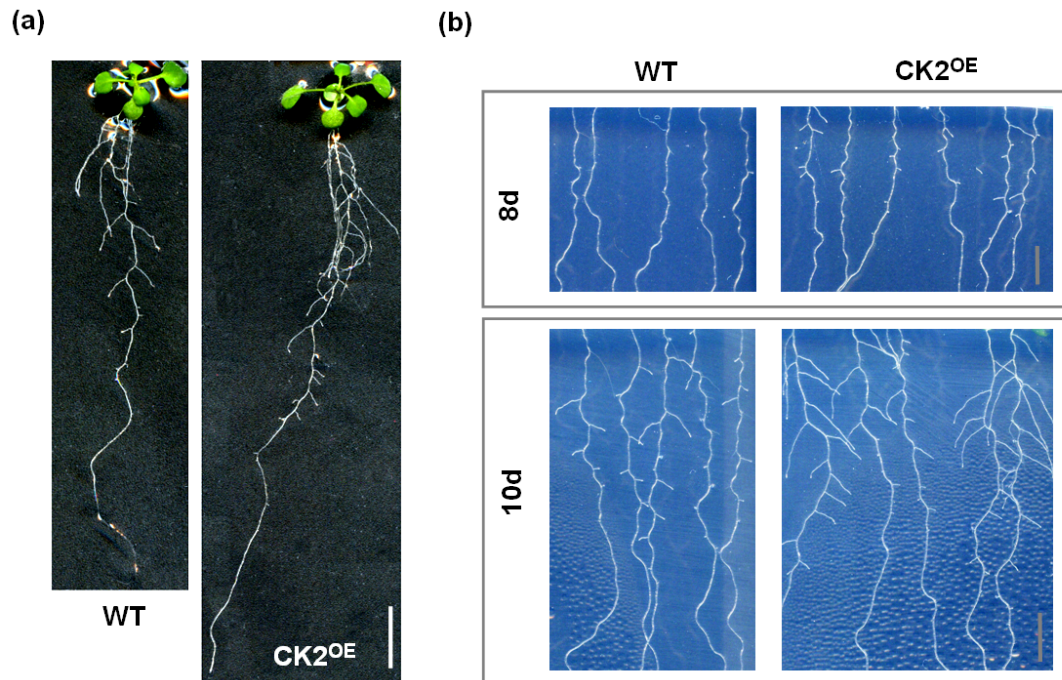43
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Figure S2. Root development of CK2α-overexpressing plants.
(a) Phenotype of 13d-old Arabidopsis seedlings. CK2[OE] plants exhibit slightly longer primary roots and increased number of lateral roots. Scale bar: 1cm. (b) Detail of lateral roots in 8d-old and 10d-old Arabidopsis seedlings. The number of emerged lateral roots is higher in 8d-old CK2[OE] seedlings than in WT plants and, the number and lenght of emerged lateral roots is increased in 10d-old CK2[OE] plants. Scale bars: 0.5 cm.
Abbreviations: WT, wild-type plants; CK2[OE], CK2α-overexpressing plants.
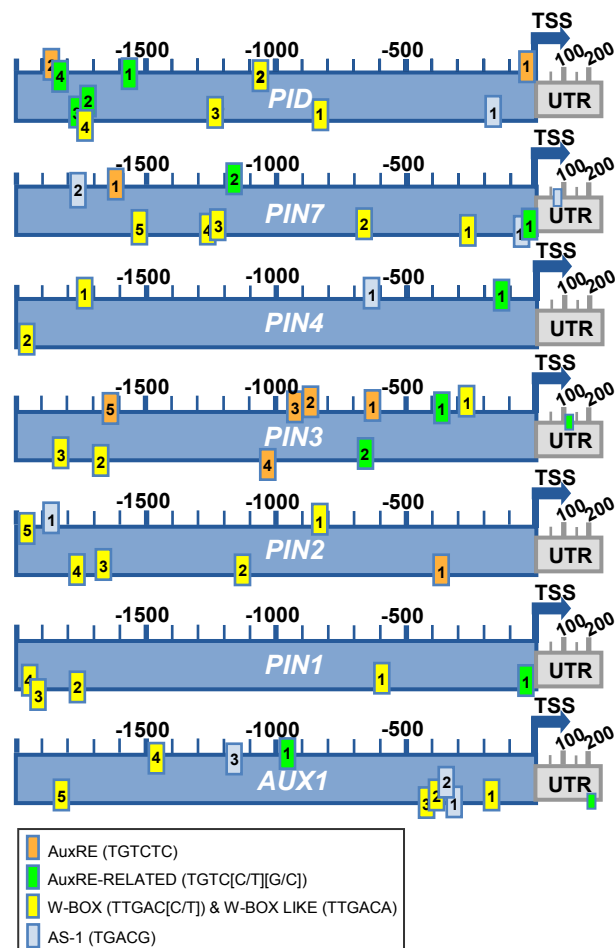
Figure S3. *Cis*-element organizations in gene promoters of *PINs*, *AUX1* and *PID*.

The -2,000 bp promoter sequences and the 5'-UTRs of five members of the *PIN* gene family (*PIN1*, *PIN2*, *PIN3*, *PIN4* and *PIN7*), as well as of *PINOID* and *AUX1* genes, are plotted in the 5' to 3' orientation. The location of specific *as-1*, W-box, and ARE *cis*-elements is shown for each gene, using a color code. The motifs in the promoter region are in numerical order according to their proximity to the transcription start site. The exact positions of the motifs are shown in Table S3. *Abbreviations*: TSS, transcription start site; UTR, untranslated region.