Running title: SnRK2s are involved in cadmium stress response

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Title: SNF1-related protein kinases type 2 are involved in plant responses to cadmium stress

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FOOTNOTES

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ABSTRACT

Cadmium ions are notorious environmental pollutants. In order to adapt to cadmium-induced deleterious effects plants have developed sophisticated defense mechanisms. However, the signaling pathways underlying the plant response to cadmium are still elusive. Our data demonstrate that SNF1-related protein kinases 2 (SnRK2s) are transiently activated during cadmium exposure and are involved in the regulation of plant response to this stress. Analysis of *Nicotiana tabacum* Osmotic Stress-Activated Protein Kinase (NtOSAK) activity in tobacco BY-2 cells indicates that reactive oxygen species (ROS) and nitric oxide, produced mainly via an L-arginine-dependent process, contribute to the kinase activation in response to cadmium. SnRK2.4 is the closest homologue of NtOSAK in *Arabidopsis thaliana.* Comparative analysis of seedling growth of *snrk2.4* knockout mutants *versus* wild type Arabidopsis suggests that SnRK2.4 is involved in the inhibition of root growth triggered by cadmium; the mutants were more tolerant to the stress. Measurements of the level of three major species of phytochelatins in roots of plants exposed to Cd^{2+} showed a similar (PC2, PC4) or lower (PC3) concentration in *snrk2.4* mutants in comparison to wild type plants. These results indicate that the enhanced tolerance of the mutants does not result from a difference in the phytochelatins level. Additionally, we have analyzed ROS accumulation in roots subjected to Cd^{2+} treatment. Our data show significantly lower Cd^{2+}-induced ROS accumulation in the mutants' roots. Concluding, the obtained results indicate that SnRK2s play a role in the regulation of plant tolerance to cadmium, most probably by controlling ROS accumulation triggered by cadmium ions.

INTRODUCTION

Cadmium is one of the most toxic soil pollutants. Cadmium ions accumulate in plants and affect, via the food chain, animal and human health. In plants, cadmium is taken up by roots and is transported to aerial organs leading to chromosomal aberrations, growth reduction and inhibition of photosynthesis, transpiration, nitrogen metabolism, nutrient and water uptake, eventually causing plant death (for review see DalCorso et al., 2008). Plants are challenged not only by cadmium ions themselves, but also by Cd^{2+}-induced harmful effects including oxidative stress (Schützendübel et al., 2001; Olmos et al., 2003; Cho and Seo, 2005; Sharma and Dietz, 2008). The extent of the detrimental effects on plant growth and metabolism depends on the level of cadmium ions present in the surrounding environment and on the plant's sensitivity to heavy metal stress.

Tolerant plants avoid heavy metal uptake and/or induce the expression of genes encoding products involved, directly or indirectly, in heavy metal binding and removal from potentially sensitive sites, by sequestration or efflux (Clemens, 2006). The best characterized heavy metal binding ligands in plants are thiol-containing compounds metallothioneins and phytochelatins (PCs), whose production is stimulated by Cd^{2+}. PCs bind metal ions and transport them to the vacuole, thus reducing the toxicity of the metal in the cytosol (for review see Cobbett, 2000, Cobbett and Goldsbrough, 2002). PCs are synthesized from reduced glutathione (GSH). Therefore, production of compounds involved in cadmium detoxification and, at the same time, in cadmium tolerance closely depends on sulfur metabolism. So far, our knowledge on the cellular processes induced by cadmium which lead to changes in sulfur metabolism in plants has been rather limited.

Protein kinases and phosphatases are considered major signal transduction elements. However, until now only a few of them have been described to be involved in cadmium stress response or sulfur metabolism. For instance, excessive amounts of cadmium or copper activate mitogen-activated protein kinases (MAPKs) in *Medicago sativa* (Jonak et al., 2004), *Oryza sativa* (Yeh et al., 2007), and *Arabidopsis thaliana* (Liu et al., 2010). Studies on rice MAPKs involved in heavy metal stress response indicate that the activity of these kinases depends on the oxidative stress induced by Cd^{2+}. Moreover, Yeh et al. suggested that the activation of MAPKs in rice by cadmium or copper required the activity of calcium dependent

protein kinase (CDPK) and phosphatidylinositol 3-kinase (PI3 kinase), since the MAPK pathways involved in cadmium and copper stress response could be inhibited by a CDPK antagonist (W7) or a PI3 kinase inhibitor (wortmannin). However, so far the function of the identified kinases in plant adaptation to heavy metal pollution has not been established. There is some information concerning an involvement of CDPK in sulfur metabolism (Liu et al., 2006). Soybean serine acetyltransferase (GmSerat2;1), the enzyme that catalyzes the first reaction in the biosynthesis of cysteine from serine, is phosphorylated by CDPK. The phosphorylation has no effect on GmSerat2;1 activity, but it renders the enzyme insensitive to the feedback inhibition by cysteine (Liu et al., 2006). There is growing evidence that SNF1-related protein kinases 2 (SnRK2s) play a role in the regulation of sulfur metabolism. Most information showing a connection between SnRK2s and sulfur metabolism comes from experiments on the lower plant *Chlamydomonas reinhardtii* (Davies et al., 1999; Irihimovitch and Stern, 2006; Gonzalez-Ballester et al., 2008; Gonzalez-Ballester et al., 2010). Chlamydomonas SNRK2.1 is considered a general regulator of S-responsive gene expression in green algae (Gonzalez-Ballester et al., 2008).

In higher plants the SnRK2 family members are known to be involved in plant response to drought, salinity and in ABA-dependent plant development (Boudsocq and Lauriere, 2005; Fujii et al., 2007; Fujii and Zhu, 2009; Nakashima et al., 2009; Fujita et al., 2009; Fujii et al., 2011; Kulik et al., 2011). Ten members of the SnRK2 family have been identified in *Arabidopsis thaliana* and in *Oryza sativa* (Boudsocq et al., 2004; Kobayashi et al., 2004). All of them, except SnRK2.9 from Arabidopsis*,* are rapidly activated by treatment with different osmolytes, such as sucrose, mannitol, sorbitol, and NaCl, and some of them also by abscisic acid (ABA). Results presented by Kimura et al. (2006) suggest that in Arabidopsis, similarly to algae, some SnRK2s are involved in the regulation of S-responsive gene expression and *O*-acetyl-L-serine accumulation under limited sulfur supply indicating that also higher plants' SnRK2s could be involved in sulfur metabolism.

As it was mentioned before, oxidative stress induced by cadmium ions significantly contributes to the metal toxicity. ROS can be produced in many different reactions in various compartments of the cell in response to cadmium (Romero-Puertas et al., 2004; Heyno et al., 2008; Tamás et al., 2009). The best characterized ROS-generating enzymes that take part in the response to cadmium are the plasma membrane-bound NADPH oxidases (Olmos et al., 2003; Romero-Puertas et al.,

2004; Garnier et al., 2006). There are some indications that plant NADPH-oxidases are phosphorylated by SnRK2s (Sirichandra et al., 2009), therefore it is highly plausible that SnRK2s play a role in the regulation of ROS accumulation in plants subjected to cadmium stress. Taking into consideration all facts mentioned above we hypothesized that SnRK2s could be involved in the plant response to stress induced by cadmium ions. To verify this conjecture, we analyzed the activity and potential role of selected SnRK2s, in tobacco cells and Arabidopsis plants, in the response to cadmium ions.

RESULTS

$CdCl_2$ Activates MAPK and NtOSAK Pathways in Tobacco BY-2 Cells

To identify protein kinases involved in the plant response to cadmium ions, as a first approach we monitored the activity of protein kinases phosphorylating myelin basic protein (MBP) in BY-2 tobacco cells exposed to increasing concentrations of $CdCl_2$ (0-1000 μM). The kinase activity in protein extracts prepared from BY-2 cells, not treated and treated with $CdCl_2$ for 30 min, was analyzed by in-gel kinase activity assay. In tobacco cells exposed to Cd^{2+} at concentrations higher than 10 μM, at least two different protein kinases phosphorylating MBP, with molecular masses of 47 and 42 kDa were activated (Figure 1A). Since the activity of both kinases was relatively high after cell treatment with 50 or 100 μM Cd^{2+} and these concentrations were frequently used by other researchers investigating the activity of protein kinases potentially involved in plant response to Cd^{2+} (Jonak et al., 2004; Yeh et al., 2007; Liu et al., 2010), 100 μM $CdCl_2$ was chosen for further experiments. The time course of the activation revealed that the kinases exhibited maximal activity when the cells were treated with 100 μM $CdCl_2$ for about 15-60 minutes (Figure 1B). Because earlier reports indicated that kinases belonging to the MAPK family were activated in similar conditions (Jonak et al., 2004; Yeh et al., 2007), we checked whether the kinases activated by Cd^{2+} in our experimental conditions were in fact MAPKs. For this purpose, we performed an immunoblotting assay using specific antibodies recognizing the double phosphorylated motif TEY present in active MAPKs. As shown in Figure 1C, cadmium ions cause activation of a 47-kDa MAPK.

NtOSAK is a member of the SnRK2 family which has been shown to be strongly activated in response to osmotic stress (Kelner et al., 2004). Because $CdCl_2$ treatment triggers oxidative (Schützendübel et al., 2001; Olmos et al., 2003; Martinez et al., 2010) and osmotic stresses (Perfus-Barbeoch et al., 2002) in plant cells, we suspected that the 42-kDa kinase activated by cadmium ions could be a SnRK2 protein kinase, most probably NtOSAK. To verify this hypothesis we monitored the activity of NtOSAK in BY-2 cells exposed to 100 µM $CdCl_2$ by immunocomplex-kinase activity assay using specific anti-NtOSAK antibodies (Figure 1D). Additionally, we analyzed the phosphorylation of Ser-158 in NtOSAK, which is required for the kinase activity (Burza et al., 2006), using specific antibodies recognizing phosphorylated Ser-158 (Figure 1E). In parallel, the protein level of NtOSAK was monitored by Western blotting with specific anti-NtOSAK antibodies recognizing a C-terminal peptide of the kinase (Figure 1F). Results of those experiments confirmed that NtOSAK is transiently activated in BY-2 cells subjected to $CdCl_2$ treatment. To be sure that NtOSAK activation depends on the presence of cadmium ions and not on the type of cadmium salt applied we analyzed the kinase activity in BY-2 cells treated with $CdSO_4$ in parallel to $CdCl_2$ treatment (Figure 1G). In both cases we observed similar activation of NtOSAK.

Additionally, we checked the effect of Cu^{2+} ions on NtOSAK activity by monitoring NtOSAK activation in BY-2 cells exposed to $CuSO_4$ (in concentration up to 200 µM). In this case the kinase activation was significantly lower than in cells treated with cadmium salts (Supplemental materials, Figure S1).

ROS and NO Contribute to NtOSAK Activation in Response to $CdCl_2$

It has previously been shown that cadmium treatment induces ROS and NO production in plant cells (Olmos et al., 2003; Garnier et al., 2006; Besson-Bard et al., 2009; De Michele et al., 2009; Arasimowicz-Jelonek et al., 2011). In order to check whether NO or ROS participate in the NtOSAK activation in response to cadmium, the kinase activity was analyzed in BY-2 cells pretreated with the NO scavenger 2-(4-carboxyphenyl)-4,5-dhydro-4,4,5,5-tetramethyl-1H-imidazol-1yl-oxy-3-oxide (cPTIO) or with the antioxidant enzyme catalase, prior to 100 µM $CdCl_2$ exposure. We observed a substantial inhibition of the Cd^{2+}-dependent NtOSAK activation in those cells, when compared to control experiment (without cPTIO or catalase

pretreatment). The results indicate that both ROS and NO contribute to the NtOSAK activation in response to cadmium stress (Figure 2A, B, C).

In plant cells NO can be produced via non-enzymatic reaction(s) or through at least two enzymatic pathways: from nitrate/nitrite by nitrate reductase (NR), and from L-arginine by enzyme(s) similar to mammalian NO synthase (NOS) (del Río et al., 2004; Besson-Bard et al., 2008). To characterize the route which leads to the Cd^{2+}-dependent NO generation and NtOSAK activation in BY-2 cells, we analyzed the kinase activity induced by 100 µM $CdCl_2$ in BY-2 cells pretreated with the mammalian NOS inhibitor *N*-nitro-L-Arg-methyl ester (L-NAME) or with the NR inhibitor tungstate before $CdCl_2$ addition. Results of those experiments revealed that L-NAME inhibited the Cd^{2+}-induced NtOSAK activation (Figure 2D), whereas this effect was not observed in cells treated with tungstate. Unexpectedly, in cells treated with tungstate, the NtOSAK activity was stimulated even in the absence of cadmium (Figure 2E). These results indicate that NtOSAK activation in response to cadmium ions involves L-Arg-derived NO.

NtOSAK Undergoes Activation in Tobacco Cells in Response to Oxidative Stress

Considering that NO and ROS are involved in the NtOSAK activation in response to cadmium ions, we addressed the question whether the kinase activity in tobacco cells is triggered by these signaling molecules. As it had previously been established that NtOSAK is activated in response to treatment with a NO donor (DEA/NO) (Lamotte et al., 2006; Wawer et al., 2010), we focused our studies on the effect of ROS on the kinase activity. To determine whether NtOSAK is activated by oxidative stress, the kinase activity was monitored in tobacco BY-2 cells exposed to 2 mM H_2O_2. Addition of H_2O_2 to BY-2 cells in suspension caused activation of NtOSAK (Figure 2F). The maximal activity of NtOSAK was observed 15 min after the addition of H_2O_2 and returned to the control value within 90 min. It should be mentioned that in some experiments the elevation of NtOSAK activity took place as late as 30 min after the stressor application and reached a maximum by 60 min (data not shown). This variability could be due to slightly different culture conditions of BY-2 cells and also to H_2O_2 instability.

SnRK2.4 and SnRK2.10 are Activated in Response to Cadmium

To investigate the role of SnRK2s in the plant response to cadmium, we turned to *Arabidopsis thaliana* as a model species, since its whole genome is sequenced and knockout mutants of several *SnRK2*s are available.

According to phylogenetical analysis, the closest homologues of NtOSAK in *A. thaliana* are SnRK2.4 and SnRK2.10 (Supplemental materials, Figure S2A). Therefore, we first studied the activity of these enzymes in Arabidopsis seedlings exposed to $CdCl_2$. The kinase activity was analyzed by immunocomplex kinase activity assay using anti-NtOSAK/SnRK2.4/SnRK2.10 antibodies raised against an N-terminal peptide of SnRK2.4, SnRK2.10, and NtOSAK. Indeed, the first 11 N-terminal amino acids of SnRK2.4, SnRK2.10, and NtOSAK are identical (Supplemental materials, figure S2B). Since in other SnRK2s, but not in any other Arabidopsis protein, sequences exhibiting high similarity to the antigen are present, the antibodies can to some extent recognize also other SnRK2s. The specificity of the antibodies used is presented in Supplemental Materials (Supplemental materials, Figure S3).

Analysis of the kinase activity by immunocomplex-activity assay showed activation of SnRK2(s) in ten-day-old Arabidopsis seedlings exposed to $CdCl_2$ and to sorbitol, used as a positive control (Figure 3A). To investigate whether the activation is due to NO produced *via* the NR or AtNOA1 pathways, we studied Cd^{2+}-dependent SnRK2 activation in hydroponically grown seedlings of *nia1nia2* (double knockout mutant of NR) and *atnoa1* Arabidopsis T-DNA insertion mutants. The *atnoa1* and *nia1nia2* mutants had previously been shown to produce less NO in response to several biotic as well as abiotic stresses (Besson-Bard et al., 2008; Zhao et al., 2009; Sun et al., 2010; Hao et al., 2010; Lozano-Juste and León, 2010; Xuan et al., 2010). In both *nia1nia2* and *atnoa1* seedlings, we observed activation of SnRK2(s) in response to $CdCl_2$ treatment (Figure 3B). These results indicate that neither NR nor AtNOA1 contribute to the SnRK2 activation in Arabidopsis seedlings exposed to Cd^{2+}.

In order to individually analyze the activation of SnRK2.4 and SnRK2.10, the closest homologues of NtOSAK, a transient expression system was applied. We produced either of the two kinases as an EGFP-fusion protein (EGFP-SnRK2.4 or EGFP-SnRK2.10), in Arabidopsis protoplasts. Changes in the kinase activity of each fusion protein in response to 200 µM $CdCl_2$ or 300 mM NaCl were monitored by in-

gel kinase activity assay using MBP as a substrate. The results indicated that both kinases were slightly activated by cadmium, albeit their activation was much lower than the activation observed in response to salinity stress (Figure 3C).

SnRK2.4 and *SnRK2.10* Expression in Response to Cd^{2+}

The level of *SnRK2.4* and *SnRK2.10* transcripts was analyzed by RT-PCR in ten-day-old Arabidopsis seedlings untreated and treated with 100 µM $CdCl_2$ for various time (0, 1, 3, 6, 12 h). We did not observe any significant changes in the amount of the *SnRK2.4* or *SnRK2.10* transcripts in plants exposed to cadmium stress for up to 12 hours (Figure 3D). Therefore, the observed transient Cd^{2+}-dependent activation of these kinases is not due to changes in the expression level of the respective genes. We also analyzed the level of *SnRK2.4* and *SnRK2.10* transcripts in roots of four-week-old Arabidopsis grown in hydroponic culture and subjected to 20 µM $CdCl_2$ for 48 h. In this case, the level of *SnRK2.4* and *SnRK2.10* transcripts was significantly higher than in untreated plants (Figure 3E). These results were confirmed by real time RT-PCR (Figure 3F); the expression level of *SnRK2.4* and *SnRK2.10* was about 150% and 175% of control, respectively.

NtOSAK and SnRK2.4 are Localized to Cytoplasm and Nucleus, whereas SnRK2.10 is Exclusively Cytoplasmic; Localization is not Influenced by Cadmium Stress

In order to determine the sub-cellular distribution of the tobacco and Arabidopsis kinases studied we investigated the localization of the EGFP-NtOSAK, EGFP-SnRK2.4 and EGFP-SnRK2.10 fusion proteins in plant protoplasts (tobacco or Arabidopsis, respectively), treated with 50 or 100 µM $CdCl_2$ for various time periods (0, 30, 60, 120 min). As shown in Figure 4, EGFP-SnRK2.10 was found exclusively in the cytoplasm, whereas EGFP-SnRK2.4 and EGFP-NtOSAK were present in the nucleus and cytoplasm. The localization of the kinases studied was not altered in response to cadmium salt. These data suggest that SnRK2.4 rather than SnRK2.10 is a functional homologue of NtOSAK in Arabidopsis. Therefore, we focused our further studies on SnRK2.4.

SnRK2.4 Contributes to Root Growth Sensitivity to $CdCl_2$

Cadmium ions strongly impair the growth of Arabidopsis seedlings. Therefore, to gain more insight into the role of SnRK2.4 in the plant response to cadmium, we analyzed cadmium-response phenotypes of two lines of *SnRK2.4* insertion mutants (*snrk2.4-1* and *snrk2.4-2*), which do not express *SnRK2.4* (Figure 5A and B), and of wild type Arabidopsis (Col-0). We measured root lengths of seedlings that were germinated for 10 days on control medium or medium supplemented with 30 or 100 µM $CdCl_2$ (Figure 5C and D). In the absence of $CdCl_2$, we did not observe any significant differences in root growth between the analyzed lines. As expected, increasing concentrations of $CdCl_2$ progressively inhibited the growth of seedling roots in all the lines. However, the roots of both insertion mutants were about 20% longer than the roots of wild type plants when grown on medium with 30 µM $CdCl_2$. On plates with 100 µM cadmium, the roots of *snrk2.4-1* and *snrk2.4-2* were, respectively, about 20% and 45% (±5%) longer than the roots of wild type seedlings. The data suggest that SnRK2.4 contributes to the inhibition of root elongation caused by cadmium ions.

SnRK2.4 Regulates Phytochelatins Accumulation in Plants Exposed to Cadmium Ions

Plants' tolerance to cadmium greatly depends on the level of thiol-containing molecules, both high molecular weight (e.g., phytochelatins and methalothioneins) as well as low-molecular-weight compounds (e.g., glutathione), in plant tissues. Taking this into consideration, we analyzed the total amount of thiol groups in roots of *snrk2.4* mutants and wild type Arabidopsis plants not treated and treated with 20 µM $CdCl_2$ for two days. The obtained results showed that the level of thiol groups is considerably higher in plants exposed to cadmium stress. However, our data did not show any significant differences between the lines studied, the two *snrk2.4* mutants and wild type plants (Figure 6A).

Subsequently, we estimated levels of GSH and of three major species of phytochelatins (PC2, PC3, and PC4) in roots of *snrk2.4* mutants and Col-0 Arabidopsis exposed or not (control) to 20 µM $CdCl_2$ for two days. The GSH level was reduced after treatment with Cd^{2+} in all lines studied (Figure 6B), however, the

reduction was slightly less pronounced in the mutants than in wild type plants. As expected, PCs were not detected in control plants (data not shown), they were detected only in plants subjected to cadmium ions. We observed some differences in the phytochelatins level between roots of the mutants and wild type plants exposed to the stressor (Figure 6C). Surprisingly the PC3 level was lower in the mutants than in wild type plants. These results indicate that lower sensitivity of *snrk2.4* plants to cadmium does not result from alteration of the total amount of thiol-containing compounds or a higher accumulation of PCs. Additionally, the analysis showed that SnRK2.4 is most probably involved in regulation of PCs synthesis in plants exposed to cadmium ions.

SnRK2.4 Regulates Level of ROS Produced in Arabidopsis Roots in Response to Cadmium Ions

Cadmium ions induce oxidative stress in plants (Schützendübel et al, 2001; Olmos et al., 2003; Garnier et al., 2006), which contributes to the metal toxicity (Cho and Seo, 2005; Sharma and Dietz, 2008). Therefore, we analyzed the level of ROS induced by cadmium treatment in roots of five-day-old Arabidopsis seedlings exposed or not to 50 µM $CdCl_2$ for 30 min. ROS level was monitored in roots of *snrk2.4-1*, *snrk2.4-2* and wild type (Col-0) plants using the fluorescent dye H_2DCFDA. In control conditions (no Cd^{2+}) the level of ROS in the mutants' roots was ca. 50% higher than in roots of the wild type seedlings. These differences were unexpected. We can speculate that in control conditions the higher ROS level in mutants than in the wild type plants can be due to the different sensitivity of the seedling lines studied to changes of the environment, mainly temperature and light, during progression of the experiment (e.g., transferring seedlings from incubator, placing them on slides, etc.). Under cadmium stress the responses of the wild type and the two mutants were dramatically different: while in Col-0 the stress brought about a substantial increase of ROS level (by ca. 80%), the mutants responded by an almost two-fold reduction of ROS level in comparison to control conditions (Figure 7).

SnRK2.4 is not Involved in Regulation of NO Production Induced by Cadmium Ions in Arabidopsis Roots

SnRK2s belong to the SNF1/AMPK-related protein kinase family. The activity of mammalian AMP-activated protein kinase (AMPK) is regulated by NO (Zhang et al., 2008). Moreover, AMPK itself plays a role in the regulation of NO production in response to several different stresses by phosphorylating eNOS and thus enhancing its activity (Chen et al., 1999; Morrow et al., 2003; Ritchie et al., 2010). Therefore, we decided to check whether SnRK2.4 is involved in the regulation of NO production in response to cadmium stress using DAF-2DA. NO level was investigated in roots of five-day-old Arabidopsis seedlings (*snrk2.4-1*, *snrk2.4-2* and wild type Col-0), after 7-hour treatment with 200 µM $CdCl_2$, with or without NO scavenger (250 µM cPTIO). A strong increase of NO level was observed in Cd^{2+} treated seedlings, when compared to control plants and the induction effect was strongly reduced in seedlings co-treated with cPTIO. However, we did not observe any differences in NO production in response to Cd^{2+} between the studied mutant lines and wild type Arabidopsis (Supplemental materials, Figure S4).

SnRK2.4 Regulates the Expression of Genes Involved in Iron Homeostasis in Plant Cells

There are strong data indicating that cadmium ions influx is closely connected with iron homeostasis. Cadmium is absorbed into plant roots mainly by iron transporter IRT1 (Iron-Responsive Transporter1) (Clemens 2001). IRT1 is a plasma-membrane cation transporter responsible for the uptake of ferrous ions, which can also transport several heavy metal ions including Cd^{2+}. IRT1 works in concert with the Fe^{3+} reductase FRO2 (Ferric Reductase Oxidase2) and their expression is under the control of the transcription factor FIT1 (Fe-deficiency Induced Transcription Factor1). An earlier paper reported that the expression of several genes was modulated by NO during Cd^{2+} treatment (Besson-Bard et al., 2009). Among them there were genes whose products are crucial for iron homeostasis: *IRT1, FRO2, FIT1 and NAS4 (*Nicotianamine Synthase4).

Since this and also other studies (Lamotte et al., 2006; Wawer et al., 2010) showed that NO participates in the activation of SnRK2s, we wondered whether SnRK2s play a role in controlling the expression of *IRT1, FRO2,* and *FIT1.* Therefore, we decided to analyze the expression of these genes in roots of *snrk2.4-1, snrk2.4-2*, and wild type Arabidopsis plants, untreated and treated with cadmium ions. Gene

expression was analyzed by real-time RT-PCR in roots of four-week-old *snrk2.4-1, snrk2.4-2*, and wild type Arabidopsis plants, untreated and treated with 20 µM $CdCl_2$ for 48 h. In untreated mutant plants, the expression of all three genes was lower by ca. 20-30%, compared with the wild type line (Figure 8). Upon cadmium treatment, the level of mRNA of *IRT1* and *FRO2* in Arabidopsis roots decreased significantly, to a different extent in each line studied, resulting in a similar level of expression of these genes in wild type and mutant plants.

DISCUSSION

Our understanding of the role of protein kinases in signaling pathways involved in the plant response to heavy metal stress is still rather elusive. The only convincing evidence is that MAPK cascades take part in the signal transduction triggered by heavy metal stress. Activation of several MAPKs in response to treatment with Cu or Cd ions has been observed in *Medicago sativa* (Jonak et al., 2004), *Arabidopsis thaliana* (Liu et al., 2010) and *Oryza sativa* (Yeh et al., 2007). Some MAPKs have been suggested to improve Cd tolerance of rice plants, since Cd-tolerant cultivars have a significantly increased MAPK activity (Yeh et al., 2007). However, there is no experimental data showing the specific role of MAPK(s) in the regulation of plant tolerance/sensitivity to heavy metal stress.

Our results indicate that treatment of BY-2 tobacco cells with Cd^{2+} at concentrations which cause MAPKs activation also stimulates SnRK2 activity. The time course of the SnRK2 activation is very similar to that observed for MAPKs**.** The MAPK activation in response to cadmium stress depends on ROS generation (Jonak et al., 2004; Yeh et al., 2007; Liu et al., 2010). The same is true for SnRK2 activation; pretreatment of tobacco BY-2 cells with catalase before addition of $CdCl_2$ significantly reduced NtOSAK (tobacco SnRK2) activity. Our results revealed that oxidative stress by itself induces SnRK2 activity in plant cells. Moreover, also NO plays a significant role in SnRK2s activation (Lamotte et al., 2006; Wawer et al., 2010, and present results). The NtOSAK activation by cadmium ions in BY-2 cells can be abolished by pretreatment of the cells with c-PTIO or inhibitors of mammalian NOS, which indicates that the NO responsible for the Cd^{2+}-dependent induction of SnRK2 activity is generated mainly *via* an L-arginine-dependent pathway involving a putative plant NOS-like enzyme. This suggestion is supported by the fact that the

induction of SnRK2 activity in response to cadmium ions was not decreased in the Arabidopsis mutants *nia1nia2* and *atnoa1*, in which enzymatic pathways involved in NO production, other than the L-arginine-dependent one, are non-functional. In fact the activation was even stronger in *nia1nia2* and *atnoa1* than in wild type Col-0 Arabidopsis. Our present data confirmed previous results indicating that NO production in response to cadmium ions in *A. thaliana* does not involve NR or NOA1 and is suppressed by mammalian NOS inhibitors (Besson-Bard et al., 2009; De Michele et al., 2009), suggesting that the Cd^{2+}-dependent NO synthesis is catalyzed by a so far unidentified enzyme. Concluding, the above results indicate that NtOSAK activation in response to Cd^{2+} treatment is triggered by ROS and NO produced in response to this stressor.

Since SnRK2s are activated in plants exposed to cadmium ions and because there are strong indications that in the lower plant *Chlamydomonas reinhardtii* SnRK2s are involved in sulfur metabolism (Davies et al., 1999; Irihimovitch and Stern, 2006; Gonzalez-Ballester et al., 2008), which is closely connected with regulation of heavy metal tolerance, we addressed the question of the role of SnRK2.4, a putative NtOSAK functional homologue in Arabidopsis, in the plant response to cadmium stress. For this purpose we applied a reverse genetics approach. First, we analyzed the effect of cadmium ions on root growth of wild type Col-0 and insertion mutants *snrk2.4-1* and *snrk2.4-2*. Roots of the studied mutants were significantly longer than roots of wild type plants grown on medium supplemented with 30 µM or 100 µM $CdCl_2$, indicating that *snrk2.4* plants are more tolerant to the stress. This result suggested that SnRK2.4 is an important element of plant response to cadmium. In plants, mainly thiol-containing compounds (phytochelatins, metallothioneins, and glutathione) are responsible for reduction of metal toxicity. Moreover, glutathione plays the role of a reducing agent in plant defense against oxidative stress, one of the most serious contributors to cadmium toxicity (for review see Jozefczak et al., 2012, Seth et al., 2012). Therefore, we analyzed the total amount of thiol groups as well as the level of major phytochelatin species and glutathione in roots of the mutants studied and in wild type plants exposed to cadmium ions. The results indicate that the *snrk2.4* mutants' enhanced tolerance to cadmium in comparison to wild type plants, observed in root growth test, is not due to a difference in the total level of thiol-containing compounds accumulating in plant tissues exposed to cadmium ions or even in the phytochelatins

concentration. The GSH level, after cadmium treatment, was only slightly higher in roots of the mutants, whereas surprisingly, levels of some PC species (especially, PC3) were lower in mutants than in Col-0 plants. These results suggest that PC synthase might be regulated by SnRK2.4 either at the level of transcription of *PCS*(s) or at the activity level. It was previously suggested that PCS could be positively controlled by post-translational modifications e.g., phosphorylation (Wang et al., 2009). Therefore, it is highly plausible that SnRK2s, activated in response to heavy metal ions, are involved in PCSs regulation.

Since oxidative stress is one of the major components of cadmium toxicity, in order to investigate the contribution of SnRK2.4 in Cd^{2+}-induced oxidative stress we monitored ROS level generated in response to cadmium in roots of *snrk2.4-1* and *snrk2.4-2* mutants and in Col-0 Arabidopsis. This analysis revealed that the ROS level is significantly lower in *snrk2.4* mutants than in wild-type seedlings. This is in accordance with a slightly higher GSH level in the mutants, however the differences in the GSH level seem to be too small to be responsible for the observed disparity. Therefore, we presume that there are some other factors accountable for differences in ROS accumulation in *snrk2.4* mutants and wild type Arabidopsis. Previous work showed that the Arabidopsis SnRK2 family member OST1/SnRK2.6 acts upstream of ROS production induced by ABA in guard cells (Mustilli et al., 2002). In a loss-of-function mutant (*ost1*), ABA treatment failed to increase the ROS level in guard cells, as it was observed in wild-type guard cells, and consequently did not induce stomatal closure. The impairment of the ABA-induced ROS production in the *ost1* mutant could be bypassed by externally applied H_2O_2. Recently, it was shown that NADPH oxidases could be SnRK2s' cellular substrates (for reviews see Hubbard et al., 2010; Umezawa et al., 2010; Joshi-Saha et al., 2011). A recombinant cytosolic part of one of Arabidopsis NADPH oxidases, AtrbohF, produced in a bacterial system is phosphorylated by SnRK2.6/OST1 *in vitro.* Moreover, the kinase interacts with AtrbohF in bimolecular fluorescence complementation (BIFC) assay, suggesting that NADPH oxidase could be a SnRK2.6/OST1 substrate *in vivo* (Sirichandra et al., 2009). AtrbohF and AtrbohD (another NADPH oxidase), which are abundant in plant roots, are involved in ROS production in response to several abiotic and biotic stresses (Sagi and Fluhr, 2006; Ma et al., 2011; Mariano et al., 2011; Suzuki et al., 2011). It was shown that phosphorylation and Ca^{2+} binding are needed for AtrbohD and AtrbohF activation (Ogasawara et al., 2008; Kimura et al., 2012). Therefore, it is

possible that SnRK2.4, and maybe also other SnRK2 kinases, participate in AtrbohD and/or AtrbohF phosphorylation and activation in plant roots exposed to a toxic concentration of cadmium salt. In this way SnRK2s can be involved in a positive feedback loop in ROS accumulation: the kinases are activated by ROS and in turn they most probably participate in ROS production.

It is well established that ROS produced by NADPH oxidases are required for activation of Ca^{2+} channels (Murata et al., 2001, Kwak et al., 2003; Foreman et al., 2003), and Ca^{2+} channels are implicated in Cd^{2+} uptake (for review see Clemens, 2006). Considering this, SnRK2 could have an impact on Cd^{2+} uptake by influencing ROS accumulation. However, we did not see any significant differences in cadmium level between the *snrk2.4* mutants and Col-0 Arabidopsis grown on medium with a toxic level of $CdCl_2$ (30 µM) (data not shown). This lack of differences in cadmium accumulation between the lines studied could be explained e.g., by the relatively high Ca^{2+} concentration in the medium; Ca^{2+} is a potent inhibitor of Cd^{2+} uptake (via Ca^{2+} channels) and its toxicity (Rodríguez-Serrano et al., 2009).

As it was mentioned before, Cd^{2+} enters plant cells efficiently through iron transporters (for review see Clemens, 2006). There is strong evidence that IRT1 is responsible for Cd^{2+} uptake by plant roots. Therefore, iron deficiency, which induces *IRT1* expression, stimulates heavy metal transport into plants (Cohen et al., 1998; Besson-Bard et al., 2009). Our results indicate that the *snrk2.4* mutants studied have a significantly lower expression of *IRT1* in comparison with wild-type plants, but only in the absence of Cd^{2+}. In roots of the *snrk2.4* mutants we observed not only reduction of *IRT1* expression, but also reduction of expression of two other genes involved in iron homeostasis and iron uptake, *FRO2* and *FIT*. We did not see this phenomenon in Cd^{2+}-treated roots; the expression of genes mentioned was basically the same in the *snrk2.4* mutants and wild type plants exposed to a toxic concentration of cadmium. Right now, we can only speculate about the reasons responsible for the lack of those differences in stressed plants. It is highly likely that in plants growing in medium with a high concentration of cadmium, several different signaling pathways are induced which are involved in regulation of *IRT1*, *FRO2* and *FIT1* expression, and those are not connected or only slightly connected with the SnRK2 pathway. Considering the role of SnRK2 in the plant response to cadmium, we should keep in mind the localization of SnRK2. Our results showed that SnRK2.4 is present both in the nucleus and in the cytoplasm and its localization does not

change upon stress. However, we do not know whether in response to cadmium both pools of the kinase (nuclear and cytoplasmic) are activated; it could well be that only the cytoplasmic pool is active, which is not involved in regulation of gene expression but is responsible for phosphorylation of cytoplasmic or membrane proteins, e.g., NADPH oxidase. On the other hand, it is possible that during growth in normal conditions a low activity of both nuclear and cytoplasmic SnRK2.4 and maybe also other SnRK2s is needed for normal plant development and iron homeostasis. Previous studies showed that NO positively regulates expression of *IRT1*, *FRO2*, and *FIT1* (Besson-Bard, et al., 2009). Since NO is a key element in NtOSAK activation (Lamotte et al., 2006, Wawer et al., 2010) and iron homeostasis is regulated by NO (for review see: Ramirez et al., 2010), we speculated that NO regulates expression of these genes at least partly by regulation of SnRK2's activity. This might indicate that to some extent SnRK2 could be involved in cadmium uptake when the level of Cd^{2+} is very low, not recognized by the plant as toxic, but not when the Cd^{2+} concentration is relatively high (in the µM range).

Concluding, we show that SnRK2s, plant-specific kinases known so far to be involved in regulation of plant development and defense against osmotic stress (Fujii and Zhu, 2009; Nakashima et al., 2009; Fujii et al., 2011), also take part in the plant response to heavy metal stress probably by influencing ROS accumulation. The obtained results are summarized in a hypothetical model of the role of SnRK2 in plant response to the stress induced by cadmium ions (Figure 9). We cannot exclude the role of SnRK2s in the regulation of other processes involved in the plant response to this stress, for example by controlling anti-oxidative enzymes' and metal transporters' activity, as well as expression of various stress-related genes. It is noteworthy that osmotic stress (water deficit and salinity) influences the plant response to heavy metal stress (Xu et al., 2010; Lefèvre et al., 2010) and that the Cd^{2+}-response signaling pathways overlap and cross-talk with different abiotic stress pathways (Dal Corso et al., 2010). It should be stressed out that the common signaling molecules, ROS and NO, triggering and regulating plant responses to various environmental conditions are key elements contributing to activation of SnRK2s studied by a variety of abiotic stresses (e.g., salinity and stress induced by heavy metal ions). Most probably therefore, SnRK2s similarly to MAPKs, which are activated by several different stresses also play a role in the plant response to heavy metal stress.

MATERIALS AND METHODS

Plant Lines and Growth Conditions

Tobacco Bright Yellow 2 (BY-2) cell suspension culture, kindly provided by Dr. Witold Filipowicz (Friedrich-Miescher Institute, Basel, Switzerlad), was cultured as described elsewhere (Nagata et al., 1992; Burza et al., 2006). The cells were subcultured every 7 days. BY-2 cells were treated with different stressors for indicated time, as described in Results, harvested by centrifugation, frozen in liquid nitrogen, and stored at -80 ^{o}C until analyzed.

Several different *Arabidopsis thaliana* lines were used: *A. thaliana* ecotype Columbia (Col-0), wild type; *atnoa1* (former *atnos1*, kindly provided by N.M. Crawford, University of California, USA); the nitric reductase (NR) double mutant *nia1nia2* (gift from C. Meyer, INRA Versailles, France); *snrk2.4-1* (SALK_080588) and *snrk2.4-2* (SALK_146522) T-DNA insertion lines were obtained from the Nottingham Arabidopsis Stock Center (NASC) (http://signal.salk.edu/cgi-bin/tdnaexpress) (Alonso et al., 2003). Homozygous plants were selected by PCR screening using gene specific primers in combination with the left-border insert specific primer (LBb1.3). The level of *SnRK2.4* expression was analyzed by RT-PCR. All primers used in this study are listed in Supplemental data 1.

Plants were grown at 24ºC / 22ºC under long day conditions (16 h-light / 8h-dark cycle) in hydroponic culture (Araponic System) using the following medium: 3.5 mM KNO_3, 2.5 mM $Ca(NO_3)_2$, 1 mM $Mg(NO_3)_2$, 5 mM NH_4NO_3, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 0.9 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM NaCl, 50 µM Fe-Na-EDTA, 0.64 µM $Cu(NO_3)_2$, 10 µM $Mn(NO_3)_2$, 0.82 µM $(NH_4)_2MoO_4$, 0.096 µM zinc acetate, 0.11 µM $CoCl_2$, 25 µM H_3BO_3, 1 mM MES pH 5.8. Roots of four-week-old plants, not treated or treated with 20 µM $CdCl_2$ for 2 days, were harvested, frozen in liquid nitrogen and stored at -80^{o}C until analyzed.

For aseptic cultures, seeds were sterilized by gentle shaking in 70% ethanol for 2 minutes and then incubating in water : bleach solution 13:1 (v:v) for 20 minutes. Finally, the seeds were extensively washed five times with sterile water. For sterile hydroponic culture about 100 seeds were imbibed at 4ºC for 5 days to synchronize germination onset and grown for 10 days in 300-ml Erlenmeyer flasks containing 100 ml of ½ Murashige and Skoog (MS) medium supplemented with ½ MS Vitamin

Solution, 43 mg/L Fe-Na-EDTA, 500 mg/L MES, 10 g/L sucrose, pH 5.7. Seedlings were treated with different stressors as indicated in Results, harvested, frozen in liquid nitrogen and stored at -80ºC until analyzed. For seedling culture on Petri plates for use in microscopic observations, seeds were sterilized as described above and plants were grown vertically in medium containing ½ MS salts, ½ MS Vitamin Solution, 20 g/L sucrose and 8 g/L agar, pH 5.7. For determination of seedlings' resistance to cadmium ions we plated sterile seeds on Petri plates on medium prepared according to Besson-Bard et al. (2009) with or without addition of $CdCl_2$ (final concentration 30 or 100 µM). For transient expression experiments protoplasts were isolated from six-day-old *Arabidopsis thaliana* T87 or tobacco BY-2 cells. Arabidopsis suspension cultures were grown in Gamborg B5 medium as described by Yamada et al. (2004). Cells were subcultured every 7 days.

All chemicals were from Sigma/Aldrich with the exception of sucrose, which was from Merck.

Protoplast Transient Expression Assay

Protoplasts were isolated and transformed *via* PEG treatment according to the protocol provided by He et al. (2007) with minor modifications recently described (Bucholc et al., 2010). After transformation Arabidopsis T87 protoplasts were suspended in WI incubation solution (0.5 M mannitol, 4 mM MES (pH 5.7), 20 mM KCl), whereas BY-2 protoplasts were transferred into K4 medium (MS salt, MS vitamins, 0.4 M sucrose, 250 mg/L xylose, 0.1 mg/L 2,4D, 0.2 mg/L BAP, 1 mg/L NAA) and incubated at 25^{o}C in the dark for two days. cDNAs encoding NtOSAK, SnRK2.4 and SnRK2.10 were inserted into the pSAT6-EGFP-C1 vector provided by Prof. T. Tzfira (University of Michigan, Ann Arbor, MI, USA). cDNAs encoding SnRK2.4 and SnRK2.10 were PCR-amplified using *Pfu* DNA polymerase and appropriate plasmids obtained from the Nottingham Arabidopsis Stock Center (NASC) as templates and cloned into pCR II- TOPO (Invitrogen). Primers are listed in Supplemental data 1. The sequences were verified by DNA sequencing and cloned as EcoRI*/*SalI (*SnRK2.4* and *SnRK2.10*) or XhoI/SalI (*NtOSAK*) fragments into pSAT6-EGFP-C1 vector. In each transformation about $2x10^5$ protoplasts were transfected with about 50 µg of plasmid DNA. The transfected protoplasts were

subjected to 200 µM $CdCl_2$ or 500 mM NaCl treatment. In control experiments, water instead of the stressor was added to the transfected protoplasts. The transformed protoplasts were used for analysis of the Cd^{2+} effect on EGFP-SnRK2.4 and EGFP-SnRK2.10 activities (measured by in gel-kinase activity assay) and for visualization of the cellular localization of EGFP-NtOSAK, EGFP-SnRK2.4, and EGFP-SnRK2.10 in response to Cd^{2+} treatment. Visualization was performed as described previously (Bucholc et al., 2010; Wawer et al., 2010).

RT-PCR Analysis

Total RNA was isolated from untreated and stressor-treated BY-2 cells or three-week-old Arabidopsis seedlings (separately roots and leaves) using TRI REAGENT (MRC, Cincinnati, OH) according to the procedure recommended by the manufacturer. cDNA was synthesized from 1 µg of total RNA using HSRT 100 kit (Sigma-Aldrich). Briefly, RNA was reverse transcribed for 60 min at 47˚C in 20 µL of reaction mixture containing 1 unit of enhanced avian reverse transcriptase, 500 µM each dNTP, 3.5 µM anchored oligo(dT) primer, 1 unit of RNase inhibitor. Two microliters of the RT reaction mixture was used for PCR in 20 µL containing 0.2 units of *Taq* DNA polymerase (EURX, Gdańsk, Poland), 200 µM each dNTP, 1.5 mM $MgCl_2$, and 625 nM appropriate primers. Routine PCR conditions were: 3 min, 94˚C (first cycle); 30s, 94˚C; 30s, 49 or 54 ˚C; 30 s, 72˚C (27 cycles); and 10 min, 72˚C (final cycle). PCR products were separated on 0.8% agarose gels and visualized by EtBr staining. Primers are listed in Supplemental data 1.

Real Time RT-PCR Analysis

Real-time PCR analysis of gene expression (*SnRK2.4*, *SnRK2.10*, *IRT1*, *FIT*, *FRO2)* was performed by two-step qPCR. Total RNA was extracted from Arabidopsis roots (control or treated for two days with 20 µM $CdCl_2$) with RNeasy Plant Mini Kit (QIAGEN) and DNase-digested with TURBO DNA-free kit (Ambion), according to standard manufacturer's protocol. RNA quality was checked on Nanodrop 1000 and 2100 Bioanalyzer (Agilent). Reverse transcription of 1 µg of RNA was performed in 20-µL reaction using SuperScript III reverse transcriptase (Invitrogen) and random pentadecamers, with some modifications of standard manufacturer's protocol.

Following RNA digestion with RNase H (Ambion), each cDNA sample was diluted 5x and stored. Expression level was assayed by quantitative PCR in a Rotorgene 3000 (Corbett Research) device, using MESA GREEN qPCR MasterMix Plus for SYBR Green I No ROX (Eurogentec). Following amplification, a meltcurve was performed in the 60-95 °C range, with 0.5°C steps. For each target gene amplification, two gene-specific primers were used (listed in Supplemental data 1) and all cDNA samples (three replicates) and standards (two replicates) were assayed in a single run. Relative gene expression in each sample was calculated using standard curve method (5-point), normalized using a geometric mean of expression values for four reference genes (*PDF2*, *SAND*, *YLS8*, *F-Box*) and scaled to the calibrator sample (Col-0 control). RNA quality analysis and test qPCR reactions proved that the material obtained for comparative studies was of high quality and free of amplification inhibitors or genomic DNA contamination (Supplemental figures S5 and S6). Detailed description of the experiment setup, analysis and primer design, according to MIQE guidelines (Bustin et al. 2009, http://www.rdml.org/miqe.php), is presented in Supplemental Protocol.

Preparation of Protein Extracts

Protein extracts were prepared as previously described (Mikołajczyk et al., 2000; Burza et al., 2006; Wawer et al., 2010).

Immunoblotting

Western blotting was performed as described previously (Burza et al., 2006). Antibodies used:

- polyclonal anti-NtOSAK antibodies raised against the C-terminal peptide (KQVKQAHESGEVRLT) of the kinase (BioGenes, Berlin, Germany);
- polyclonal anti-NtOSAK/SnRK2.4/SnRK2.10 antibodies raised against the N-terminal peptide (MDKYELVKDIG) of these kinases (BioGenes, Berlin, Germany);
- phospho-specific polyclonal antibodies raised in rabbit against the phosphopeptide (KS(P)TVGT) and purified by affinity chromatography (BioGenes, Berlin, Germany);
- antibodies recognizing active MAPKs - phospho - p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cell Signaling Technology).

Immunoprecipitation

Immunoprecipitation was performed as described previously (Mikołajczyk et al., 2000; Burza et al., 2006; Wawer et al., 2010) using anti-C-terminal NtOSAK or anti-N-terminal NtOSAK/SnRK2.4/SnRK2.10 antibodies. Briefly, Protein A-agarose (10 μL per sample) was incubated for 2 hours with antibodies (24 μg for anti-C-terminal NtOSAK or 120 μg for anti-N-terminal NtOSAK/SnRK2.4/SnRK2.10 per sample) at 4^{o}C with gentle shaking. After incubation agarose beads were pelleted by brief centrifugation and unbound antibodies were removed by triple washing protein A-agarose with immunoprecipitation buffer. The equal volume of protein A-agarose with bound antibodies was added to protein extract (150 or 300 μg in the case of BY-2 cells or Arabidopsis plants, respectively) and incubated for 4 hours at 4^{o}C with gentle shaking. Agarose beads-protein complexes were pelleted by brief centrifugation, washed three times with 1 mL of immunoprecipitation buffer and two times with 1 mL of 20 mM Tris-HCl pH 7.5 buffer.

Immunocomplex Kinase Activity Assay

After immunoprecipitation Laemmli sample buffer was added to the pelleted protein complexes attached to protein A-agarose and the sample was heated at 95°C for 3 min with vigorous shaking. After brief centrifugation the supernatant was analyzed by in-gel kinase activity assay.

In-gel Kinase Activity Assay

In-gel kinase activity assays were performed according to Zhang and Klessig (1997).

Determination of thiol-group content

The content of thiol groups [SH] was analyzed by modified Ellman test (Ellmam, 1959) according to the procedure described by Wawrzyński et al. (2006). Roots of

four-week-old Arabidopsis plants of wild type, *snrk2.4-1,* and *snrk2.4-2* lines grown in hydroponic culture, non-treated and treated with 20 µM $CdCl_2$ for 48 hours, were analyzed. About 100 mg of root tissue was powdered in liquid nitrogen and after homogenization the tissue was treated with 500 µL of cold 0.1 M HCl with vigorous mixing. After treatment samples were centrifuged (10 min, 20 000 x g). Aliquots of 200 µL of supernatant, 775 µL of 0.5 M K_2HPO_4, and 25 µL of DTNB (5,5'-dithio-2-nitrobenzoic acid) (Sigma) were mixed together and incubated for 2 min at room temperature. After incubation the optical density was measured at 412 nm. The amount of thiols was calculated from difference between the OD of the sample with and without DTNB as nmoles per gram of fresh weight.

The level of PCs and GSH was determined in plant leaves and roots according to the procedure described previously (Wojas et al., 2008). 200-300 mg of previously frozen powdered plant material were homogenized in 1.78 mL of cold 6.3 mM diethylenetriaminepenta-acetic acid (DTPA), 100 µL of 1 M NaOH, and 100 µL of 6M $NaBH_4$ (in 0.1 M NaOH). N-acetyl-L-cysteine at final concentration 10 µM was added to each sample as an internal standard. The homogenized samples were centrifuged (5 min, 10 000 x g) and 250 µL of the obtained extracts were mixed with 10 µL of 20 mM monobromobimane and 450 µL of HEPPS buffer, pH 8.2 containing 6.3 mM DTPA. Derivatization was performed at 45ºC in the dark. The reaction was stopped after 30 min with 300 µL of 1 M methanesulphonic acid. Finally the samples were filtered through 0.22 µm filters and stored at 4ºC in the dark until HPLC analysis. Thiol-compounds were separated on Nova –Pak C_{18} column (Waters) at 37ºC and were eluted with methanol-water gradient, both with 0.1 % TFA (trifluoro-acetic acid). The injection volume was 20 µL. For calibration GSH at concentration from 5 up to 20 µM was used.

The data were integrated using Waters Millenium Software.

Reactive Oxygen Species Detection

ROS production in Arabidopsis roots was analyzed using 2',7'-dichlorodihydrofluoresceine diacetate (H_2DCFDA, Invitrogen) basically according to the procedure described in (Murata et al., 2001). Five-day-old *Arabidopsis thaliana* seedlings of *snrk2.4-1* and *snrk2.4-2* mutants and wild type (Col-0) were incubated in ½ MS medium without (control) or with 50 µM $CdCl_2$ for 30 minutes, rinsed with

medium for 2 minutes, stained with 20 µg/mL propidium iodide (PI, Invitrogen) for 2 minutes, rinsed with medium, and stained with H_2DCFDA (30 µM) for 20 min. The excess of the dye was removed by three washes with the medium. Whole seedlings were carefully placed on slides with home-made chambers preventing damage and drying. Images were collected with a 20x (NA 0.75) Plan Fluor multi-immersion objective mounted on an inverted epifluorescence TE 2000E microscope (Nikon) coupled with an EZ-C1 confocal laser scanning head (Nikon). Fluorescence images are z-stack projections assembled from ten optical sections collected with 10 µm steps made with the standard EZ-C1 Nikon software. The fluorescence of H_2DCFDA was excited with blue light at 488 nm emitted by a 40 mW argon-ion laser (Melles Griot). H_2DCFDA fluorescence was detected with a 515/30 nm band-pass filter and rendered in false green. The fluorescence of PI was excited with green light at 543 nm emitted by a 1 mW He-Ne laser (Melles Griot) and was detected with a 590 nm long-pass filter and rendered in false red. All confocal parameters (laser power, gain, etc.) and conditions were the same during each experiment. NIS-Elements AR 3.0 software was used to quantify the total intensity of H_2DCFDA fluorescence signal in all images rendered from z-stack projections. Each experimental variant was repeated at least three times with 6 to 10 z-stacks collected in each series.

Nitric Oxide Detection

NO production in Arabidopsis roots was analyzed according to the method described by Besson-Bard et al. (2009) using 4,5-diaminofluorescein diacetate (DAF-2DA, Sigma-Aldrich) fluorescent dye. Five-day-old seedlings were incubated in 1 µM DAF-2DA in 50 mM Tris-HCl pH 7.5 solution for 2 hours in darkness. Then, roots were washed three times with distilled water (to remove excess of the dye) and transferred into 50 mM Tris-HCl pH 7.5 (control) or 50 mM Tris-HCl pH 7.5 supplemented with 200 µM $CdCl_2$. The fluorescence of DAF-2T was observed after 30 minutes or 7 hours of incubation using a Leica epifluorescence microscope model DMRB. Images were collected with an RGB 3CCD camera (Sony) and for DAF-2T fluorescence observation a standard GFP filter was used. Pictures were analyzed by visilog 5.4 (Noesi) software and the fluorescence was quantified using the NIS-Elements BR 3.0 software.

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FIGURE LEGENDS:

Figure 1. MAPK and SnRK2 are activated in plant cells in response to cadmium stress.

Six-day-old BY-2 suspension cell cultures were treated with various concentrations of $CdCl_2$ (0-1000 μM) (A) or $CdSO_4$ (0-200 μM) (G) for 30 min, or with 100 μM $CdCl_2$ for various time (0-90 min) (B-F). Protein kinase activity in cells untreated and treated with the stressor was monitored by in-gel kinase activity assay with MBP as a substrate (A-B, D, and G), or by western blotting (C, E). D and G, activity of NtOSAK analyzed by immunocomplex kinase activity assay using specific anti-C-terminal NtOSAK antibodies. C, immunoblot probed with anti-phospho – p44/42 MAPK antibodies. E, immunoblot probed with anti-Ser-158(P) antibodies. F, NtOSAK

protein level determined by western blotting with specific anti-C-terminal NtOSAK antibodies.

Figure 2. Cadmium induces NtOSAK activation in NO- and ROS-dependent manner.

BY-2 cells were treated with 100 µM $CdCl_2$ without (A) or with pretreatment with: 500 µM cPTIO for 1 h (B), 1000 U/mL catalase for 30 min (C), 300 µM L-NAME for 10 min (D), 1 mM sodium tungstate for 30 min or 24 h (E). NtOSAK activity in BY-2 cells was detected by immunocomplex in-gel kinase activity assay using specific anti-C-terminal NtOSAK antibodies and MBP as a substrate. Additionally, NtOSAK activity was monitored by immunocomplex kinase activity assay in BY-2 cells treated with 2 mM H_2O_2 for various time (F). NtOSAK protein level determined by western blotting with specific anti-C-terminal NtOSAK antibodies in protein extracts before immunoprecipitation (♦).

Figure 3. Effect of cadmium ions on SnRK2.4 and SnRK2.10 activity and on *SnRK2.4* and *SnRK2.10* transcript level.

Ten-day-old seedlings of *Arabidopsis thaliana* wild type Col-0, as well as insertion mutants *nia1nia2* and *atnoa1* were treated with 750 mM sorbitol or with different concentration of $CdCl_2$: 150 µM, 300 µM or 500 µM - Col-0 (A) and 300 µM - *nia1nia2* and *atnoa1* (B). Activity of SnRK2.4/SnRK2.10 in seedling extracts was monitored by immunocomplex in-gel kinase activity assay using specific antibodies against N-terminal peptide of both kinases and MBP as a substrate. (C), *EGFP-SnRK2.4* and *EGFP-SnRK2.10* were transiently expressed in Arabidopsis T87 protoplasts. Protoplasts were treated with 200 µM $CdCl_2$ or 300 mM NaCl for 30 min and activity of EGFP-SnRK2.4 and EGFP-SnRK2.10 was analyzed by in-gel kinase activity assay with MBP as a substrate. (D), ten-day-old Col-0 Arabidopsis seedlings were treated with 100 µM $CdCl_2$ for various time (0-12 h), total RNA was isolated and transcript level of *SnRK2.4* and *SnRK2.10* was analyzed by semi-quantitative RT-PCR. As a control *Actin2* transcript level was monitored. (E), four-week-old Col-0 Arabidopsis plants grown in hydroponic culture were treated with 20 µM cadmium chloride for two days and transcript level of *SnRK2.4 and SnRK2.10* was monitored in plant roots by RT-PCR. The results were confirmed by qRT-PCR (F).

Figure 4. Localization of NtOSAK, SnRK2.10, and SnRK2.4 before and after cadmium treatment.

Tobacco protoplast were prepared from BY-2 cells and transformed with EGFP-NtOSAK construct. The kinase localization was monitored before and after 30 min treatment with 50 µM $CdCl_2$. In both conditions NtOSAK localized to cytoplasm and nucleus (A). Arabidopsis protoplasts were prepared from T87 cells and transformed with EGFP-SnRK2.4, EGFP-SnRK2.10 or EGFP constructs. Localization of the fluorescent proteins was observed before or after 30-min treatment with 50 µM $CdCl_2$. EGFP-SnRK2.10 protein was observed only in cytoplasm in both conditions, whereas EGFP-SnRK2.4 was localized simultaneously to cytoplasm and nucleus in control and in cadmium-containing medium. n - indicates position of the nucleus.
Data represent one of several independent experiments showing similar results.

Figure 5. SnRK2.4 regulates root growth in response to cadmium ions.

Sites of T-DNA insertions in *SnRK2.4* gene (A). *SnRK2.4* transcript levels in leaves of *snrk2.4-1* (SALK_080588) and *snrk2.4-2* (SALK_146522), and Col-0 determined by semi-quantitative RT-PCR (B). Seeds of wild type Arabidopsis and both mutants were sterilized and sown on square Petri plates on control media or media supplemented with 30 or 100 µM $CdCl_2$. Seeds were vernalized for 3 days at 4^oC in darkness and germinated in standard germination condition. After 14 days of growth, root length was measured by ImageJ free software (C). Values correspond to means ±SD of three independent experiments. Asterisk means statistically significant difference between root length of the mutants and wt plants ($p<0.05$). (D) Image of seedlings after 14 days of growing on control media or media supplemented with 30 or 1000 µM $CdCl_2$ (bar =1 cm). Data represent one of three independent experiments showing similar results.

Figure 6. Analysis of the level of thiol-containing compounds in roots of *snrk2.4* mutants and wild type Arabidopsis plants.

Four Four week-old *A. thaliana* Col-0 wild type mutant plants grown in hydroponic culture were treated with 20 µM $CdCl_2$ for two days. Non-protein thiol level was measured by Ellman's test (A). Concentration of GSH (B) and major PC species (C) in cadmium-treated plants was analyzed in roots according to method described by

Wojas et al., 2008. Asterisk means statistically significant difference in the concentration of GSH and PCs between wt and mutants according to ANOVA One Way followed by Dunnets test ($p<0.05$). Values correspond to means ±SD *(n=3)*.

Figure 7. SnRK2.4 is involved in regulation of ROS level in Arabidopsis seedlings exposed to Cd^{2+}.

Five-day-old seedlings of *snrk2.4-1* and *snrk2.4-2* mutants and wild type Col-0 were incubated for 30 minutes in ½ MS medium (control) or medium with 50 µM $CdCl_2$, rinsed with medium and stained with PI (20 µg/mL) and H_2DCFDA (30 µM), as described in Materials and Methods. Stained roots were observed in inverted epifluorescence microscope coupled with EZ-C1 confocal laser scanning head. Fluorescent and DIC images are z-stacks projected from ten collected images. A, wild type (1, 2), *snrk2.4.1* (3, 4), and *snrk2.4.2* (5, 6) roots incubated in control (1, 3, 5) or cadmium - containing medium (2, 4, 6), stained with H_2DCFDA (DCF fluorescence in ROS presence), PI (nuclei in dead cells stained), and both images merged with DIC images. B, total intensity of DCF fluorescence in all images rendered from z-stack projections calculated with NIS-Elements AR 3.0 software. Data represent the mean ±SD of 18 to 30 images collected from three independent experiments. Asterisk means statistically significant difference between ROS level of mutant and wt plants ($p<0.05$).

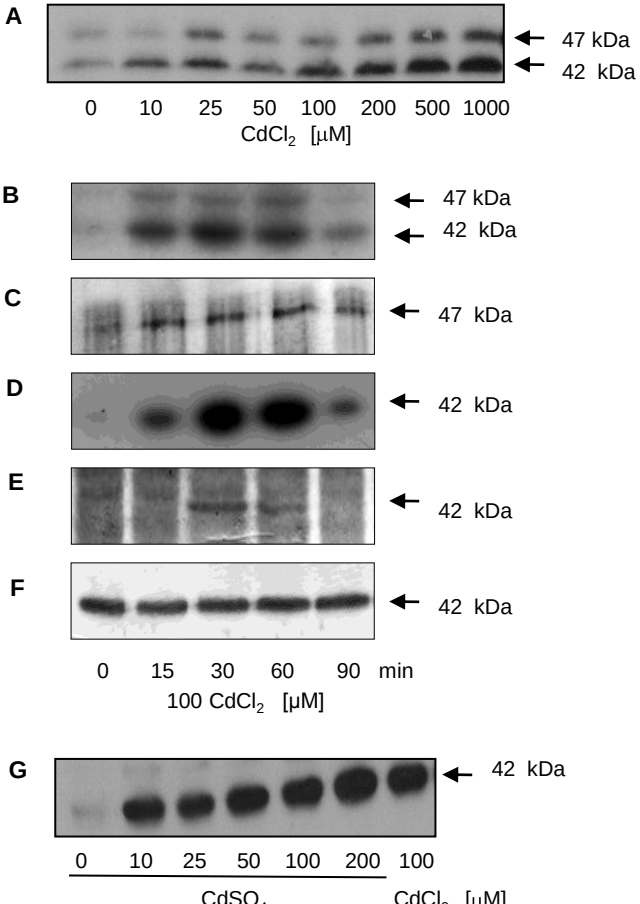
Figure 8. Effect of *SnRK2.4* gene mutation on expression of genes involved in iron transport and homeostasis.

Transcript level was monitored by qRT-PCR in roots of four-week-old plants, not treated or treated with 20 µM $CdCl_2$ for 48 h (+$CdCl_2$) and normalized against geometric mean of four houskeeping genes. In *snrk2.4-1* and *snrk2.4-2* knockout lines expression of each gene is plotted relative to that in non-treated wild-type plants (Col-0). Standard deviation bars represent SD of three replicates of each sample within experiment.

Figure 9. A hypothetical model for SnRK2 role in Arabidopsis roots exposed to Cd^{2+}

Cadmium ions induce NO and ROS accumulation in plant roots, which contribute to SnRK2 activation. Active SnRK2 stimulates Cd^{2+}-dependent ROS accumulation in

plant cells, most probably by phosphorylation and activation of NADPH oxidase(s). SnRK2 kinases contribute to the increase of the level of PCs presumably by regulating the activity or expression level of PCS. Enhanced Cd^{2+}-induced PC synthesis resulting in an increased metal tolerance might, however, cause further accumulation of ROS, as a consequence of GSH depletion.



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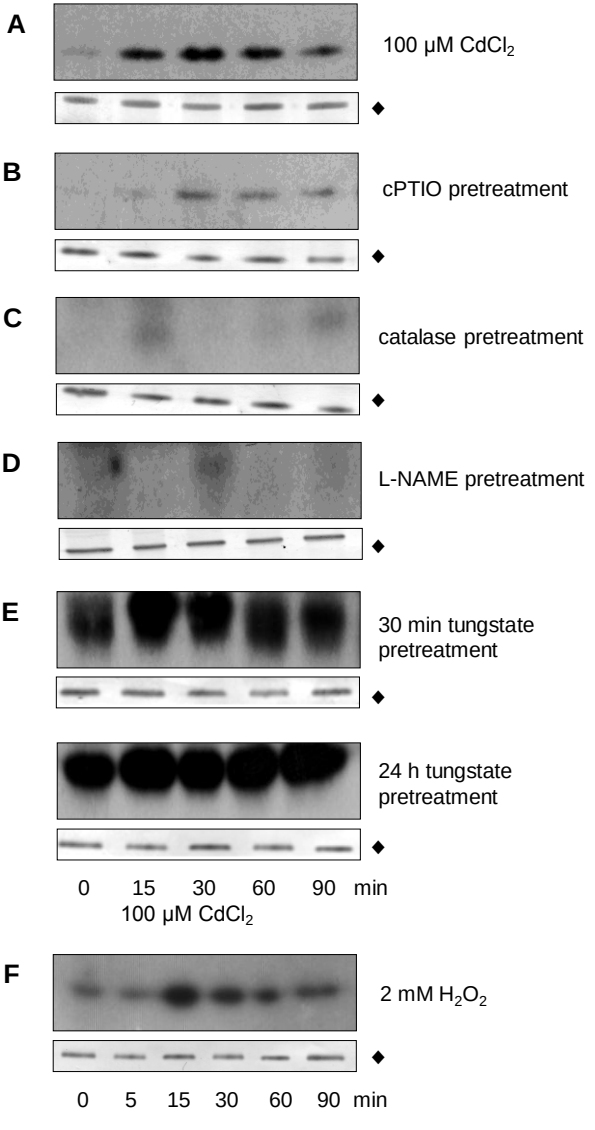

Figure 2. Cadmium induces NtOSAK activation in NO- and ROS-dependent manner.

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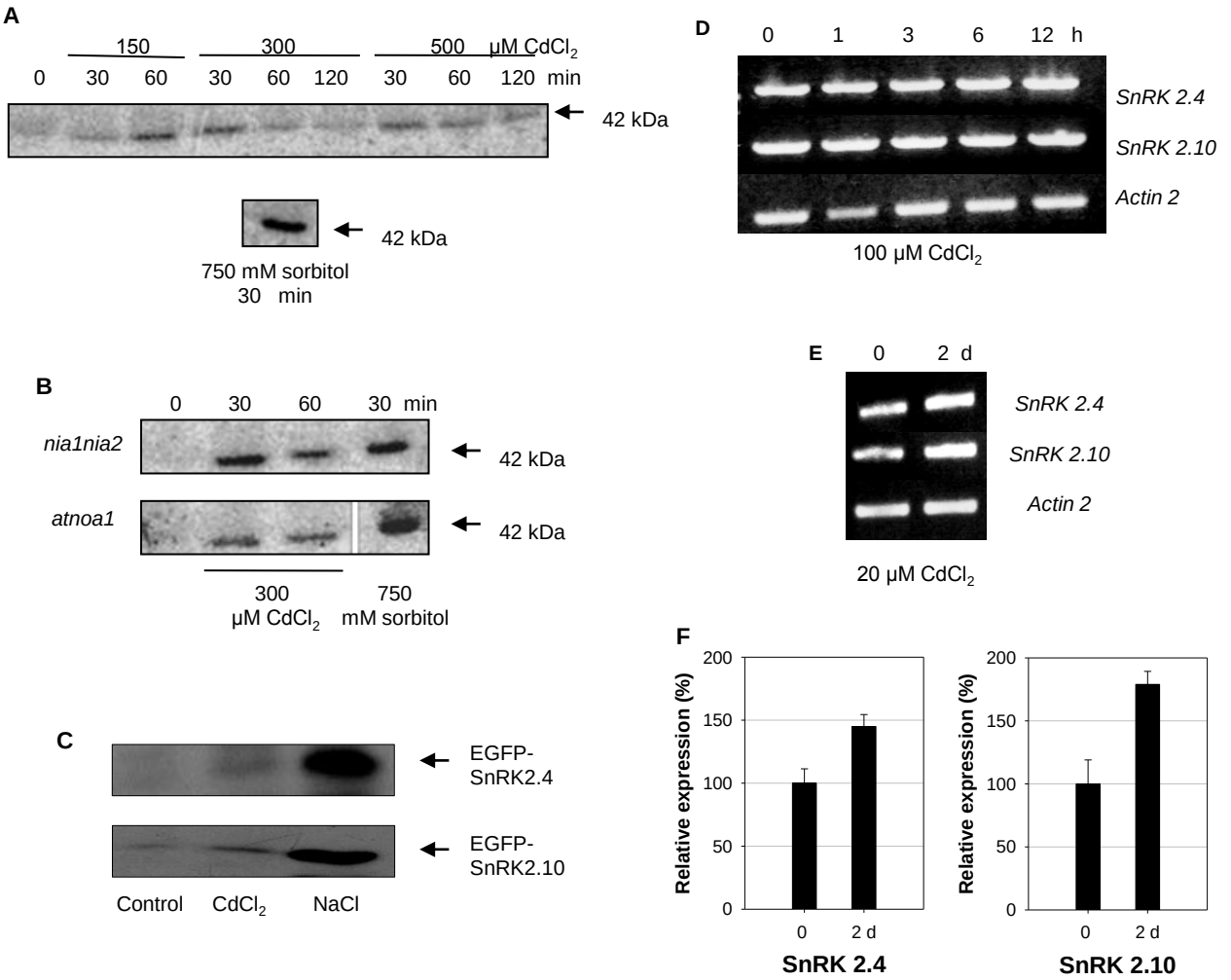

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Ten-day-old seedlings of *Arabidopsis thaliana* wild type Col-0, as well as insertion mutants *nia1nia2* and *atnoa1* were treated with 750 mM sorbitol or with different concentration of $CdCl_2$: 150 μM, 300 μM or 500 μM - Col-0 (A) and 300 μM - *nia1nia2* and *atnoa1* (B). Activity of SnRK2.4/SnRK2.10 in seedling extracts was monitored by immunocomplex in-gel kinase activity assay using specific antibodies against N-terminal peptide of both kinases and MBP as a substrate. (C), *EGFP-SnRK2.4* and *EGFP-SnRK2.10* were transiently expressed in Arabidopsis T87 protoplasts. Protoplasts were treated with 200 μM $CdCl_2$ or 300 mM NaCl for 30 min and activity of EGFP-SnRK2.4 and EGFP-SnRK2.10 was analyzed by in-gel kinase activity assay with MBP as a substrate. (D), ten-day-old Col-0 Arabidopsis seedlings were treated with 100 μM $CdCl_2$ for various time (0-12 h), total RNA was isolated and transcript level of *SnRK2.4* and *SnRK2.10* was analyzed by semi-quantitative RT-PCR. As a control *Actin2* transcript level was monitored. (E), four-week-old Col-0 Arabidopsis plants grown in hydroponic culture were treated with 20 μM cadmium chloride for two days and transcript level of *SnRK2.4 and SnRK2.10* was monitored in plant roots by RT-PCR. The results were confirmed by qRT-PCR (F).

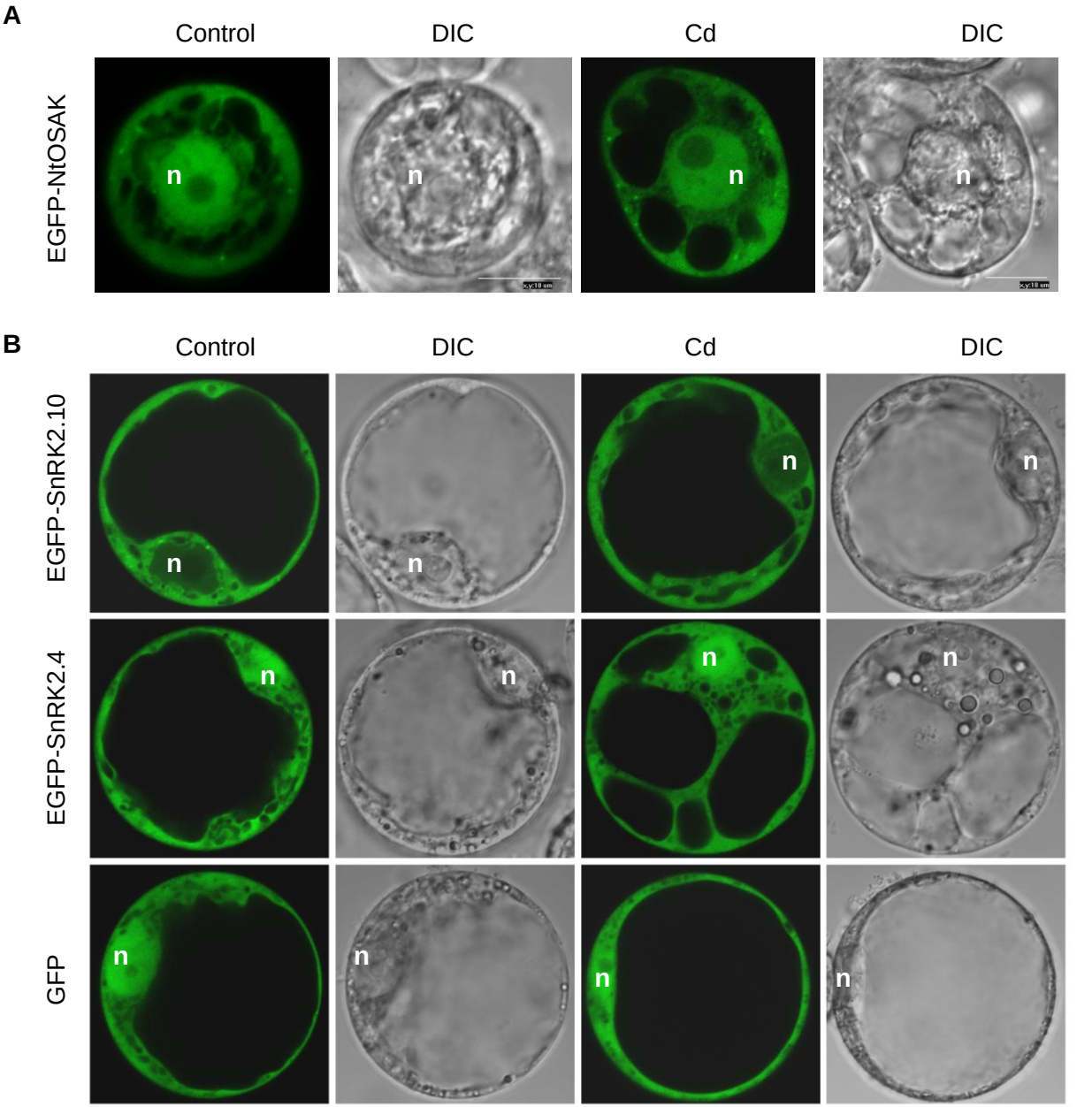


Figure 4. Localization of NtOSAK, SnRK2.10, and SnRK2.4 before and after cadmium treatment.

Tobacco protoplast were prepared from BY-2 cells and transformed with EGFP-NtOSAK construct. The kinase localization was monitored before and after 30 min treatment with 50 μM $CdCl_2$. In both conditions NtOSAK localized to cytoplasm and nucleus (A). Arabidopsis protoplasts were prepared from T87 cells and transformed with EGFP-SnRK2.4, EGFP-SnRK2.10 or EGFP constructs. Localization of the fluorescent proteins was observed before or after 30-min treatment with 50 μM $CdCl_2$. EGFP-SnRK2.10 protein was observed only in cytoplasm in both conditions, whereas EGFP-SnRK2.4 was localized simultaneously to cytoplasm and nucleus in control and in cadmium -containing medium. n - indicates position of the nucleus.

Data represent one of several independent experiments showing similar results.

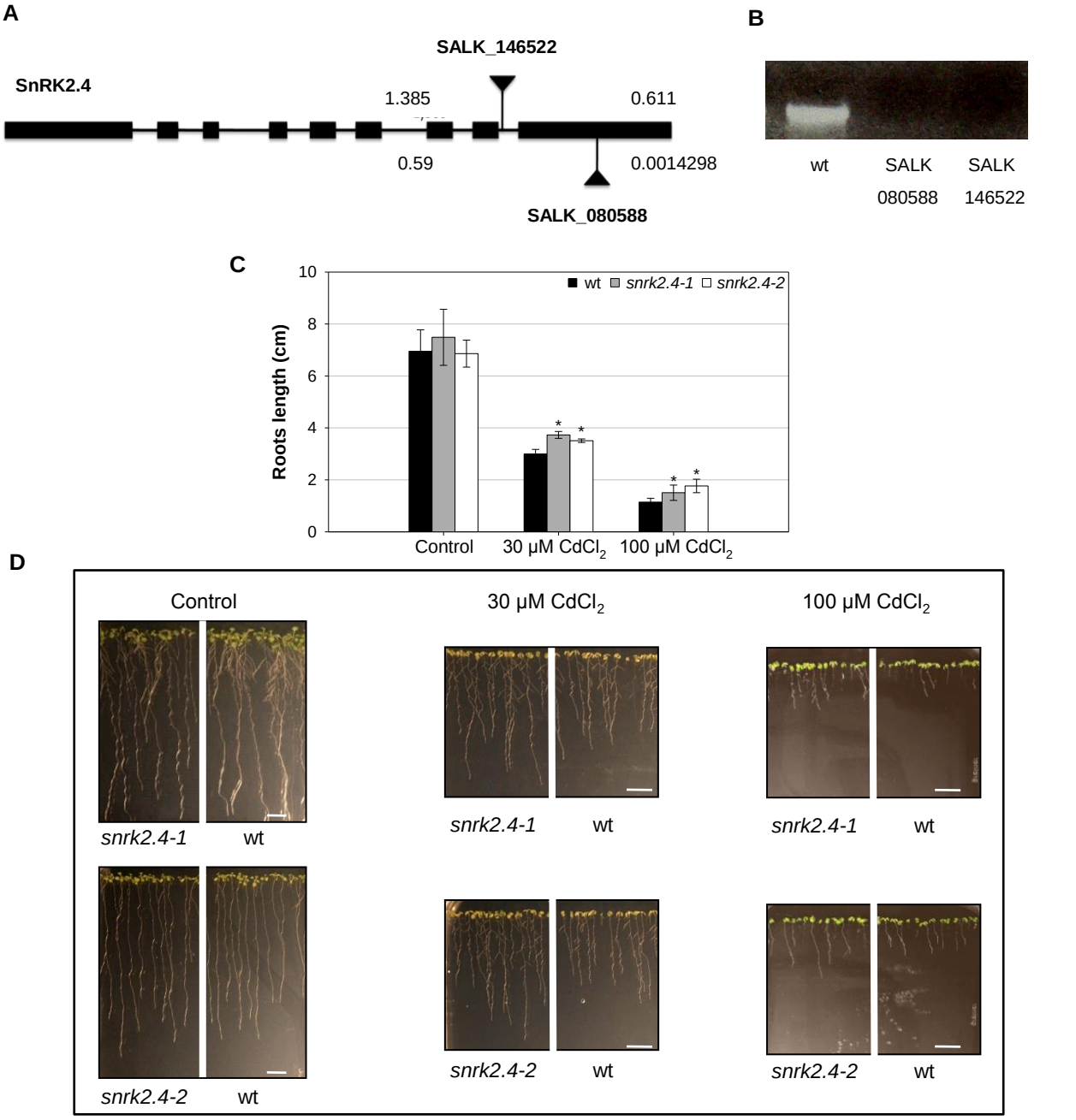


Figure 5. SnRK2.4 regulates root growth in response to cadmium ions.

Sites of T-DNA insertions in *SnRK2.4* gene (A). *SnRK2.4* transcript levels in leaves of *snrk2.4-1* (SALK_080588) and *snrk2.4-2* (SALK_146522), and Col-0 determined by semi-quantitative RT-PCR (B). Seeds of wild type Arabidopsis and both mutants were sterilized and sown on square Petri plates on control media or media supplemented with 30 or 100 μM $CdCl_2$. Seeds were vernalized for 3 days at 4ºC in darkness and germinated in standard germination condition. After 14 days of growth, root length was measured by ImageJ free software (C). Values correspond to means ±SD of three independent experiments. Asterisk means statistically significant difference between root length of the mutants and wt plants ($p<0.05$). (D) Image of seedlings after 14 days of growing on control media or media supplemented with 30 or 1000 μM $CdCl_2$ (bar =1 cm). Data represent one of three independent experiments showing similar results.

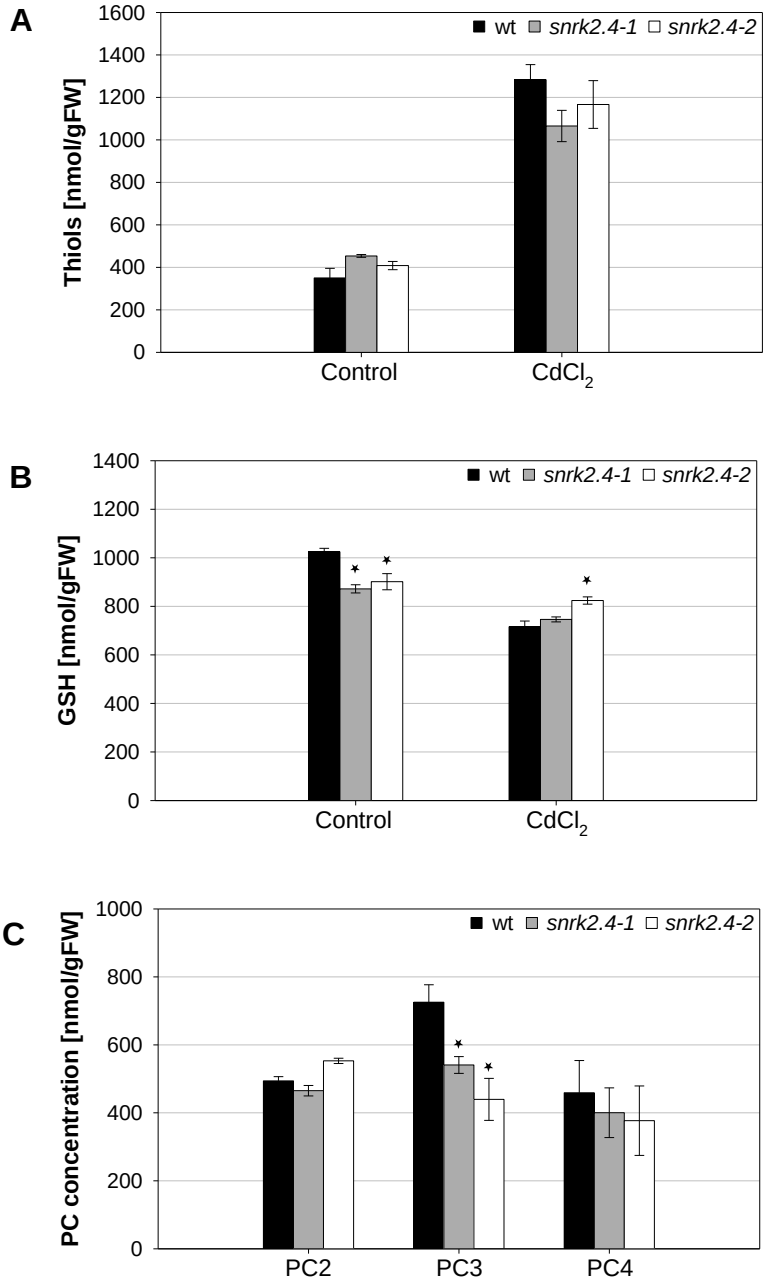


Figure 6. Analysis of the level of thiol-containing compounds in roots of *snrk2.4* mutants and wild type Arabidopsis plants.

Four week-old *A. thaliana* Col-0 wild type mutant plants grown in hydroponic culture were treated with 20 μM $CdCl_2$ for two days. Non-protein thiol level was measured by Ellman's test (A). Concentration of GSH (B) and major PC species (C) in cadmium-treated plants was analyzed in roots according to method described by Wojas et al., 2008. Asterisk means statistically significant difference in the concentration of GSH and PCs between wt and mutants according to ANOVA One Way followed by Dunnets test ($p<0.05$). Values correspond to means ±SD *(n=3)*.

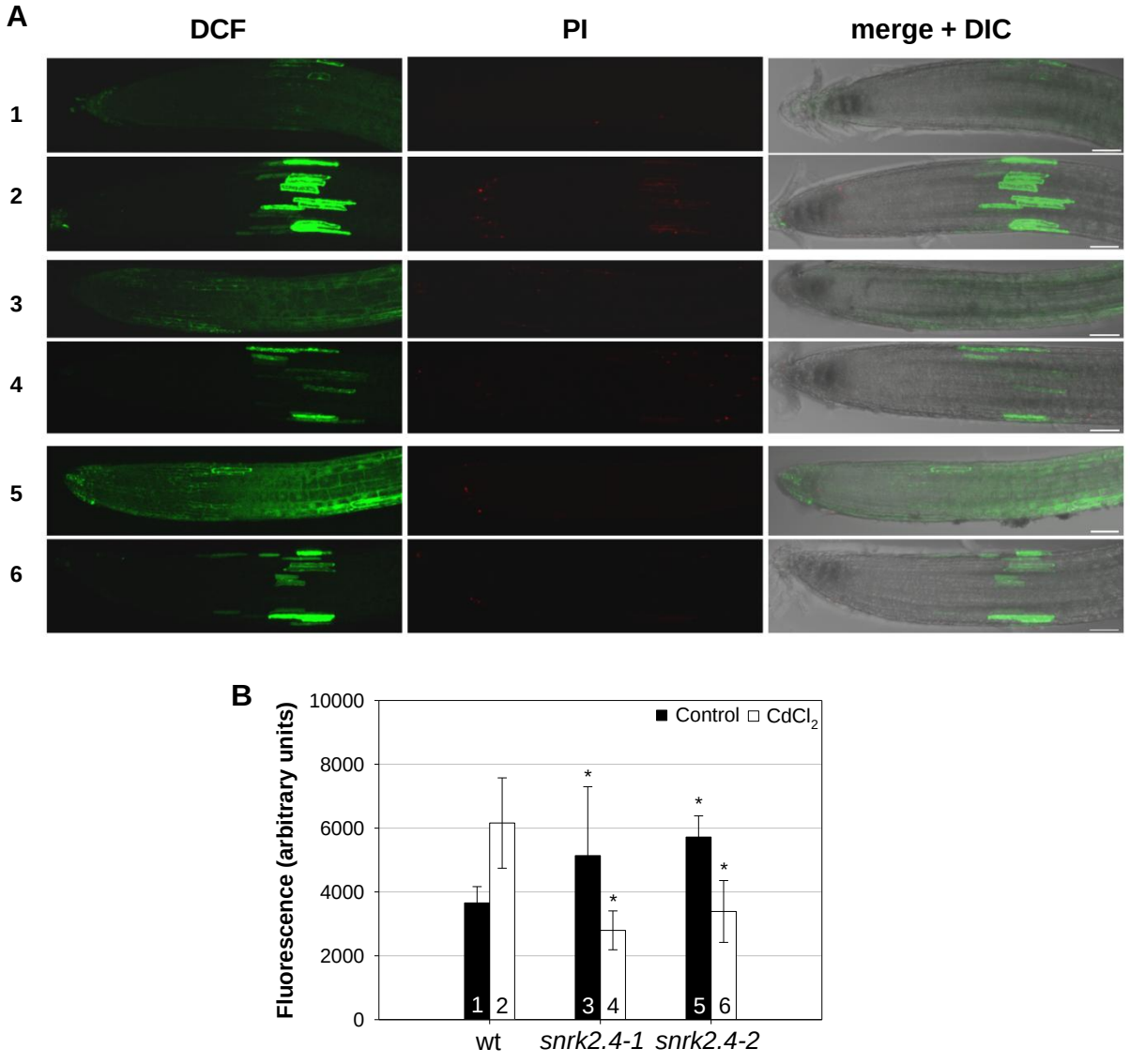
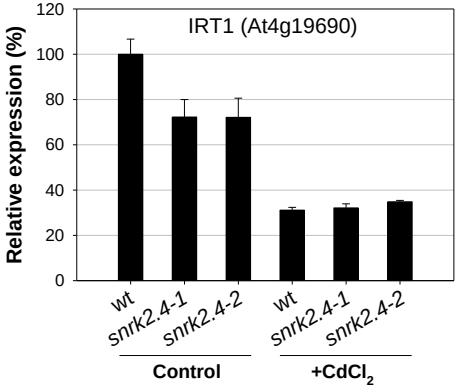
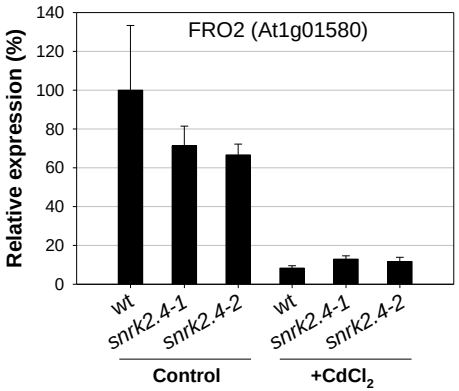


Figure 7. SnRK2.4 is involved in regulation of ROS level in Arabidopsis seedlings exposed to Cd^{2+}.

Five-day-old seedlings of *snrk2.4-1* and *snrk2.4-2* mutants and wild type Col-0 were incubated for 30 minutes in ½ MS medium (control) or medium with 50 µM $CdCl_2$, rinsed with medium and stained with PI (20 µg/mL) and H_2DCFDA (30 µM), as described in Materials and Methods. Stained roots were observed in inverted epifluorescence microscope coupled with EZ-C1 confocal laser scanning head. Fluorescent and DIC images are z-stacks projected from ten collected images. A, wild type (1, 2), *snrk2.4.1* (3, 4), and *snrk2.4.2* (5, 6) roots incubated in control (1, 3, 5) or cadmium - containing medium (2, 4, 6), stained with H_2DCFDA (DCF fluorescence in ROS presence), PI (nuclei in dead cells stained), and both images merged with DIC images. B, total intensity of DCF fluorescence in all images rendered from z-stack projections calculated with NIS-Elements AR 3.0 software. Data represent the mean ±SD of 18 to 30 images collected from three independent experiments. Asterisk means statistically significant difference between ROS level of mutant and wt plants ($p<0.05$).





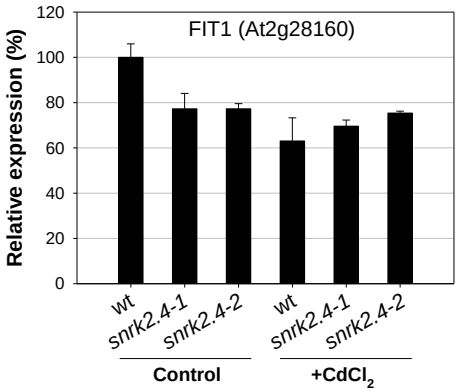


Figure 8. Effect of *SnRK2.4* gene mutation on expression of genes involved in iron transport and homeostasis.

Transcript level was monitored by qRT-PCR in roots of four-week-old plants, not treated or treated with 20 μM $CdCl_2$ for 48 h (+$CdCl_2$) and normalized against geometric mean of four housekeeping genes. In *snrk2.4-1* and *snrk2.4-2* knockout lines expression of each gene is plotted relative to that in non-treated wild-type plants (Col-0). Standard deviation bars represent SD of three replicates of each sample within experiment.

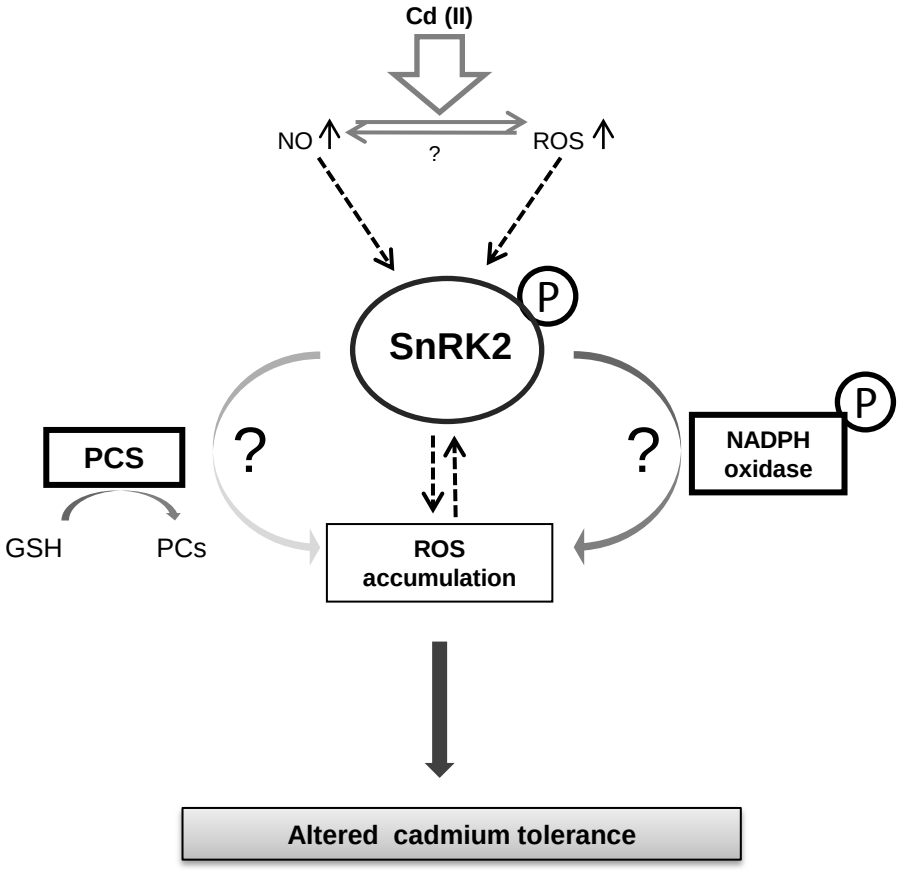


Figure 9. A Hypothetical model for SnRK2 role in Arabidopsis roots exposed to Cd^{2+}

Cadmium ions induce NO and ROS accumulation in plant roots, which contribute to SnRK2 activation. Active SnRK2 stimulates Cd^{2+}-dependent ROS accumulation in plant cells, most probably by phosphorylation and activation of NADPH oxidase(s). SnRK2 kinases contribute to the increase of the level of PCs presumably by regulating the activity or expression level of PCS. Enhanced Cd^{2+}-induced PC synthesis resulting in an increased metal tolerance might, however, cause further accumulation of ROS, as a consequence of GSH depletion.