Title: Phosphoproteomic Analysis Reveals that Dehydrins ERD10 and ERD14 are Phosphorylated by SNF1-related Protein Kinase 2.10 in Response to Osmotic Stress

Short running title: Phosphorylation of Dehydrins ERD10 and ERD14 by SnRK2.10

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Abstract

SNF1-related protein kinases 2 (SnRK2s) regulate the plant responses to abiotic stresses, especially water deficits. They are activated in plants subjected to osmotic stress, and some of them are additionally activated in response to enhanced concentrations of abscisic acid (ABA) in plant cells. The SnRK2s that are activated in response to ABA are key elements of ABA signaling that regulate plant acclimation to environmental stresses and ABA-dependent development. Much less is known about the SnRK2s that are not activated by ABA, albeit several studies have shown that these kinases are also involved in response to osmotic stress. Here, we show that one of the *Arabidopsis thaliana* ABA-non-activated SnRK2s, SnRK2.10, regulates not only the response to salinity but also the plant sensitivity to dehydration. Several potential SnRK2.10 targets phosphorylated in response to stress were identified by a phosphoproteomic approach, including the dehydrins ERD10 and ERD14. Their phosphorylation by SnRK2.10 was confirmed *in vitro*. Our data suggest that the phosphorylation of ERD14 within the S-segment is involved in the regulation of dehydrin subcellular localization in response to stress.

Introduction

Drought and salinization are the major environmental challenges for plants. All plants have the ability to sense environmental cues and activate the signaling pathways responsible for the induction of plant responses. However, plants differ significantly with respect to their tolerance to various stresses. The SNF1-related protein kinases 2 (SnRK2s) are indispensable to the plant reaction to water deficits (for review see Umezawa et al., 2010; Kulik et al., 2011; Fujii and Zhu, 2012; Yoshida et al., 2015; Zhu, 2016). SnRK2s are plant-specific enzymes that are rapidly and transiently activated in response to osmotic stress. They have been classified into three groups based on their phylogenetic analysis; the classification correlates with their response to abscisic acid (ABA) (Boudsocq et al., 2004; Kobayashi et al., 2004). Group 1 comprises kinases not activated in response to ABA, group 2 kinases are not activated (e.g., in *Oryza sativa*) or only weakly activated by ABA (e.g., in *Arabidopsis thaliana*), and group 3 kinases are strongly activated in response ABA. To date, the mechanism of activation and the physiological role have mainly been investigated for the ABA-activated SnRK2s (from group 3). It has been established that these kinases are key components of ABA signaling pathways, both in plant development (seed maturation and germination) (Fujii et al., 2007; Fujii and Zhu, 2009; Nakashima et al., 2009) and in response to water deficits (Fujii and Zhu, 2009; Fujita et al., 2009). Several independent experimental approaches have been used to identify ABA-activated SnRK2 target proteins. Thus, OST1/SnRK2.6/SRKE phosphorylates ion channels involved in stomatal movements: SLAC1 (Slow Anion Channel-Associated 1 - Geiger et al., 2009; Lee et al., 2009), KAT1 (K^+ channel - Sato et al., 2009), the NADPH oxidase RbohF (Respiratory burst oxidase homolog protein F - Sirichandra et al., 2009), the aquaporin PIP2;1 (Plasma membrane Intrinsic Protein2;1 - Grondin et al., 2015), BRM (SWI/SNF chromatin-remodeling ATPase BRAHMA - Peirats-Llobet et al., 2016) and numerous transcription factors regulating the expression of ABA-responsive genes (Kobayashi et al., 2005; Furihata et al., 2006; Yoshida et al., 2015).

Comparative phosphoproteomic studies between an Arabidopsis triple *snrk2.2/2.3/2.6* (also known as *srk2dei*) mutant deficient in all three ABA-activated SnRK2s (SnRK2.2/SRK2D, SnRK2.3/SRK2I, and SnRK2.6/SRK2E) and wild-type plants treated with ABA (Umezawa et al., 2013; Wang et al., 2013) or subjected to

desiccation (Umezawa et al., 2013) allowed the identification of several new targets of those kinases. Studies of both groups have confirmed that the ABA-activated kinases phosphorylate AREB-type transcription factors, several protein kinases and RNA- or DNA-binding proteins. Moreover, some novel potential SnRK2 targets (e.g., the proteins involved in flowering time regulation and chloroplast functioning; Wang et al., 2013) have been identified. Among the SnRK2.2/2.3/2.6 targets, Umezawa et al. (2013) identified a protein named SNS1 (from SnRK2-substrate 1). SNS1 is conserved in higher plants. An *sns1* knockout mutant exhibited the ABA-hypersensitive phenotype, indicating that SNS1 is a negative regulator of ABA signaling at the postgermination stage (Umezawa et al., 2013).

There are several indications that kinases from group 2 (SnRK2.7 and SnRK2.8) are also involved in stress signaling. They play a role in drought response (Umezawa et al., 2004; Mizoguchi et al., 2010), mainly by regulating the expression of stress-response genes. The cellular targets of SnRK2.7 and SnRK.8 comprise various transcription factors involved in abiotic stress responses (Mizoguchi et al., 2010; Kim et al., 2012). Additionally, several 14-3-3 proteins and enzymes (e.g., glyoxalase I, adenosine kinase I, and ribose 5-phosphate isomerase) have been found to be phosphorylated by SnRK2.8 (Shin et al., 2007). It has been shown that SnRK2.8 is also involved in biotic stress response. Recently, Lee et al. (2015) showed that SnRK2.8 phosphorylates Nonexpresser of Pathogenesis-Related genes 1 (NPR1), which is involved in systemic acquired resistance in response to pathogen infection. The phosphorylation of NPR1 catalyzed by SnRK2.8 is necessary for its nuclear import.

Much less is known about the role of the members of group 1 of the SnRK2 family - the kinases not activated in plants upon ABA-treatment. Several reports have indicated an involvement of these kinases in the response to osmotic stress. Thus, the ABA-non-activated kinases SnRK2.4 and SnRK2.10 regulate the root architecture in response to salinity (McLoughlin et al., 2012): SnRK2.4 regulates primary root growth, and SnRK2.10 regulates lateral root number under stress conditions. An analysis of multiple *snrk2* knockout mutants showed that plants deficient in kinases from groups 2 and 1 are affected by osmotic stress even more strongly than is the *snrk2.2/2.3/2.6* triple mutant (Fujii et al., 2011), indicating that ABA-non-activated SnRK2s also regulate plant tolerance to osmotic stress. However, the accumulation of proline induced by osmotic stress in the *snrk2.1/2.4/2.5/2.9/2.10*

mutant was higher, whereas in *snrk2.2/2.3/2.6*, it was significantly lower than that in the wild-type plants (Fujii et al., 2011), which suggests that the roles of the ABA-activated and the ABA-non-activated SnRK2s in the regulation of plant tolerance to osmotic stress have to be, to some extent, different. Recently, published data showed that the ABA-non-activated SnRK2s regulate mRNA decay under osmotic stress (Soma et al., 2017). Using a coimmunoprecipitation approach, VARICOSE (VCS), an mRNA decapping activator, has been identified as an SnRK2.1 cellular partner. SnRK2.1 and other ABA-non-activated SnRK2s phosphorylate VCS, and the phosphorylation has a substantial effect on mRNA decay. To date, no other *bona fide* cellular targets of the ABA-non-activated SnRK2s have been found.

In vitro screening of peptides phosphorylated by recombinant SnRK2.10 has revealed that its phosphorylation consensus site is LXRXXS (Vlad et al., 2008). An analysis of Arabidopsis protein databases has indicated that such sequences are present in several proteins involved in stress response, e.g., dehydrin LEA (At2g21490), dehydrin Xero 1 (At3g50980), and glutathione peroxidase 6 (At4g11600) (Vlad et al., 2008). However, the phosphorylation of those proteins by SnRK2.10 *in vivo* has not yet been confirmed.

SnRK2.10 is unique among the Arabidopsis ABA-non-activated SnRK2s as it is the only one that localizes exclusively to the cytoplasm, whereas all the other members of this group are found in both the cytoplasm and the nucleus (Kulik et al., 2012; Soma et al., 2017, and Supplemental Figure S1). This suggests that its role may not be fully comparable to other SnRK2s.

Here, to establish the role of SnR2.10 in the plant response to environmental stresses, we identified several of its potential targets phosphorylated in response to salinity stress and analyzed the phosphorylation of two of them, ERD10 (Early Responsive to Dehydration 10) and ERD14, in detail.

Materials and Methods

Plant material and growth conditions

The *Arabidopsis thaliana* lines used in this work were all derivatives of Col-0: Col-0-wild type; T-DNA insertion lines: single mutants *snrk2.4-1* (*SALK_080588*), *snrk2.4-2* (*SALK_146522*), *snrk2.10-1* (*WiscDsLox233E9*) and *snrk2.10-3* (*SAIL_698_C05*)

(Sessions et al, 2002; Alonso et al., 2003; Woody et al., 2007)*;* a double mutant *snrk2.4/10* (*SALK_080588*/*WiscDsLox233E9*)*;* a quadruple mutant *snrk2.1/2.4/2.5/2.10* (*SAIL_519_C01*/*SALK_080588*/*SALK_075624*/ *WiscDsLox233E9*); and SnRK2.10-GFP expressing lines. The mutants *snrk2.4-1, snrk2.10-1,* and *snrk2.4/2.10*, as well as the *SnRK2.10-GFP* expressing lines, were kindly provided by Prof. Christa Testerink, the University of Amsterdam. The quadruple mutant described here was obtained by crossing *SAIL_519_C01, SALK_080588, SALK_075624*, and *WiscDsLox233E9* mutants (Supplemental Figure S2).

For the phosphoproteomic and gene expression analyses, the plants were grown in hydroponic culture (Araponics system) as described by Kulik et al. (2012). The roots of the 5-week-old plants that were not treated or treated with 250 mM NaCl for 30 min (for phosphoproteomic analysis) or 150 mM NaCl for up to 6 days (for gene expression analysis) were harvested, frozen in liquid nitrogen and stored at -80ºC until analysis.

For the in-gel kinase activity assay, the Arabidopsis seedlings were grown in sterile hydroponic culture in flasks as described by Kulik et al. (2012). Two-week-old plants were treated with 250 mM NaCl for 10 min, harvested by sieving and frozen in liquid nitrogen. The plant material was kept at -80°C until analysis.

For the transient expression assays, the *Nicotiana benthamiana* plants were grown in soil in a growth chamber under 60% relative humidity and with a day/night regime of 16 h light (23°C) / 8 h dark (19°C).

The Arabidopsis T87 cell line used for protoplast isolation was grown in Gamborg B5 medium as described by Yamada et al. (2004).

Sample preparation for MS analysis

Total protein extracts were prepared according to the method described by Tsugita and Kamo (1999) with modifications. To approximately 300 mg of ground root powder, 1 mL of prechilled 10% (w/v) trichloroacetic acid (TCA) / 0.07% dithiothreitol (DTT) in acetone was added, and the samples were incubated at -20ºC overnight. Then, they were centrifuged at 14 000 rpm for 15 min, and the supernatants were discarded. The pellets were washed three times by suspension in ice-cold acetone containing 0.07% DTT and centrifugation as above. Next, the pellets were dried at

room temperature in a SpeedVac for 10 min and suspended in 300 µL of lysis buffer [30 mM Tris, pH 8.5, 7 M urea, 2 M thiourea, 4% CHAPS and PhosStop; (Roche)], incubated overnight at 4ºC, and centrifuged as above. Then, the supernatants were collected.

Mass spectrometry

The dissolved samples were subjected to an in-solution trypsin digestion procedure. The proteins were reduced with 50 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) at 60°C for 30 min, alkylated with 200 mM methyl methanethiosulfonate (MMTS) at room temperature for 15 min, and digested overnight with trypsin (Sequencing Grade Modified Trypsin - Promega V5111). The peptide mixtures were analyzed by LC/MS (liquid chromatography coupled to tandem mass spectrometry) using a Nano-Acquity LC system (Waters) and an Orbitrap Velos or Q Exactive mass spectrometer (Thermo Electron Corp., San Jose, CA) as detailed below. The samples for the phosphorylation site analysis were split in two; approximately 20% of the total volume was directly analyzed by LC/MS for protein identification, and the remaining 80% was subjected to the enrichment of the phosphorylated peptides on titanium dioxide as described previously (Graczyk et al., 2011). Briefly, the peptides were diluted in 80% acetonitrile (AcN), 5% trifluoroacetic acid (TFA), and 1 M phthalic acid and incubated with titanium dioxide beads (GL Sciences). To remove the nonphosphorylated peptides, the beads were washed with 80% AcN and 0.1% TFA. The phosphorylated peptides were eluted with ammonium hydroxide (2.5%), pH 10.5.

The peptide mixture was applied to an RP-18 trap (nanoACQUITY Symmetry® C18 – Waters 186003514) using 0.1% TFA as the mobile phase and then transferred to a nano-HPLC RP-18 column (nanoACQUITY BEH C18 - Waters 186003545) using an AcN gradient (0% - 35% AcN in 180 min) in the presence of 0.05% formic acid with a flow rate of 250 mL/min. The column outlet was coupled directly to the ion source of the spectrometer working in the regime of data dependent MS to MS/MS switch. To ensure a lack of cross-contamination from previous samples, a blank run preceded each analysis.

The data were processed by Mascot Distiller followed by Mascot Search (Matrix Science, London, UK, on-site license) against the SwissProt database with the decoy database search enabled option and the taxonomy restricted to

Arabidopsis thaliana. The search parameters for the precursor and product ion mass tolerances were 15 ppm and 0.6 Da, respectively; enzyme specificity, trypsin; missed cleavage sites allowed, 1; fixed modification of cysteine by methylthio; variable modification of methionine oxidation and serine, threonine and tyrosine phosphorylation. The Mascot Search results were internally calibrated with in-house MScan software (proteom.ibb.waw.pl) as described previously (Mikula et al., 2010). The calibrated data were re-searched with the corrected mass tolerance values. The peptides with a Mascot score exceeding the identity threshold value, which corresponds to a false discovery rate (FDR) value <1%, calculated by the Mascot procedure were considered positively identified. Additionally, the phosphorylated peptides were curated manually.

Expression and purification of recombinant proteins

The recombinant kinases SnRK2.4, SnRK2.10, SnRK2.6 and SnRK2.8 were prepared as described previously (Bucholc et al., 2011).

Full-length cDNAs for ERD10 and ERD14 were PCR-amplified using specific primers (listed in Supplemental Table 5) and cloned as EcoRI/SalI fragments into a pGEX-4T-1 vector (Amersham Biosciences). All PCR reactions were performed using high-fidelity Phusion polymerase (Thermo Fisher Scientific) and verified by DNA sequencing. The GST-tagged dehydrins were expressed in *E. coli* BL21 at 37ºC for 3 hours and purified using glutathione-Sepharose 4B beads (GE Healthcare) according to the manufacturer's instructions.

Protein kinase activity assays

In-solution kinase activity assay

The kinase activity assay in solution was performed as described previously (Bucholc et al., 2011) with minor modifications. The recombinant kinases (approximately 1-2 µg) were incubated with 4 µg of Myelin Basic Protein (MBP) or 5 µg of recombinant ERD10/ERD14 and with 50 µM of ATP supplemented with 1 µCi of [γ-^{32}P]ATP in kinase buffer (25 mM Tris-HCl, pH 7.5, 30 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT) in a final volume of 25 µL. After 30 min of incubation at 30°C, the reactions were stopped by the addition of Laemmli sample buffer. After boiling the samples for 5 min, the proteins were separated by SDS-PAGE. The phosphorylated proteins were visualized by autoradiography.

In-gel kinase activity assay

In-gel kinase activity assays were performed according to Zhang and Klessig (1997) using recombinant GST-ERD10 or GST-ERD14, instead of MBP, at concentrations of 0.3-0.4 mg/ml.

Determination of phosphorylation sites by MS analysis

For the LC/MS analysis of the proteins phosphorylated *in vitro,* the phosphorylation was performed as above (in-solution kinases activity assay) but without [γ-^{32}P]ATP. The reaction was stopped via the precipitation of the proteins with chloroform/methanol according to Wessel and Fuge (1984).

Site-directed mutagenesis

Site-directed mutagenesis was performed using the Quick Change II Site-Directed Mutagenesis Kit (Agilent) and the primers listed in Supplemental Table 5. The mutated cDNA was verified by sequencing and transformed into *E. coli* BL21. The expression and purification of the mutated proteins were performed as described above.

Rosette water status measurement

The Arabidopsis plants of the appropriate genotype were grown for 5-6 weeks under short day conditions (8 h light at 22°C / 16 h dark at 20°C) in a CLF PlantClimatics chamber incubator and watered copiously one day before harvest. The Cut Rosette Water Loss (CRWL) was determined as described previously by Bouchabke et al. (2008) with minor modifications. Freshly cut rosettes were weighed immediately, incubated in windless conditions under constant temperature (22-24°C) and weighed five times hourly. After overnight drying at 70°C to a constant mass, the rosettes were weighed for dry mass, and water loss was calculated.

For the relative water content (RWC) determination of the rosettes of the plants grown as described above, the procedure used by Ellouzi et al. (2013) was applied.

Drought tolerance test

The Arabidopsis plants were grown in pots for 17 days under long-day conditions (16 h light at 22°C / 8 h dark at 20°C) and for an additional 2 weeks without watering.

After that time, the plants were watered. Pictures were taken before rewatering and on the next day of rewatering.

Transient expression in *Nicotiana benthamiana* leaves and *Arabidopsis thaliana* protoplasts

Constructs for the intracellular localization of the proteins studied and for the BiFC assays were prepared using the Gateway® Cloning System. The construction of the pENTR ®-D/TOPO™ vector with SnRK2.4, SnRK2.6 and SnRK2.8 cDNAs was described previously (Krzywińska et al, 2016). SnRK2.10, ERD10, ERD10S106A, ERD10S106E, ERD14, ERD14S79A, and ERD14S79E cDNA was PCR-amplified and cloned into the pENTR®-D/TOPO™ vector. Then, the required cDNA was recombined into pSITE-2CA and pSITE II n-EYFP-N1 or pSITE II c-EYFP-C1 vectors (Martin et al., 2009) by a Gateway LR reaction and transformed into the *Agrobacterium tumefaciens* strain GV3101.

For the transient expression of the constructs in *N. benthamiana* leaves, fresh overnight cultures of *A. tumefaciens* containing the appropriate binary plasmids were spun down and washed twice with sterile water. To perform the localization experiments, the bacteria were resuspended in sterile water and brought to a final density of 4×10^8 cfu/mL (OD600 ~ 0.4). For the bimolecular fluorescence complementation (BiFC) assays, the appropriate bacterial suspensions were adjusted to 8×10^8 cfu/mL and mixed in a 1:1 ratio before infiltration. Leaves of 4- to 5-week-old *N. benthamiana* plants were infiltrated with the bacterial suspension using a needleless syringe. The leaves were harvested and analyzed under a confocal microscope 2 days after agroinfiltration.

Protoplasts were isolated from the T87 cells and transformed with the appropriate plasmids according to He et al. (2007) with minor modifications. In each transformation, approximately $5x10^5$ protoplasts were transfected with 20 µg of plasmid DNA. After transformation, the protoplasts were suspended in WI solution (0.5 M mannitol, 4 mM MES, pH 5.7, 20 mM KCl) and incubated at 21ºC in the dark for approximately 16 h.

Construction and selection of transgenic *Arabidopsis thaliana* plants

The pSITE-2CA plasmids containing cDNA encoding GFP-ERD14 or GFP-ERD14S79E (described above) were transformed into Col-0 Arabidopsis plants by

the floral dip method using the *A. tumefaciens* strain GV3101 as previously described by Clough & Bent (1998) and Zhang at al. (2006). The selection of the transgenic lines was performed on ½ MS agar plates supplemented with kanamycin (50 µg/mL) according to Harrison et al. (2006).

Confocal laser scanning microscopy

The subcellular localization of the fluorescent fusion proteins was evaluated using a Nikon C1 confocal system built on a TE2000E platform and equipped with a 60× Plan-Apochromat oil immersion objective (Nikon Instruments B.V. Europe, Amsterdam, The Netherlands). The fluorescence of the GFP/YFP fusion proteins was excited with a Sapphire 488 nm laser (Coherent, Santa Clara, CA, USA) and observed at 515/530 nm. The 543 nm line of a He-Ne laser (Melles Griot, NY, USA) with a 650 nm long pass filter was used for chlorophyll detection. The confocal images were processed and analyzed using EZ-C1 3.60 Nikon FreeViewer software.

Gene expression analysis

RNA was extracted from 100 mg of frozen material using TRI Reagent (MRC) according to the manufacturer's instructions. Genomic DNA contamination was removed with the Rapid Out DNA Removal kit (Thermo Fisher Scientific). Reverse transcription was performed on 1 µg of pure RNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The resulting cDNAs were diluted ten-fold with ultra-pure water and 1 µL (corresponding to 5 ng of RNA) was assayed by qPCR in a Step One Plus system (Applied Biosystems) using GoTaq® qPCR Master Mix (Promega). The expression levels were calculated relative to the housekeeping genes *EF-1α* and *TIP41* for roots and *UBC21* and *PDF2* for leaves (Czechowski et al., 2005) using a relative standard curve method. For each sample, a target quantity of the gene of interest was determined by interpolating the value from a standard curve made from serial dilutions of the pooled cDNAs from individual technical replications. The value from the standard curve was then divided by the target quantity of the housekeeping gene. A list of primers used in this study is presented in Supplemental Table 5.

Western blotting

Western blotting using anti-dehydrin antibodies (AS07 206, Agrisera) was performed according to the protocol recommended by the manufacturer.

Results

Identification of Potential SnRK2.10 Targets by Phosphoproteomic Approach

To identify the cellular targets of SnRK2.10 phosphorylated in response to stress, we compared the sets of phosphoproteins isolated from the roots of five-week-old Arabidopsis plants: wild type (wt), *snrk2.10-1* knockout mutant (KO), and two transgenic Arabidopsis expressing *35S:GFP-SnRK2.10* (OE) subjected or not to salt stress (treatment with 250 mM NaCl for 30 min). Four independent experiments were performed. The proteins were digested with trypsin, and the tryptic phosphopeptides were enriched by affinity chromatography on TiO_2 and analyzed by liquid chromatography-tandem mass spectrometry (LC/MS). As a result, 1715 phosphopeptides were identified (Supplemental Table 1). We found 114 phosphopeptides (representing 95 proteins) that, according to the results of MS/MS fragmentation, were at least 2 times more often identified in the roots of wt or OE plants subjected to salt stress than in the roots of nontreated plants. Moreover, these phosphorylations were absent in the *snrk2.10* mutant (Supplemental Tables 2 and 3). Therefore, we assume that the list may contain proteins phosphorylated by SnRK2.10 directly or by kinase(s) downstream of SnRK2.10. Among them, there were RNA binding proteins, protein kinases, phosphatases, transcription and translation factors, and late embryogenesis abundant (LEA) proteins, including dehydrins. This list also included SnRK2.10 itself, as expected. Gene Ontology (GO) annotation indicates that the majority of the identified proteins play a role in the response to diverse environmental stresses (Supplemental Figure S3, Supplemental Table 4).

To identify the overrepresented sequence motifs phosphorylated by SnRK2.10 (or some kinases under control of SnRK2.10) in response to salinity, the identified phosphopeptides were analyzed using the Motif-X algorithm (Schwartz and Gygi, 2005; Chou and Schwartz, 2011). In this group, two major phosphorylation motifs were extracted: -pS-P- and R-x-x-pS, where x can be any amino acid (Figure 1). The second motif is a well-known SnRK2 (as well as other SnRKs) phosphorylation motif (Kelner et al., 2004; Vlad et al., 2008), while –pSP- represents the mitogen-activated

protein kinase (MAPK) target motif. The –pS-P- motif has also been identified in several phosphoproteomic studies performed to find proteins phosphorylated in Arabidopsis in response to ABA in the SnRK2.2/2.3/2.6 pathway (Umezawa et al., 2013; Wang et al., 2013) and in the SnRK1 pathways triggered by energy deprivation (Nukarinen et al., 2016) or by submergence (Cho et al., 2016). These results and our results indicate that in response to stress, the SnRKs most likely directly or indirectly regulate some of the MAPK family members. Moreover, our results showed that several other sequences are phosphorylated in a SnRK2-dependent manner in Arabidopsis plants subjected to salt stress. The results of Umezawa et al. (2013) and Wang et al. (2013) also showed other motifs whose phosphorylation was dependent on ABA-activated SnRK2s.

Our further studies focused on two acidic dehydrins (dehydration proteins), ERD10 and ERD14, which were identified in our phosphoproteomic analysis as possible targets of SnRK2.10. We have chosen these proteins because of their participation in plant protection against salinity and water deficits (for review see Hanin et al., 2011; Kosová et al., 2014) and because dehydrins have been considered before as SnRK2.10 targets based on its substrate specificity (Vlad et al., 2008). The ERD10 and ERD14 phosphopeptides, which were enhanced under salinity stress and identified in our phosphoproteomic analysis, are listed in Table 1.

SnRK2.10 is Not Involved in the Regulation of ERD10 or ERD14 Accumulation in Arabidopsis Plants in Response to Salinity

Dehydrins accumulate in response to salinity stress; therefore, we estimated the dehydrin protein level in wt and *snrk2.10* plants exposed to 250 mM NaCl for various lengths of time using anti-dehydrin antibodies (Supplemental Figure S4a) to ensure that the observed differences in the level of phosphopeptides representing dehydrins reflected changes in their phosphorylation status and not differences in their protein level. Additionally, we analyzed the impact of SnRK2.10 on ERD10 and ERD14 accumulation in Arabidopsis plants (wt and the *snrk2.10* mutants) subjected to salinity stress (150 mM NaCl up to 6 days) at both the transcript and protein levels. No significant differences were observed between those lines regarding the ERD10 and ERD14 transcript and protein levels, neither in roots nor in leaves, regardless of the duration of the exposure to NaCl. In some experiments, we observed a slightly lower expression of *ERD10* and *ERD14* in the *snrk2.10* mutant lines in comparison to

411 that in wt; however, these differences were not statistically significant. This shows
412 that SnRK2.10 is unlikely to significantly modulate the level of those two dehydrins
413 under salinity stress (Supplemental Figures S4b and S4c).
414
415 **ERD10 and ERD14 are Phosphorylated by ABA-non-Activated SnRK2s**
416 To verify whether the two dehydrins, ERD10 and ERD14, could indeed be
417 phosphorylated by SnRK2.10, we produced recombinant GST-ERD10 and GST-
418 ERD14 in *E. coli* and used them in an *in vitro* phosphorylation assay with SnRK2s
419 representing different groups: SnRK2.10 and SnRK2.4 from group 1, SnRK2.8 from
420 group 2, and SnRK2.6 from group 3. Both dehydrins were strongly phosphorylated by
421 SnRK2.10 and SnRK2.4, significantly less by SnRK2.8, and negligibly by SnRK2.6
422 (Figure 2a), indicating that ERD10 and ERD14 might indeed be the physiological
423 targets of group 1 SnRK2s and possibly also of some other kinases but not of the
424 SnRK2s activated by ABA.
425 To identify the dehydrin residues phosphorylated by SnRK2.10 *in vitro*, the
426 proteins used for the *in vitro* phosphorylation assay were digested with trypsin, and
427 the tryptic peptides were analyzed by LC/MS. Five phosphopeptides were found for
428 ERD10 and three for ERD14 (Figure 2b, Table 2, and Supplemental Figure S5). To
429 establish which of them represent the main phosphorylation sites, we substituted the
430 serines/threonines identified by LC/MS in all repetitions of the experiment (T49 and
431 S106 for ERD10 and S79 for ERD14) with alanines and analyzed the
432 phosphorylation of the mutated forms of dehydrins by recombinant SnRK2.10. The
433 substitution of S106 in ERD10 and S79 in ERD14 (both located in the KLHRSxSSS
434 sequence at the beginning of the S-segment) caused a significant reduction in
435 phosphorylation by SnRK2.10, while the substitution of T49 in ERD10 did not (Figure
436 2c), indicating that S106 and S79 in ERD10 and ERD14, respectively, are the main
437 SnRK2.10 phosphorylation sites *in vitro*. Despite the fact that the *in vitro* studies
438 predicted S106 to be the main SnRK2.10 phosphorylation site in ERD10 (and S79 in
439 ERD14), the phosphoproteomic analysis of the *in vivo* phosphorylated proteins
440 (Table 1, Supplemental Table 1 and 2) failed to identify corresponding
441 phosphopeptides. We believe that this result was because the ERD10
442 phosphopeptide ^{104}SNSSSSSSSDEEGEDGEK^{121} is strongly acidic and, as such,
443 would be detected with very low efficiency owing to so-called ionization suppression.
444 A similar situation occurs for the ERD14 phosphopeptide

^{77}SDSSSSSSSEEEGSDGEK^{95}. However, in the phosphoproteomic studies performed by Umezawa et al. (2013), the ERD14 peptide ^{101}LHRSDSSSSSSSEEEGSDGE^{120} was found to be strongly phosphorylated at various residues (including S79) in response to desiccation. However, it was only when the authors used an isotope labeling with ^{16}O/^{18}O approach (Umezawa et al., 2013, supplemental data), whereas, when the label-free method (the method we used) was applied neither this phosphopeptide nor the ERD10 phosphopeptide ^{104}SNSSSSSSSDEEGEDGEK^{121} were identified.

ABA-non-activated SnRK2s are the Major Kinases Phosphorylating ERD10 and ERD14 in Arabidopsis Seedlings in Response to Salinity

To visualize kinases that might phosphorylate ERD10 and ERD14 in Arabidopsis seedlings exposed to salinity, we conducted an in-gel protein kinase activity assay with GST-ERD10 or GST-ERD14 incorporated into the gel. We analyzed the phosphorylation of GST-ERD10 and GST-ERD14 by proteins extracted from 2-week-old Arabidopsis seedlings not exposed and exposed to salinity stress. For analysis, we used the following Arabidopsis lines: wt, single (*snrk2.10-1*) and multiple (*snrk2.4/2.10* and *snrk2.1/2.4/2.5/2.10*) mutants deficient in SnRK2.10 and some other ABA-non-activated SnRK2s. Both dehydrins were clearly phosphorylated by 40-42 kDa kinases present in the extract from the wt seedlings treated with 250 mM NaCl for 10 min (Figure 2d and Supplemental Figure S6). This phosphorylation was not found when the extracts from the seedlings not treated with NaCl were analyzed and was significantly decreased when the extracts from all mutants were studied, especially *snrk2.1/4/5/10*. The results indicate that SnRK2.10 and other ABA-non-activated SnRK2s phosphorylate ERD10 and ERD14, and they seem to be the major kinases phosphorylating these dehydrins in response to salinity. However, even in the *snrk2.1/4/5/10* mutant, some kinase activity phosphorylating ERD10 and ERD14 was still present (Supplemental Figure S6), indicating that in addition to ABA-non-activated SnRK2s, there are also other kinases involved in the phosphorylation of dehydrins.

SnRK2.10 is Involved in Plant Response to Dehydration

The ERD14 peptide ^{101}LHRSDSSSSSSSEEEGSDGE^{120}, identified by our study as being phosphorylated *in vitro* by SnRK2.10, has been found to be strongly

phosphorylated in response to desiccation in phosphoproteomic studies of Umezawa et al. (2013). This result suggests that SnRK2.10 might be involved in the regulation of plant sensitivity to water deficits, especially since SnRK2s from group 1 (SnRK2.1, SnRK2.4, SnRK2.5, and SnRK2.10) are activated in Arabidopsis plants subjected to drought stress (Soma et al., 2017). We decided to check whether SnRK2.10 and/or SnRK2.4 (a kinase closely related to SnRK2.10) are involved in the plant response to dehydration. To this end, we measured water loss in detached rosettes of 6-week-old plants differing in their SnRK2 status (Col-0 wt and *snrk2.4* and *snrk2.10* knockout mutants). The water loss was higher in *snrk2.10* mutants (in *snrk2.10-1* this difference was particularly significant) than in *snrk2.4* or wild-type plants (Figure 3a), indicating that SnRK2.10, but not SnRK2.4, is involved in the response to dehydration. The RWC of the detached rosettes of all tested plant lines were equal at the beginning of the experiment (time 0 min) in control conditions (Supplemental Figure S6a).

Additionally, we analyzed the survival of the Col-0 wt and the *snrk2.10* knockout mutants under water deprivation conditions (watering was withdrawn for 14 days) and after rewatering. The results showed that the *snrk2.10-1* mutant was more sensitive to dehydration than were wt plants (Figure 3b). However, the *snrk2.10-3* mutant was nearly indistinguishable from wt plants with respect to drought survival (Figure 3b). These data are in line with the water loss results in the detached rosettes as described above. The differences between the phenotypes of the mutants might be due to the differences in the localization of T-DNA insertion; in *snrk2.10-1*, the insertion is localized within the sixth exon of the *SnRK2.10* gene, whereas in *snrk2.10-3,* it is located at the end of the last exon (Supplemental Figure S7a). This suggests that even though the whole *SnRK2.10* transcript is absent in the *snrk2.10-3* mutant (Supplemental Figure S7b), a shorter version of the transcript (and possibly a truncated version of the protein) might still be present. In the case of the *snrk2.10-1* mutant, the insertion is localized within the region encoding the kinase domain. Therefore, the functional kinase cannot be produced.

Dehydrins ERD10 and ERD14 Interact with SnRK2.10 *in planta*

To determine whether the dehydrins interact with SnRK2.10 *in planta,* we used the bimolecular fluorescence complementation (BiFC) assay. SnRK2.10 together with ERD10 or ERD14 (each fused to the complementary nonfluorescent fragments of the

yellow fluorescence protein, YFP) were transiently produced in the *N. benthamiana* leaves. We observed interactions between the kinase and both dehydrins in the cytoplasm (Figure 4), which confirms that ERD10 and ERD14 interact with SnRK2.10 *in planta*.

The Phosphorylation of ERD14 Affects its Subcellular Localization

It is well known that at least some dehydrins are phosphorylated and that this modification influences their interaction with other proteins and membranes and might affect their subcellular localization (for review see Rorat, 2006). We investigated the effect of ERD10 and ERD14 phosphorylation on their subcellular localization using several independent approaches. One of the approaches was a transient expression system. *N. benthamiana* leaves were agroinfiltrated with plasmids encoding the wt and mutated forms of dehydrins containing the phosphomimetic substitution (ERD10S106E and ERD14S79E) in fusion with EGFP. Additionally, the nonphosphorylatable forms of the proteins (EGFP-ERD10S106A and EGFP-ERD14S79A) were expressed. The subcellular localization of the dehydrins was monitored by confocal microscopy. The results showed that all three forms of EGFP-ERD10 were localized exclusively in the cytoplasm (Figure 5), indicating that the phosphorylation of S106 has no effect on the subcellular localization of ERD10. The EGFP-ERD14 wild type and EGFP-ERD14S79A were also localized in the cytoplasm, whereas EGFP-ERD14S79E was localized in the cytoplasm and nucleus, suggesting that the phosphorylation of S79 might regulate the subcellular localization of ERD14 (Figure 5). Because SnRK2s (catalyzing phosphorylation of S79) are activated in response to salinity and dehydration, we analyzed the subcellular localization of EGFP-ERD10, EGFP-ERD14 and their mutated forms in agroinfiltrated *N. benthamiana* leaves exposed to 250 mM NaCl. In the leaves exposed to salt stress, all three forms of EGFP-ERD10 were observed exclusively in the cytoplasm, confirming that ERD10 localizes to the cytoplasm and that phosphorylation of S106 has no effect on its localization (Figure 5). Surprisingly, even though NaCl should trigger EGFP-ERD14 phosphorylation, we did not observe the nuclear localization of EGFP-ERD14 upon exposure to NaCl. EGFP-ERD14, similar to EGFP-ERD14S79A, was present in the cytoplasm before and after the salt treatment. Only the EGFP-ERD14S79E variant was present in the nuclei (Figure 5). Because of heterologous expression, detection of ERD14 phosphorylation in tobacco

leaves could have been difficult; therefore, we decided to analyze the localization of EGFP-ERD14 and EGFP-ERD14S79E transiently expressed in Arabidopsis protoplasts that were not treated and treated with 250 mM NaCl. Under control conditions (before NaCl treatment), we observed that EGFP-ERD14 was present in approximately 35% of the analyzed protoplasts in both the cytoplasm and nucleus, whereas in 65% of the analyzed protoplasts, it was present only in the cytoplasm (Figure 6a). The percentage of the protoplasts with the nuclear localization of EGFP-ERD14 was increased by approximately 5% after NaCl application. EGFP-ERD14S79E was localized in the nucleus in all the examined cells, both under control and salinity stress conditions (Figure 6a). Notably, in both the Arabidopsis protoplasts and the *N. benthamiana* leaves exposed to NaCl, we observed EGFP-ERD14 within the membrane of large vesicles resembling “bulbs”, as described previously by Saito et al. (2002; 2011) (Figure 5b and 6a), suggesting that EGFP-ERD14 is involved in salt-induced membrane remodeling.

To confirm that S79 phosphorylation triggers ERD14 nuclear localization, we generated Arabidopsis transgenic plants expressing EGFP-ERD14 or EGFP-ERD14S79E. Although the transgene expression was under the control of the 35S promoter, EGFP-ERD14 and EGFP-ERD14S79E were mainly visible in the young roots. In control conditions, GFP-ERD14 was present in the root’s cell proliferation and cell elongation zones exclusively in the cytoplasm. Only in the differentiation zone, apart from the cytoplasm, was EGFP-ERD14 occasionally present in the nuclei. Following salt application, we observed the EGFP-ERD14 signal in the nuclei of not only the root’s cell differentiation zone but also in the elongation zone. In the differentiation zone, the nuclear localization of EGFP-ERD14 was observed in more cells than it was in the elongation zone (Figure 6b). However, we were not able to observe EGFP-ERD14 in the nuclei of highly proliferating cells. EGFP-ERD14 was present in these cells only in the cytoplasm and in close proximity to membranes. The results presented by McLoughlin et al. (2012) showed that in transgenic plants expressing *SnRK2.10–YFP* under the control of the *SnRK2.10* promoter, SnRK2.10–YFP was not detectable in the root tip. It was predominantly present in the distal root tissue. Our results indicate that the nuclear localization of ERD14 coincides with the presence of SnRK2.10.

The localization of EGFP-ERD14S79E was not dependent on the root zone or experimental conditions as it was always present in both the cytoplasm and the nucleus (Figure 5 and 6).

Moreover, as in the case of the transiently expressed EGFP-ERD14, following salt application, we observed the presence of EGFP-ERD14 close to the plasma membrane and in the membrane structures resembling “bulbs” (Figure 6a).

Discussion

SnRK2s are plant-specific kinases involved in the response to osmotic stress caused by drought or salinity. Group 1 kinases of the SnRK2 family are activated rapidly upon hyperosmotic stress (Burza et al., 2006; McLoughlin et al., 2012; Soma et al., 2017), indicating that they likely have a key role in the response to this stress. However, detailed information concerning this issue is still limited. To fill this gap, we undertook the identification of proteins phosphorylated by one of the ABA-non-activated SnRK2s, SnRK2.10. This particular kinase was chosen since it is clearly involved in the salinity stress response (McLoughlin et al., 2012), and it is the only group 1 SnRK2 with an exclusively cytoplasmic localization; the others localize to the cytoplasm and nucleus (Kulik et al., 2012; Soma et al., 2017 and Supplemental Figure S1). We, therefore, reasoned that the role of SnRK2.10 would not fully overlap that of the other Arabidopsis SnRK2s. This assumption is in agreement with the results of McLoughlin et al. (2012), who showed different functions of SnRK2.10 and SnRK2.4 in roots in response to salinity, and our data presented here indicate that SnRK2.10, but not SnRK2.4, plays a protective role in the plant response to drought. The data presented by Soma et al. (2017) (in the supplemental material) showing that drought survival and water loss rates were similar in *snrk2.1/4/5/10* mutant and wild-type plants combined with our present data showing a higher water loss from the rosettes of a *snrk2.10* mutant when compared with that of the wild type or a *snrk2.4* mutant additionally suggest that SnRK2.10 plays a role that does not fully overlap with the other SnRK2s.

By comparing the phosphoproteome of wt, *snrk2.10*, and Arabidopsis overexpressing *GFP-SnRK2.10,* we identified 95 proteins likely phosphorylated from the result of SnRK2.10 activity in response to salinity stress. Among them were DNA-

and RNA-binding proteins, protein kinases and phosphatases, several enzymes involved in plant metabolism, and dehydrins.

Dehydrins attracted our attention because they play an important role not only during the last phase of embryogenesis and the desiccation stage of seed development (Kalemba and Pukacka, 2007) but also in the plant response and acclimation to harsh environmental conditions, especially dehydration caused by drought, cold, freezing or salinity (for review see Hanin et al., 2011; Kosová et al., 2014). Dehydrins constitute group 2 of the late embryogenesis abundant (LEA) protein family. They are intrinsically disordered proteins that accumulate in high levels in plant cells in response to abiotic stresses and that play a protective role as molecular chaperones for membranes, proteins and nucleic acids (for review see Hara, 2010; Hanin et al., 2011; Graether and Boddington, 2014; Liu et al., 2017). Dehydrins are divided into five subgroups: K_n, SK_n, K_nS, Y_nSK_n, and Y_nK_n, where K is the sequence EKKGIME/DKIKEKLPG (or a similar sequence) rich in basic amino acids characteristic of all dehydrins (present in 1 to 11 copies), S is a serine-rich segment (with a stretch of 4-10 serine residues), and Y is the segment that contains the (V/T)D(E/Q)YGNP motif.

Dehydrins are highly phosphorylated proteins, and some data indicate that not only their level but also their phosphorylation status is important for stress tolerance. The accumulation of the phosphorylated form of wheat DHN-5 dehydrin (closely related to maize Rab17) was observed in a variety of Tunisian durum resistant to salt and drought stress, while in the sensitive variety, it was weakly detected (Brini et al., 2007). This suggests a positive role for DHN-5 dehydrin phosphorylation in the plant response to osmotic stress. Phosphorylation of *Thellungiella salsuginea* dehydrins TsDHN-1 and TsDHN-2 has been shown to be important for stabilizing the cytoskeleton under stress conditions (Rahman et al., 2011), and a phosphoproteomic analysis performed by Yang et al. (2013) in the root tips of *Phaseolus vulgaris* L. showed that phosphorylation of several dehydrins was significantly enhanced under polyethylene glycol-induced osmotic stress.

An analysis of SnRK2.10 substrate specificity has indicated that some dehydrins might be its targets (Vlad et al., 2008). Our phosphoproteomic data showed enhanced phosphorylation of two acidic dehydrins belonging to the K_nS subgroup in response to salinity and suggested that SnRK2.10 catalyzes this phosphorylation. Phosphorylated peptides derived from dehydrins ERD10 and

ERD14 were observed in the root extracts of wild-type plants and plants expressing GFP-SnRK2.10 but not in the extracts of the *snrk2.10* mutant. In-gel kinase activity assays with GST-ERD10 and GST-ERD14 as substrates confirmed their phosphorylation by SnRK2.10 and other ABA-non-activated SnRK2s in response to salt stress.

GST-ERD10 and GST-ERD14 were also strongly phosphorylated by SnRK2.10 *in vitro*, slightly less affected by another ABA-non-activated kinase, SnRK2.4, and practically not affected by the ABA-dependent kinase, SnRK2.6. The analysis of *in vitro* phosphorylation by SnRK2.10 showed several phosphorylated residues, and site-directed mutagenesis defined one preferentially phosphorylated residue in each of the dehydrins studied that was localized in a cluster of serines of the S-segment. In both ERD14 and ERD10, the preferentially phosphorylated serine lies in the sequence KLHRSxSSS, which is in full agreement with the SnRK2.10 phosphorylation consensus described by Vlad et al. (2008). Notably, the sequence LHRSxS(4-10)E/D(3) is conserved in all dehydrins.

There are numerous studies concerning the phosphorylation of the S-segment of dehydrins (for a review, see Rorat, 2006; Hanin et al., 2011; Graether and Boddington, 2014) claimed to be catalyzed by CK2 protein kinase (Alsheikh et al., 2003 and 2005). However, it has not been proven that CK2 is the only kinase that phosphorylates dehydrins *in vivo*; quite the opposite. Phosphorylation of maize dehydrin Rab17 (Responsive to ABA 17), also known as DHN1, in plant cells is performed not only by CK2 but also by another kinase(s) (not yet identified) (Riera et al., 2004). The tomato TAS14 protein, a homolog of maize Rab17, is phosphorylated *in vivo* by at least two kinases, CK2 and a kinase whose substrate specificity resembles that of cAMP-dependent protein kinase (PKA); *in vitro* PKA efficiently phosphorylated Rab17 (Godoy et al., 1994). It should be noted that PKA recognizes and phosphorylates the R/K-X-X-S/T motif, which is also efficiently phosphorylated by SnRK2s. Therefore, we suggest that both CK2 and SnRK2 might phosphorylate dehydrins in their S-segment in response to osmotic stress (salinity or dehydration). Since the ABA-non-activated SnRK2s are activated very rapidly in response to osmotic stress (SnRK2.4/SnRK2.10 are fully active within the first few minutes following stressor application; McLoughlin et al., 2012), SnRK2.10 and possibly some other SnRK2s likely phosphorylate dehydrins at the very early stages of the plant response to stress. This modification could trigger subsequent phosphorylation

carried out by, e.g., CK2. It has been shown that phosphorylation in the S-segment of Arabidopsis ERD10 and ERD14 (Alsheikh et al., 2003; 2005), as well as of celery vacuolar-associated dehydrin-like protein VCaB45 (Heyen et al., 2002), promotes binding of bivalent metal ions, especially calcium. Those authors analyzed the calcium binding properties of recombinant ERD10 and ERD14 phosphorylated *in vitro* by CK2 (Alsheikh et al., 2003; 2005) or VCaB45 isolated from plants not treated or treated with phosphatase and rephosphorylated with CK2 (Heyen et al., 2002). Their results clearly showed enhanced calcium binding by phosphorylated ERD10 and ERD14. In the case of VCaB45, the binding was the strongest for the protein isolated from the plants not treated with phosphatase and was practically abolished for dephosphorylated VCaB45. The phosphorylation by CK2 only partially restored the calcium binding ability of the protein (Heyen et al., 2002), indicating that *in vivo* VCaB45 is phosphorylated by another kinase(s). The authors suggested that in the phosphorylated state, the dehydrins ERD10, ERD14 and VCaB45 could act as calcium buffers since their Ca^{2+} binding capacity was rather high, or they could play a role as calcium-dependent chaperones, similar to calreticulin and calnexin (Nigam et al., 1994; Michalak et al., 2002). Since enhanced ERD10 and ERD14 phosphorylation has been observed in plants exposed to desiccation (Umezawa et al., 2013) or salt stress (our results), we can expect that the dehydrins phosphorylation by SnRK2s might modulate calcium signaling in response to osmotic stress.

Beside calcium, zinc and iron are also strongly bound by phosphorylated ERD10 and ERD14 (Alsheik, 2005). There is evidence that zinc and other divalent cations promote DNA binding by dehydrins (Hara et al., 2009). Therefore, even though DNA binding by ERD14 has not been shown, we can consider that phosphorylation might regulate subcellular localization of the dehydrin and have an impact on its possible nucleic acid binding ability.

Several reports have indicated that phosphorylation of the S-segment is important for the nuclear targeting of dehydrins and for the regulation of their association with membranes (Rorat, 2006; Hanin et al., 2011; Graether and Boddington, 2014). It is widely accepted that dehydrins are localized in various cellular compartments, mainly in the cytoplasm and nucleus but also in mitochondria or chloroplasts (for review see Graether and Boddington, 2014). It has been shown that the localization of some dehydrins depends on their phosphorylation. The best

example is the maize dehydrin Rab17 from the YSK_2 group. Goday et al. (1994) have shown that the phosphorylation status of maize Rab17 correlates with its nuclear localization. Rab17 phosphorylated in the S-segment is transported to the nucleus (Jensen et al., 1998; Riera et al., 2004). The results of Riera et al. (2004) have revealed that Rab17 phosphorylation delayed seed germination in salinity stress conditions. The subcellular localization of acidic dehydrins SK_2 and SK_3 is controversial. ERD10 and ERD14 have been described as cytosolic (Rorat, 2006; Candat et al., 2014; Cedeno et al., 2017) even though several programs predicted their cytosolic/nuclear localization (Candat et al., 2014). Dehydrin DHN24 from *Solanum sogarandinum*, which is similar to ERD14, has also been considered an exclusively cytoplasmic protein (Rorat, 2006). Only recently has it been shown that DHN24 is present not only in the cytoplasm but also in the nucleus and the microsomal fraction (Szabala et al., 2014). Our results suggest that phosphorylation of S79, the first serine of the serine stretch in the S-segment, plays a role in the transport of ERD14 from the cytoplasm to the nucleus. We found that the phosphorylation of S79 is catalyzed by SnRK2.10 (or other ABA-non-activated SnRK2s), but we do not exclude its phosphorylation by protein kinases belonging to other families or that phosphorylation by SnRK2s enhances phosphorylation by other kinases. Moreover, we do not exclude that phosphorylation of other serines in the S-segment might also be involved in the regulation of the subcellular localization of ERD14. Candat et al. (2014) observed an exclusively cytoplasmic localization of LEA proteins (including ERD10 and ERD14), but their experiments were performed under normal osmolarity conditions only. Cedeno et al. (2017) analyzed ERD10 and EDR14 localization in control, cold, and mild osmotic stress conditions; however, the osmotic stress applied was too weak to cause efficient SnRK2 activation. Moreover, they used a heterologous expression system for the production of Arabidopsis proteins: transient expression in *N. benthamiana* leaves. We also did not observe the translocation of EGFP-ERD14 to the nucleus expressed in *N. benthamiana* leaves upon salt application. Furthermore, even when EGFP-ERD14 was transiently expressed in Arabidopsis protoplasts or stably expressed in Arabidopsis plants, we did not observe nuclear localization in response to salt in every protoplast/cell monitored but only in some of them. In the transgenic plants expressing EGFP-ERD14, we did not observe the nuclear localization of EGFP-ERD14 in the highly proliferating cells of the root tip either before or after the salt treatment. It should be

emphasized here that SnRK2.10 is absent from root tips (McLoughlin et al., 2012). We were able to observe the nuclear localization of EGFP-ERD14 only in some (not all) of the cells of the root's elongation and differentiation zones in the seedlings expressing EGFP-ERD14 subjected to salt stress. In plant cells, most likely only a small pool of dehydrins is phosphorylated and transported to the nucleus, even in response to stress, while their majority stays in the cytoplasm to protect membrane and cytoplasmic proteins. In contrast, in all systems studied, we observed the nuclear localization of the mutated variant of EGFP-ERD14, EGFP-ERD14S79E, where S79 was substituted with glutamic acid, indicating that phosphorylation within the S-segment might trigger dehydrin transport to the nucleus. We can conjecture that ERD14 localized in the nucleus could protect DNA/RNA against oxidative stress generated in response to osmotic stress.

Additionally, EGFP-ERD14 was present in "bulb-like" structures that were much more numerous in NaCl-treated Arabidopsis and tobacco cells than in the control ones. Saito et al. (2002; 2011) described similar structures formed by mobile continuous vacuolar membranes. It is worth mentioning that celery dehydrin VCaB45 is associated with vacuolar membranes (Heyen et al., 2002), and ERD14 is the only Arabidopsis dehydrin recognized by anti-VCaB45 antibodies (Heyen et al., 2002), suggesting similarities between these two proteins. Our results indicate that in response to salinity, ERD14 associates with specific membranous structures, but this issue needs further study.

In conclusion, we have demonstrated that SnRK2.10 is involved in the Arabidopsis response not only to salinity but also to dehydration and have identified its numerous target proteins, including dehydrins ERD10 and ERD14. These dehydrins are phosphorylated by SnRK2.10 and possibly also by other ABA-non-activated SnRK2s in response to stress. The major sites of SnRK2.10 phosphorylation in both dehydrins were identified. The SnRK2 phosphorylation sites are present in all dehydrins, suggesting the universal nature of this modification. We also showed that ERD14 phosphorylation in the S-segment might be involved in its nuclear import. Although, the physiological role of ERD14 and ERD10 phosphorylation in the plant response to abiotic stresses remains unclear, we can assume that it has an impact on their chaperone activity and, as a consequence, membrane, protein and possibly also nucleic acids stability. Moreover,

phosphorylation might create some specificity in the selection of dehydrin targets in response to salinity or drought. Further studies should verify these assumptions.

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Conflict of Interest

The authors claim no conflict of interest.

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Table 1. The phosphopeptides derived from ERD10 and ERD14 identified by the phosphoproteomic approach as being potentially phosphorylated by SnRK2.10

Accesion number	Protein name	Peptide position	Phosphopeptide	Mass
At1g20450	ERD10	204 - 230	KPEDSQVVNTTPLVETAT<u>T</u>PIADIPEEK	3000.4580
At1g76180	ERD14	131 - 157	KPEDG<u>S</u>AVAAAPVVVPPPVEEAHPVEK	2798.3891

Table 2. The phosphopeptides derived from ERD10 and ERD14 phosphorylated by SnRK2.10 *in vitro*

Dehydrin	Phosphorylated peptide	Peptide position	Phosphorylation site position			
ERD10	VATEE<u>S</u>SAPEIK	17 - 28	S22		S23	
	TQI<u>S</u>EPESFVAK	58 - 69	S61		S65	
	SN<u>**S**</u>SSSSSSDEEGEDGEK	104 - 121	**S106**		S107	
	EEVKPQETT<u>**T**</u>LASEFEHK	40 - 57	**T49**			
	KPEDSQVVN<u>TT</u>PLVETA<u>T</u>PIADIPEEK	204 - 230	S208	T213	T214	T221
ERD14	VATEESSAEV<u>T</u>DR	16 - 28	S21		T26	
	SD<u>**S**</u><u>SS</u>SSSSEEEGSDGEK	77 - 95	**S79**		S78	S79
	KPEDGSAVAAAPVVVPPPVEEAHPVE	131 - 157	S136			

The results represent four independent experiments. The phosphorylation sites identified are underlined; those identified in all four experiments are bolded.

Figure Legends

Figure 1. The phosphorylation motifs identified within putative SnRK2.10 targets and/or proteins phosphorylated in an SnRK2.10-dependent manner in *Arabidopsis thaliana* roots exposed to salinity stress.
The phosphorylation motifs were identified using the Motif-X algorithm.

Figure 2. ERD10 and ERD14 are Phosphorylated by SnRK2.10 *in vitro*
(a) *In vitro* phosphorylation of GST-ERD10 and GST-ERD14 by SnRK2.4, SnRK2.10, SnRK2.6, or SnRK2.8. The kinases and the dehydrins studied were produced in *E. coli* and used for *in vitro* phosphorylation assays. Phosphorylation of the dehydrins (4 µg, each) or MBP (2 µg) (as a universal kinase substrate used as the kinase activity control) by the recombinant SnRK2s (1 - 2 µg) was monitored by in solution kinases activity assay described in “Material and Methods”. The reaction products were separated by SDS-PAGE, and GST-ERD10, GST-ERD14, and MBP phosphorylation were determined by autoradiography. The representative results from one of three independent experiments are shown. (b) MS spectra of phosphopeptides from ERD10 and ERD14 dehydrins phosphorylated *in vitro* by SnRK2.10. The phosphorylated residues were identified by LC/MS after *in vitro* phosphorylation of recombinant GST-ERD10 and GST-ERD14 by SnRK2.10 (see Table 2). The reaction was performed as described above in (a) but without radioactive ATP. (c) Analysis of phosphorylation of the wild-type and mutated forms of ERD10 and ERD14 (ERD10S106A, ERD10T49A, and ERD14S79A) by SnRK2.10. The reaction was performed and analyzed as described in (a). (d) In-gel kinase activity assay of 2-week-old Arabidopsis seedlings of wt, *snrk2.10-1*, *snrk2.4/2.10*, and *snrk2.1/2.4/2.5/2.10* knockout mutants. The plants were not treated or treated for 10 min with 250 mM NaCl. The extracts were subjected to an in-gel kinase activity assay using GST-ERD10 or GST-ERD14 as the substrate. The representative results from one of three independent experiments are shown. Autorad, autoradiograph; CBB, Coomassie Brilliant Blue.

Figure 3. SnRK2.10 Impacts Plant Sensitivity to Water Deficit
(a) The lack of SnRK2.10 enhances water loss from detached Arabidopsis rosettes. The whole rosettes from six-week-old Arabidopsis plants were cut off and weighed.

Then, they were incubated in windless conditions at 24°C for 5 h and weighed every hour. Finally, the rosettes were dried at 70°C overnight and weighed. The cut rosette water loss (CRWL) was calculated. The representative results from one of four independent experiments are shown. Eight plants were used for each line per experiment. For the statistical analysis, a t-test was applied. The asterisks indicate significant differences from the wild type (*P < 0.05; **P < 0.01, ***P < 0.001). The average values ± SE are shown. (b) The lack of SnRK2.10 reduces the survival of an Arabidopsis plant under drought conditions. The Arabidopsis plants were grown in pots for 17 days under long day conditions and for an additional 2 weeks without watering. The pictures were taken before watering was stopped (Before drought), after two weeks without water (Drought), and one day after rewatering (Rewatering). Ten pots were used for each line per experiment. Representative plants are presented.

Figure 4. SnRK2.10 Interacts with ERD10 and ERD14 *in planta*

N. benthamiana. leaves were co-transformed with pairs of plasmids encoding nEYFP–SnRK2.10 with cEYFP–ERD10 or cEYFP–ERD14. For the negative control, nEYFP–SnRK2.10 was co-expressed with cEYFP. BF indicates bright field, bar = 10 μm. The data represent one of three independent experiments showing similar results.

Figure 5. Subcellular Localization of EGFP-ERD10 and EGFP-ERD14 and Their Mutated Forms Transiently Expressed in *N. benthamiana* leaves

(a) Subcellular localization of EGFP-ERD10 and EGFP-ERD14 and their mutated forms transiently expressed in *N. benthamiana* leaves in control conditions or under salinity stress. *N. benthamiana* leaves were transformed with plasmids encoding EGFP-ERD10, EGFP-ERD14 or their mutated forms, EGFP-ERD10S106E, EGFP-ERD10S106A, EGFP-ERD14S79E or EGFP-ERD14S79A, and their localization was analyzed.

(b) Subcellular localization of EGFP-ERD14 produced in *Nicotiana benthamiana* leaves (in control conditions or exposed to salinity stress) in the cortical cytoplasm. “Bulb-like” structures are marked with white arrows. BF indicates bright field, bar = 10 μm.

Figure 6. Subcellular Localization of EGFP-ERD14 In Response to Salt Stress

(a) Subcellular localization of EGFP-ERD14 and EGFP-ERD14S79E expressed in Arabidopsis protoplasts. The protoplasts isolated from T87 cells were transformed with plasmids encoding EGFP-ERD14 or EGFP-ERD14S79E. The localization of chimeric proteins was studied before and after exposure to 250 mM NaCl. The percentage of nuclei containing expressed proteins was calculated from three independent experiments (approximately 50 protoplasts were analyzed in each experiment).

(b) Subcellular localization of EGFP-ERD14 and EGFP-ERD14S79E stably expressed in Arabidopsis. The localization of EGFP-ERD14 and EGFP-ERD14S79E was monitored in different types of root cells, in the proliferation zone (proliferation), elongation zone (elongation), and differentiation zone (differentiation), expressing the proteins studied before and after exposure to 250 mM NaCl (bar = 10 μm).

The data represent one of three independent experiments showing similar results.

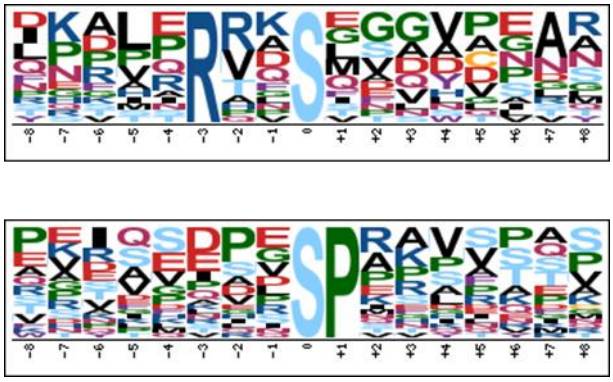


Figure 1

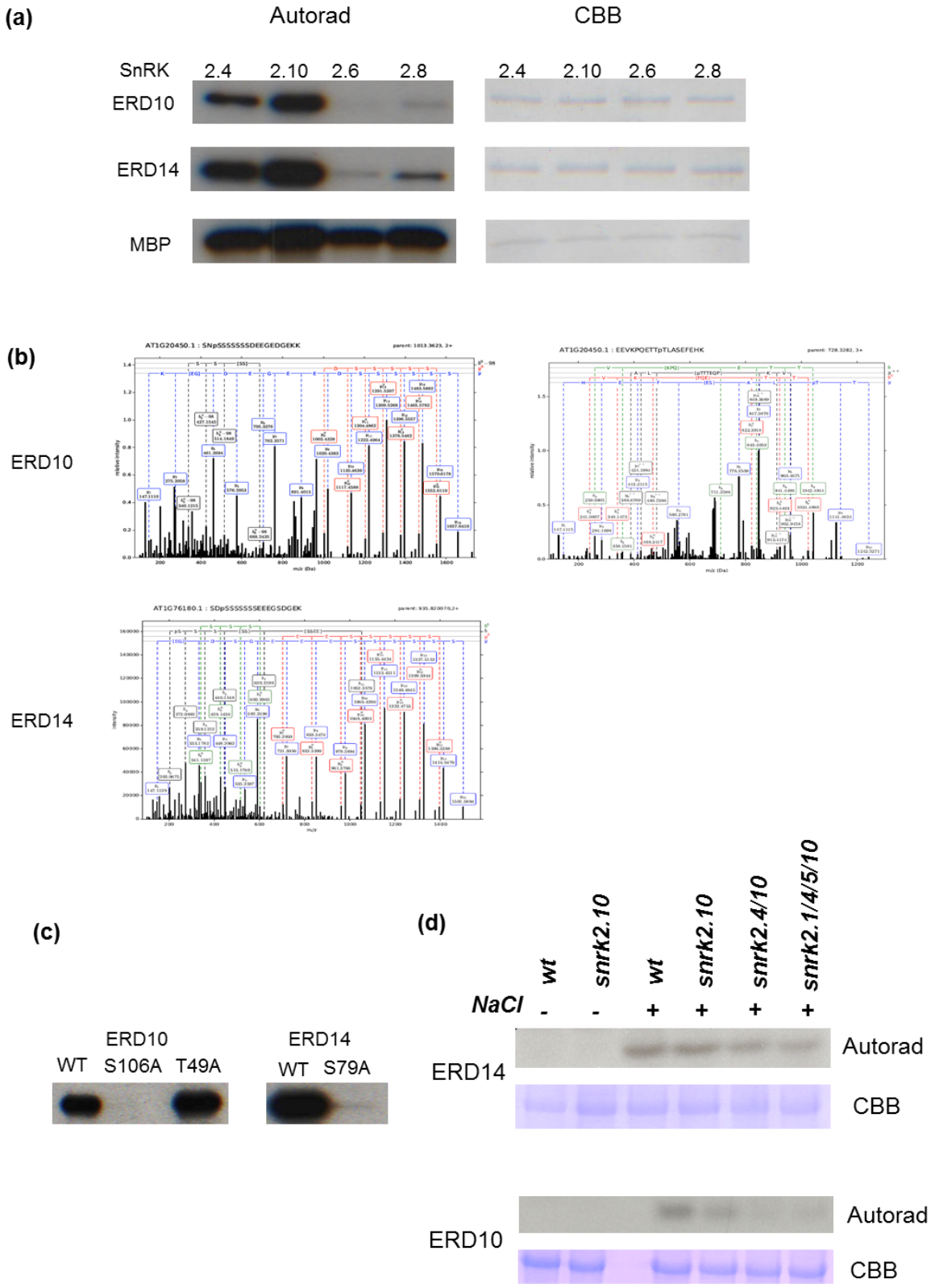
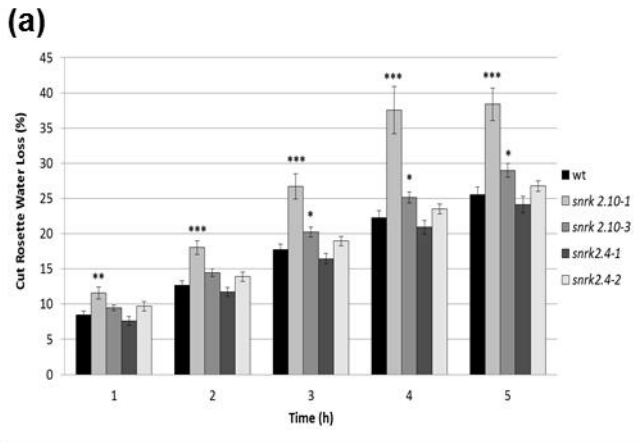
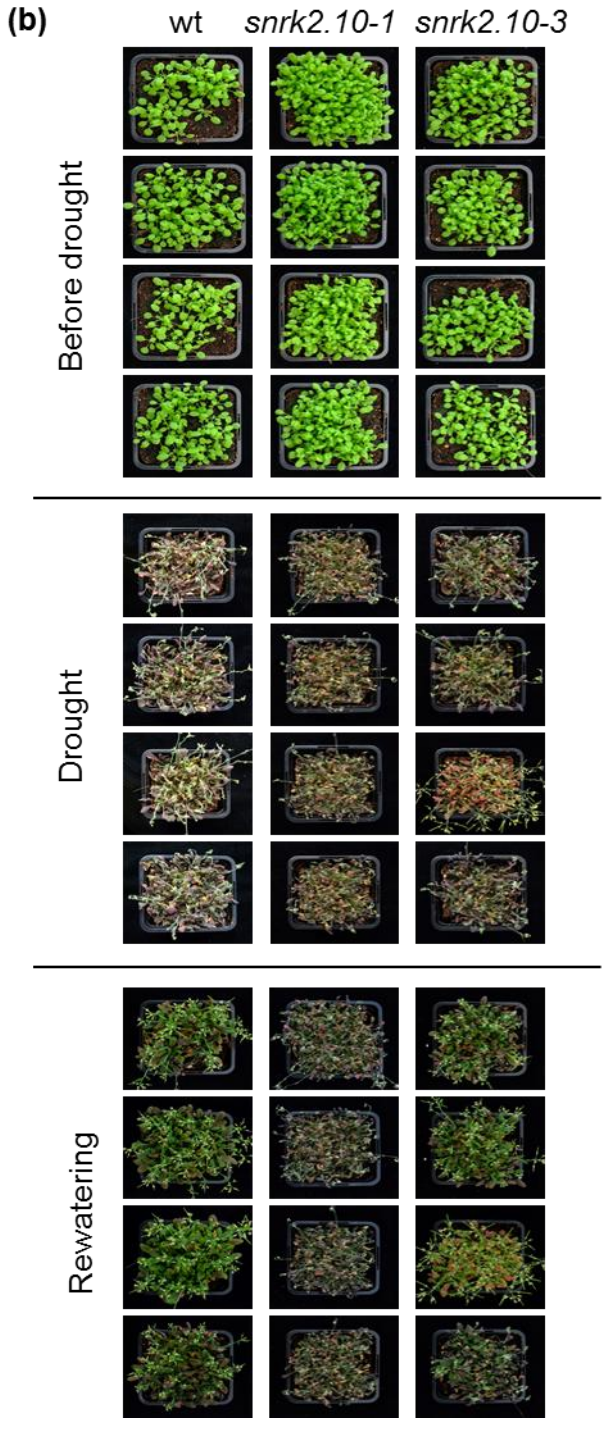

(a)
Autorad
CBB
SnRK
2.4
2.10
2.6
2.8
ERD10
ERD14
MBP
(b)
ERD10
ERD14
(c)
ERD10
WT
S106A
T49A
ERD14
WT
S79A
(d)
NaCl
wt
snrk2.10
wt
snrk2.10
snrk2.4/10
snrk2.1/4/5/10
ERD14
Autorad
CBB
ERD10
Autorad
CBB

Figure 2





1191 Figure 3

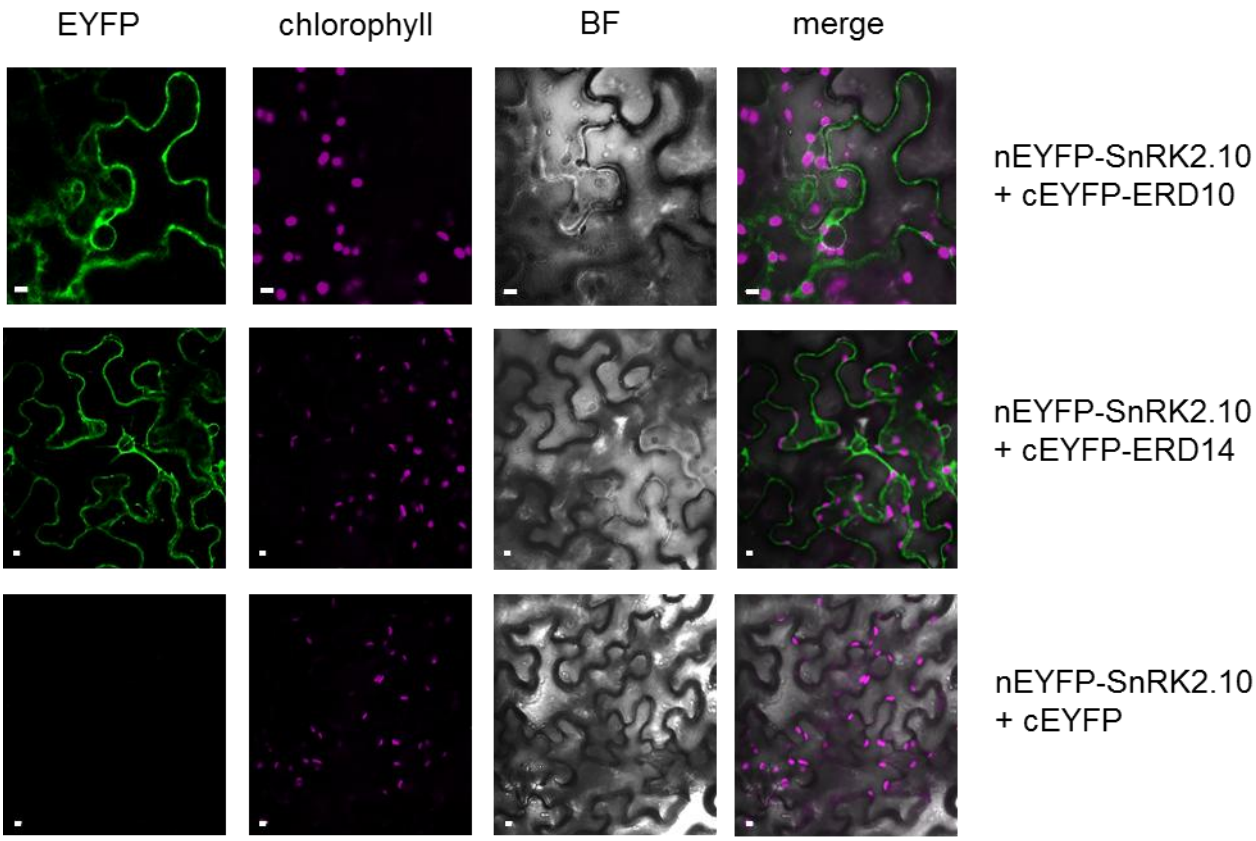

EYFP
chlorophyll
BF
merge
nEYFP-SnRK2.10
+ cEYFP-ERD10
nEYFP-SnRK2.10
+ cEYFP-ERD14
nEYFP-SnRK2.10
+ cEYFP

Figure 4

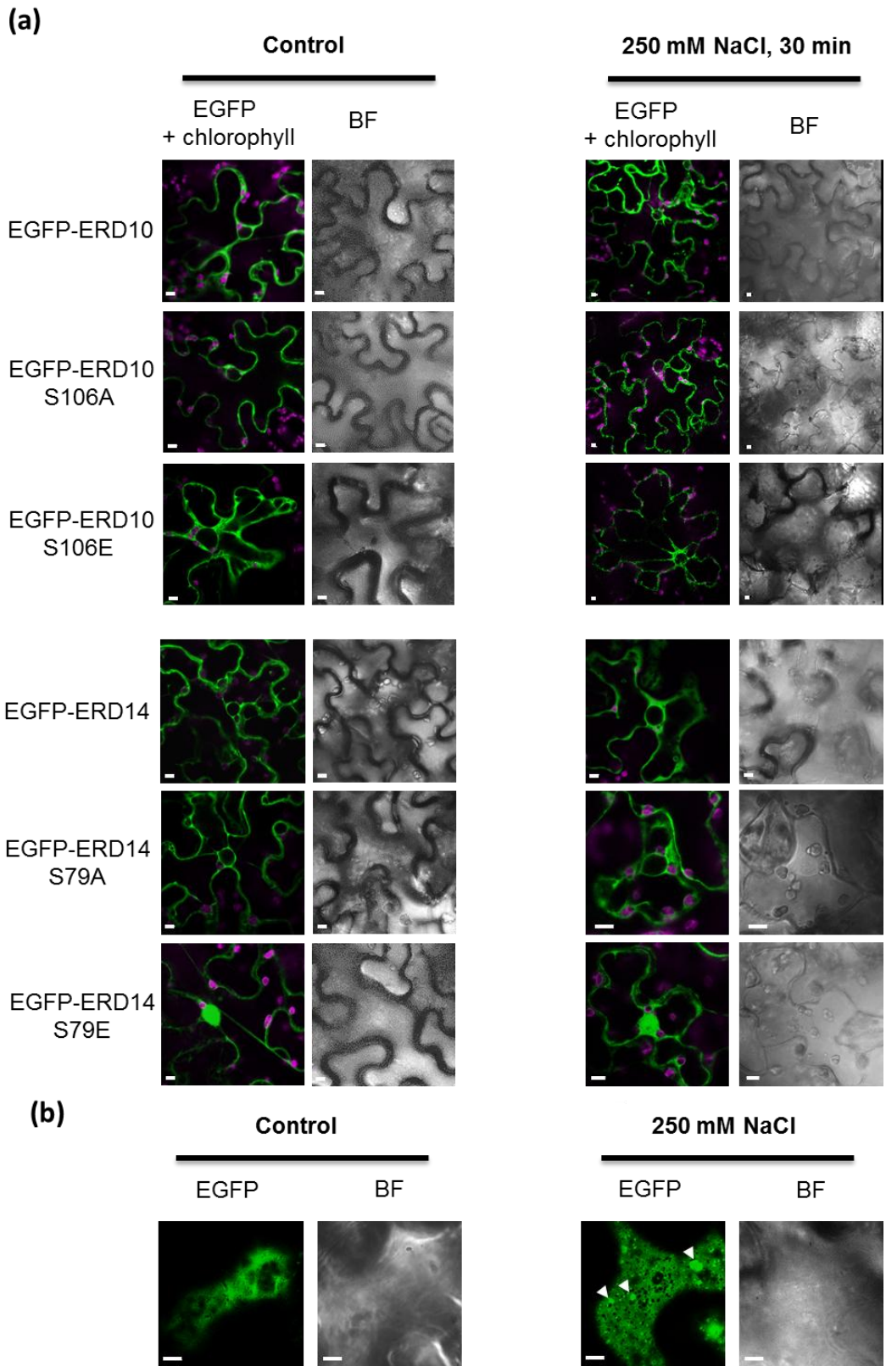

(a)
Control
250 mM NaCl, 30 min
EGFP + chlorophyll
BF
EGFP + chlorophyll
BF
EGFP-ERD10
EGFP-ERD10 S106A
EGFP-ERD10 S106E
EGFP-ERD14
EGFP-ERD14 S79A
EGFP-ERD14 S79E
(b)
Control
250 mM NaCl
EGFP
BF
EGFP
BF

Figure 5

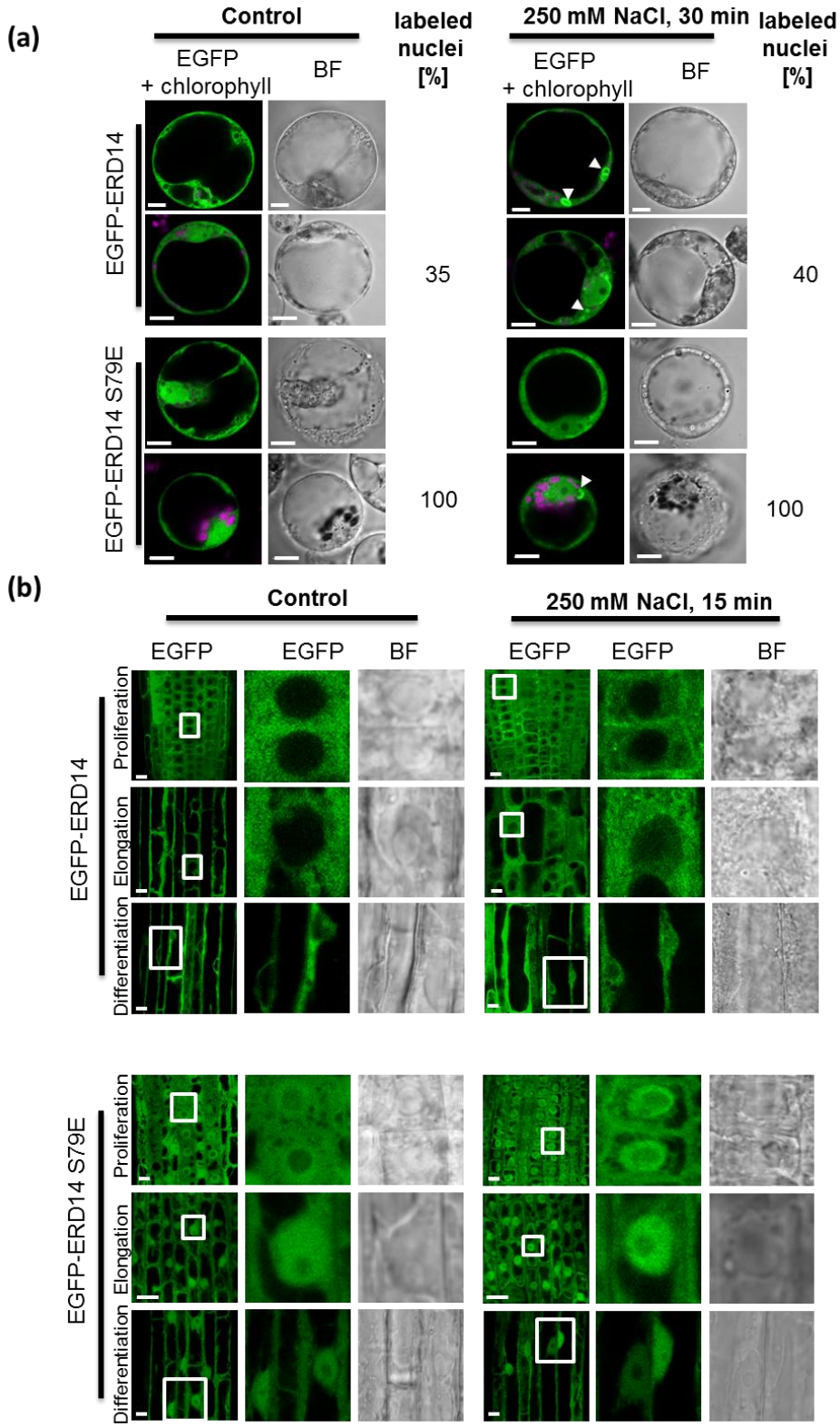

(a)
Control
250 mM NaCl, 30 min
EGFP + chlorophyll
BF
labeled nuclei [%]
EGFP-ERD14
EGFP-ERD14 S79E
35
40
100
100
(b)
Control
250 mM NaCl, 15 min
EGFP
EGFP
BF
Proliferation
Elongation
Differentiation

Figure 6