

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

3
4 **Short running title:** Phosphorylation of Dehydrins ERD10 and ERD14 by SnRK2.10

5
6 Justyna Maszkowska, Janusz Dębski, Anna Kulik, Michał Kistowski, Maria Bucholc,
7 Małgorzata Lichocka, Maria Klimecka, Olga Sztatelman, Katarzyna Patrycja
8 Szymańska, Michał Dadlez, Grażyna Dobrowolska

9 Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawińskiego
10 5a, 02-106 Warsaw, Poland

11
12 Corresponding authors: Grażyna Dobrowolska, Institute of Biochemistry and
13 Biophysics, Polish Academy of Sciences, Pawińskiego 5a, 02-106 Warsaw, Poland
14 dobrowol@ibb.waw.pl

15 and Justyna Maszkowska Institute of Biochemistry and Biophysics, Polish Academy
16 of Sciences, Pawińskiego 5a, 02-106 Warsaw, Poland
17 j.maszkowska@ibb.waw.pl

18

19 **Abstract**

20

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

36

37

38

39 Introduction

40

41 Drought and salinization are the major environmental challenges for plants. All plants
42 have the ability to sense environmental cues and activate the signaling pathways
43 responsible for the induction of plant responses. However, plants differ significantly
44 with respect to their tolerance to various stresses. The SNF1-related protein kinases
45 2 (SnRK2s) are indispensable to the plant reaction to water deficits (for review see
46 Umezawa et al., 2010; Kulik et al., 2011; Fujii and Zhu, 2012; Yoshida et al., 2015;
47 Zhu, 2016). SnRK2s are plant-specific enzymes that are rapidly and transiently
48 activated in response to osmotic stress. They have been classified into three groups
49 based on their phylogenetic analysis; the classification correlates with their response
50 to abscisic acid (ABA) (Boudsocq et al., 2004; Kobayashi et al., 2004). Group 1
51 comprises kinases not activated in response to ABA, group 2 kinases are not
52 activated (e.g., in *Oryza sativa*) or only weakly activated by ABA (e.g., in *Arabidopsis*
53 *thaliana*), and group 3 kinases are strongly activated in response ABA. To date, the
54 mechanism of activation and the physiological role have mainly been investigated for
55 the ABA-activated SnRK2s (from group 3). It has been established that these kinases
56 are key components of ABA signaling pathways, both in plant development (seed
57 maturation and germination) (Fujii et al., 2007; Fujii and Zhu, 2009; Nakashima et al.,
58 2009) and in response to water deficits (Fujii and Zhu, 2009; Fujita et al., 2009).
59 Several independent experimental approaches have been used to identify ABA-
60 activated SnRK2 target proteins. Thus, OST1/SnRK2.6/SRKE phosphorylates ion
61 channels involved in stomatal movements: SLAC1 (Slow Anion Channel-Associated
62 1 - Geiger et al., 2009; Lee et al., 2009), KAT1 (K⁺ channel - Sato et al., 2009), the
63 NADPH oxidase RbohF (Respiratory burst oxidase homolog protein F - Sirichandra
64 et al., 2009), the aquaporin PIP2;1 (Plasma membrane Intrinsic Protein2;1 - Grondin
65 et al., 2015), BRM (SWI/SNF chromatin-remodeling ATPase BRAHMA - Peirats-
66 Llobet et al., 2016) and numerous transcription factors regulating the expression of
67 ABA-responsive genes (Kobayashi et al., 2005; Furihata et al., 2006; Yoshida et al.,
68 2015).

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

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

83 There are several indications that kinases from group 2 (SnRK2.7 and
84 SnRK2.8) are also involved in stress signaling. They play a role in drought response
85 (Umezawa et al., 2004; Mizoguchi et al., 2010), mainly by regulating the expression
86 of stress-response genes. The cellular targets of SnRK2.7 and SnRK2.8 comprise
87 various transcription factors involved in abiotic stress responses (Mizoguchi et al.,
88 2010; Kim et al., 2012). Additionally, several 14-3-3 proteins and enzymes (e.g.,
89 glyoxalase I, adenosine kinase I, and ribose 5-phosphate isomerase) have been
90 found to be phosphorylated by SnRK2.8 (Shin et al., 2007). It has been shown that
91 SnRK2.8 is also involved in biotic stress response. Recently, Lee et al. (2015)
92 showed that SnRK2.8 phosphorylates Nonexpresser of Pathogenesis-Related genes
93 1 (NPR1), which is involved in systemic acquired resistance in response to pathogen
94 infection. The phosphorylation of NPR1 catalyzed by SnRK2.8 is necessary for its
95 nuclear import.

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

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

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

124 SnRK2.10 is unique among the Arabidopsis ABA-non-activated SnRK2s as it
125 is the only one that localizes exclusively to the cytoplasm, whereas all the other
126 members of this group are found in both the cytoplasm and the nucleus (Kulik et al.,
127 2012; Soma et al., 2017, and Supplemental Figure S1). This suggests that its role
128 may not be fully comparable to other SnRK2s.

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

133

134

135 **Materials and Methods**

136

137 **Plant material and growth conditions**

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

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

150 For the phosphoproteomic and gene expression analyses, the plants were
151 grown in hydroponic culture (Araponics system) as described by Kulik et al. (2012).
152 The roots of the 5-week-old plants that were not treated or treated with 250 mM NaCl
153 for 30 min (for phosphoproteomic analysis) or 150 mM NaCl for up to 6 days (for
154 gene expression analysis) were harvested, frozen in liquid nitrogen and stored at -
155 80°C until analysis.

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

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

163

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

166

167 **Sample preparation for MS analysis**

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

175 room temperature in a SpeedVac for 10 min and suspended in 300 μ L of lysis buffer
176 [30 mM Tris, pH 8.5, 7 M urea, 2 M thiourea, 4% CHAPS and PhosStop; (Roche)],
177 incubated overnight at 4°C, and centrifuged as above. Then, the supernatants were
178 collected.

179

180 **Mass spectrometry**

181 The dissolved samples were subjected to an in-solution trypsin digestion procedure.
182 The proteins were reduced with 50 mM Tris(2-carboxyethyl)phosphine hydrochloride
183 (TCEP) at 60°C for 30 min, alkylated with 200 mM methyl methanethiosulfonate
184 (MMTS) at room temperature for 15 min, and digested overnight with trypsin
185 (Sequencing Grade Modified Trypsin - Promega V5111). The peptide mixtures were
186 analyzed by LC/MS (liquid chromatography coupled to tandem mass spectrometry)
187 using a Nano-Acquity LC system (Waters) and an Orbitrap Velos or Q Exactive mass
188 spectrometer (Thermo Electron Corp., San Jose, CA) as detailed below. The
189 samples for the phosphorylation site analysis were split in two; approximately 20% of
190 the total volume was directly analyzed by LC/MS for protein identification, and the
191 remaining 80% was subjected to the enrichment of the phosphorylated peptides on
192 titanium dioxide as described previously (Graczyk et al., 2011). Briefly, the peptides
193 were diluted in 80% acetonitrile (AcN), 5% trifluoroacetic acid (TFA), and 1 M phthalic
194 acid and incubated with titanium dioxide beads (GL Sciences). To remove the
195 nonphosphorylated peptides, the beads were washed with 80% AcN and 0.1% TFA.
196 The phosphorylated peptides were eluted with ammonium hydroxide (2.5%), pH
197 10.5.

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

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

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

220

221 **Expression and purification of recombinant proteins**

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

224 Full-length cDNAs for ERD10 and ERD14 were PCR-amplified using specific primers
225 (listed in Supplemental Table 5) and cloned as EcoRI/Sall fragments into a pGEX-
226 4T-1 vector (Amersham Biosciences). All PCR reactions were performed using high-
227 fidelity Phusion polymerase (Thermo Fisher Scientific) and verified by DNA
228 sequencing. The GST-tagged dehydrins were expressed in *E. coli* BL21 at 37°C for 3
229 hours and purified using glutathione-Sepharose 4B beads (GE Healthcare) according
230 to the manufacturer's instructions.

231

232 **Protein kinase activity assays**

233 In-solution kinase activity assay

234 The kinase activity assay in solution was performed as described previously (Bucholc
235 et al., 2011) with minor modifications. The recombinant kinases (approximately 1-2
236 µg) were incubated with 4 µg of Myelin Basic Protein (MBP) or 5 µg of recombinant
237 ERD10/ERD14 and with 50 µM of ATP supplemented with 1 µCi of [γ -³²P]ATP in
238 kinase buffer (25 mM Tris-HCl, pH 7.5, 30 mM MgCl₂, 5 mM EGTA, 1 mM DTT) in a
239 final volume of 25 µL. After 30 min of incubation at 30°C, the reactions were stopped
240 by the addition of Laemmli sample buffer. After boiling the samples for 5 min, the
241 proteins were separated by SDS-PAGE. The phosphorylated proteins were
242 visualized by autoradiography.

243 In-gel kinase activity assay
244 In-gel kinase activity assays were performed according to Zhang and Klessig (1997)
245 using recombinant GST-ERD10 or GST-ERD14, instead of MBP, at concentrations of
246 0.3-0.4 mg/ml.

247

248 **Determination of phosphorylation sites by MS analysis**

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

253

254 **Site-directed mutagenesis**

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

260

261 **Rosette water status measurement**

262 The Arabidopsis plants of the appropriate genotype were grown for 5-6 weeks under
263 short day conditions (8 h light at 22°C / 16 h dark at 20°C) in a CLF PlantClimatics
264 chamber incubator and watered copiously one day before harvest. The Cut Rosette
265 Water Loss (CRWL) was determined as described previously by Bouchabke et al.
266 (2008) with minor modifications. Freshly cut rosettes were weighed immediately,
267 incubated in windless conditions under constant temperature (22-24°C) and weighed
268 five times hourly. After overnight drying at 70°C to a constant mass, the rosettes
269 were weighed for dry mass, and water loss was calculated.

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

272

273 **Drought tolerance test**

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

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

278

279 **Transient expression in *Nicotiana benthamiana* leaves and *Arabidopsis*** 280 ***thaliana* protoplasts**

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

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

300 Protoplasts were isolated from the T87 cells and transformed with the
301 appropriate plasmids according to He et al. (2007) with minor modifications. In each
302 transformation, approximately 5×10^5 protoplasts were transfected with 20 µg of
303 plasmid DNA. After transformation, the protoplasts were suspended in WI solution
304 (0.5 M mannitol, 4 mM MES, pH 5.7, 20 mM KCl) and incubated at 21°C in the dark
305 for approximately 16 h.

306

307 **Construction and selection of transgenic *Arabidopsis thaliana* plants**

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

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

314

315 **Confocal laser scanning microscopy**

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

324

325 **Gene expression analysis**

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

341

342 **Western blotting**

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

345

346 **Results**

347

348 **Identification of Potential SnRK2.10 Targets by Phosphoproteomic Approach**

349 To identify the cellular targets of SnRK2.10 phosphorylated in response to stress, we
350 compared the sets of phosphoproteins isolated from the roots of five-week-old
351 Arabidopsis plants: wild type (wt), *snrk2.10-1* knockout mutant (KO), and two
352 transgenic Arabidopsis expressing *35S::GFP-SnRK2.10* (OE) subjected or not to salt
353 stress (treatment with 250 mM NaCl for 30 min). Four independent experiments were
354 performed. The proteins were digested with trypsin, and the tryptic phosphopeptides
355 were enriched by affinity chromatography on TiO₂ and analyzed by liquid
356 chromatography-tandem mass spectrometry (LC/MS). As a result, 1715
357 phosphopeptides were identified (Supplemental Table 1). We found 114
358 phosphopeptides (representing 95 proteins) that, according to the results of MS/MS
359 fragmentation, were at least 2 times more often identified in the roots of wt or OE
360 plants subjected to salt stress than in the roots of nontreated plants. Moreover, these
361 phosphorylations were absent in the *snrk2.10* mutant (Supplemental Tables 2 and 3).
362 Therefore, we assume that the list may contain proteins phosphorylated by
363 SnRK2.10 directly or by kinase(s) downstream of SnRK2.10. Among them, there
364 were RNA binding proteins, protein kinases, phosphatases, transcription and
365 translation factors, and late embryogenesis abundant (LEA) proteins, including
366 dehydrins. This list also included SnRK2.10 itself, as expected. Gene Ontology (GO)
367 annotation indicates that the majority of the identified proteins play a role in the
368 response to diverse environmental stresses (Supplemental Figure S3, Supplemental
369 Table 4).

370 To identify the overrepresented sequence motifs phosphorylated by SnRK2.10
371 (or some kinases under control of SnRK2.10) in response to salinity, the identified
372 phosphopeptides were analyzed using the Motif-X algorithm (Schwartz and Gygi,
373 2005; Chou and Schwartz, 2011). In this group, two major phosphorylation motifs
374 were extracted: -pS-P- and R-x-x-pS, where x can be any amino acid (Figure 1). The
375 second motif is a well-known SnRK2 (as well as other SnRKs) phosphorylation motif
376 (Kelner et al., 2004; Vlad et al., 2008), while -pSP- represents the mitogen-activated

377 protein kinase (MAPK) target motif. The -pS-P- motif has also been identified in
378 several phosphoproteomic studies performed to find proteins phosphorylated in
379 Arabidopsis in response to ABA in the SnRK2.2/2.3/2.6 pathway (Umezawa et al.,
380 2013; Wang et al., 2013) and in the SnRK1 pathways triggered by energy deprivation
381 (Nukarinen et al., 2016) or by submergence (Cho et al., 2016). These results and our
382 results indicate that in response to stress, the SnRKs most likely directly or indirectly
383 regulate some of the MAPK family members. Moreover, our results showed that
384 several other sequences are phosphorylated in a SnRK2-dependent manner in
385 Arabidopsis plants subjected to salt stress. The results of Umezawa et al. (2013) and
386 Wang et al. (2013) also showed other motifs whose phosphorylation was dependent
387 on ABA-activated SnRK2s.

388 Our further studies focused on two acidic dehydrins (dehydration proteins),
389 ERD10 and ERD14, which were identified in our phosphoproteomic analysis as
390 possible targets of SnRK2.10. We have chosen these proteins because of their
391 participation in plant protection against salinity and water deficits (for review see
392 Hanin et al., 2011; Kosová et al., 2014) and because dehydrins have been
393 considered before as SnRK2.10 targets based on its substrate specificity (Vlad et al.,
394 2008). The ERD10 and ERD14 phosphopeptides, which were enhanced under
395 salinity stress and identified in our phosphoproteomic analysis, are listed in Table 1.

396

397 **SnRK2.10 is Not Involved in the Regulation of ERD10 or ERD14 Accumulation** 398 **in Arabidopsis Plants in Response to Salinity**

399 Dehydrins accumulate in response to salinity stress; therefore, we estimated the
400 dehydrin protein level in wt and *snrk2.10* plants exposed to 250 mM NaCl for various
401 lengths of time using anti-dehydrin antibodies (Supplemental Figure S4a) to ensure
402 that the observed differences in the level of phosphopeptides representing dehydrins
403 reflected changes in their phosphorylation status and not differences in their protein
404 level. Additionally, we analyzed the impact of SnRK2.10 on ERD10 and ERD14
405 accumulation in Arabidopsis plants (wt and the *snrk2.10* mutants) subjected to
406 salinity stress (150 mM NaCl up to 6 days) at both the transcript and protein levels.
407 No significant differences were observed between those lines regarding the ERD10
408 and ERD14 transcript and protein levels, neither in roots nor in leaves, regardless of
409 the duration of the exposure to NaCl. In some experiments, we observed a slightly
410 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 KLHRSxSS
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 ¹⁰⁴SNSSSSSSSDEEGEDGEK¹²¹ 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

445 ⁷⁷SDSSSSSSSSEEEGSDGEK⁹⁵. However, in the phosphoproteomic studies
446 performed by Umezawa et al. (2013), the ERD14 peptide
447 ¹⁰¹LHRSDSSSSSSSSEEEGSDGE¹²⁰ was found to be strongly phosphorylated at
448 various residues (including S79) in response to desiccation. However, it was only
449 when the authors used an isotope labeling with ¹⁶O/¹⁸O approach (Umezawa et al.,
450 2013, supplemental data), whereas, when the label-free method (the method we
451 used) was applied neither this phosphopeptide nor the ERD10 phosphopeptide
452 ¹⁰⁴SNSSSSSSSDEEGEDGEK¹²¹ were identified.

453

454 **ABA-non-activated SnRK2s are the Major Kinases Phosphorylating ERD10 and** 455 **ERD14 in Arabidopsis Seedlings in Response to Salinity**

456 To visualize kinases that might phosphorylate ERD10 and ERD14 in Arabidopsis
457 seedlings exposed to salinity, we conducted an in-gel protein kinase activity assay
458 with GST-ERD10 or GST-ERD14 incorporated into the gel. We analyzed the
459 phosphorylation of GST-ERD10 and GST-ERD14 by proteins extracted from 2-week-
460 old Arabidopsis seedlings not exposed and exposed to salinity stress. For analysis,
461 we used the following Arabidopsis lines: wt, single (*snrk2.10-1*) and multiple
462 (*snrk2.4/2.10* and *snrk2.1/2.4/2.5/2.10*) mutants deficient in SnRK2.10 and some
463 other ABA-non-activated SnRK2s. Both dehydrins were clearly phosphorylated by
464 40-42 kDa kinases present in the extract from the wt seedlings treated with 250 mM
465 NaCl for 10 min (Figure 2d and Supplemental Figure S6). This phosphorylation was
466 not found when the extracts from the seedlings not treated with NaCl were analyzed
467 and was significantly decreased when the extracts from all mutants were studied,
468 especially *snrk2.1/4/5/10*. The results indicate that SnRK2.10 and other ABA-non-
469 activated SnRK2s phosphorylate ERD10 and ERD14, and they seem to be the major
470 kinases phosphorylating these dehydrins in response to salinity. However, even in
471 the *snrk2.1/4/5/10* mutant, some kinase activity phosphorylating ERD10 and ERD14
472 was still present (Supplemental Figure S6), indicating that in addition to ABA-non-
473 activated SnRK2s, there are also other kinases involved in the phosphorylation of
474 dehydrins.

475

476 **SnRK2.10 is Involved in Plant Response to Dehydration**

477 The ERD14 peptide ¹⁰¹LHRSDSSSSSSSSEEEGSDGE¹²⁰, identified by our study as
478 being phosphorylated *in vitro* by SnRK2.10, has been found to be strongly

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

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

508

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

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

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

517

518 **The Phosphorylation of ERD14 Affects its Subcellular Localization**

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

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

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

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

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

585

586 Discussion

587

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

609 By comparing the phosphoproteome of wt, *snrk2.10*, and Arabidopsis
610 overexpressing *GFP-SnrK2.10*, we identified 95 proteins likely phosphorylated from
611 the result of SnRK2.10 activity in response to salinity stress. Among them were DNA-

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

614 Dehydrins attracted our attention because they play an important role not only
615 during the last phase of embryogenesis and the desiccation stage of seed
616 development (Kalemba and Pukacka, 2007) but also in the plant response and
617 acclimation to harsh environmental conditions, especially dehydration caused by
618 drought, cold, freezing or salinity (for review see Hanin et al., 2011; Kosová et al.,
619 2014). Dehydrins constitute group 2 of the late embryogenesis abundant (LEA)
620 protein family. They are intrinsically disordered proteins that accumulate in high
621 levels in plant cells in response to abiotic stresses and that play a protective role as
622 molecular chaperones for membranes, proteins and nucleic acids (for review see
623 Hara, 2010; Hanin et al., 2011; Graether and Boddington, 2014; Liu et al., 2017).
624 Dehydrins are divided into five subgroups: K_n , SK_n , K_nS , Y_nSK_n , and Y_nK_n , where K is
625 the sequence EKKGIME/DKIKEKLPG (or a similar sequence) rich in basic amino
626 acids characteristic of all dehydrins (present in 1 to 11 copies), S is a serine-rich
627 segment (with a stretch of 4-10 serine residues), and Y is the segment that contains
628 the (V/T)D(E/Q)YGNP motif.

629 Dehydrins are highly phosphorylated proteins, and some data indicate that not
630 only their level but also their phosphorylation status is important for stress tolerance.
631 The accumulation of the phosphorylated form of wheat DHN-5 dehydrin (closely
632 related to maize Rab17) was observed in a variety of Tunisian durum resistant to salt
633 and drought stress, while in the sensitive variety, it was weakly detected (Brini et al.,
634 2007). This suggests a positive role for DHN-5 dehydrin phosphorylation in the plant
635 response to osmotic stress. Phosphorylation of *Thellungiella salsuginea* dehydrins
636 TsDHN-1 and TsDHN-2 has been shown to be important for stabilizing the
637 cytoskeleton under stress conditions (Rahman et al., 2011), and a phosphoproteomic
638 analysis performed by Yang et al. (2013) in the root tips of *Phaseolus vulgaris* L.
639 showed that phosphorylation of several dehydrins was significantly enhanced under
640 polyethylene glycol-induced osmotic stress.

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

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

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

661 There are numerous studies concerning the phosphorylation of the S-segment
662 of dehydrins (for a review, see Rorat, 2006; Hanin et al., 2011; Graether and
663 Boddington, 2014) claimed to be catalyzed by CK2 protein kinase (Alsheikh et al.,
664 2003 and 2005). However, it has not been proven that CK2 is the only kinase that
665 phosphorylates dehydrins *in vivo*; quite the opposite. Phosphorylation of maize
666 dehydrin Rab17 (Responsive to ABA 17), also known as DHN1, in plant cells is
667 performed not only by CK2 but also by another kinase(s) (not yet identified) (Riera et
668 al., 2004). The tomato TAS14 protein, a homolog of maize Rab17, is phosphorylated
669 *in vivo* by at least two kinases, CK2 and a kinase whose substrate specificity
670 resembles that of cAMP-dependent protein kinase (PKA); *in vitro* PKA efficiently
671 phosphorylated Rab17 (Godoy et al., 1994). It should be noted that PKA recognizes
672 and phosphorylates the R/K-X-X-S/T motif, which is also efficiently phosphorylated
673 by SnRK2s. Therefore, we suggest that both CK2 and SnRK2 might phosphorylate
674 dehydrins in their S-segment in response to osmotic stress (salinity or dehydration).
675 Since the ABA-non-activated SnRK2s are activated very rapidly in response to
676 osmotic stress (SnRK2.4/SnRK2.10 are fully active within the first few minutes
677 following stressor application; McLoughlin et al., 2012), SnRK2.10 and possibly some
678 other SnRK2s likely phosphorylate dehydrins at the very early stages of the plant
679 response to stress. This modification could trigger subsequent phosphorylation

680 carried out by, e.g., CK2. It has been shown that phosphorylation in the S-segment of
681 Arabidopsis ERD10 and ERD14 (Alsheikh et al., 2003; 2005), as well as of celery
682 vacuolar-associated dehydrin-like protein VCaB45 (Heyen et al., 2002), promotes
683 binding of bivalent metal ions, especially calcium. Those authors analyzed the
684 calcium binding properties of recombinant ERD10 and ERD14 phosphorylated *in*
685 *vitro* by CK2 (Alsheikh et al., 2003; 2005) or VCaB45 isolated from plants not treated
686 or treated with phosphatase and rephosphorylated with CK2 (Heyen et al., 2002).
687 Their results clearly showed enhanced calcium binding by phosphorylated ERD10
688 and ERD14. In the case of VCaB45, the binding was the strongest for the protein
689 isolated from the plants not treated with phosphatase and was practically abolished
690 for dephosphorylated VCaB45. The phosphorylation by CK2 only partially restored
691 the calcium binding ability of the protein (Heyen et al., 2002), indicating that *in vivo*
692 VCaB45 is phosphorylated by another kinase(s). The authors suggested that in the
693 phosphorylated state, the dehydrins ERD10, ERD14 and VCaB45 could act as
694 calcium buffers since their Ca²⁺ binding capacity was rather high, or they could play a
695 role as calcium-dependent chaperones, similar to calreticulin and calnexin (Nigam et
696 al., 1994; Michalak et al., 2002). Since enhanced ERD10 and ERD14
697 phosphorylation has been observed in plants exposed to desiccation (Umezawa et
698 al., 2013) or salt stress (our results), we can expect that the dehydrins
699 phosphorylation by SnRK2s might modulate calcium signaling in response to osmotic
700 stress.

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

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

714 example is the maize dehydrin Rab17 from the YSK₂ group. Goday et al. (1994) have
715 shown that the phosphorylation status of maize Rab17 correlates with its nuclear
716 localization. Rab17 phosphorylated in the S-segment is transported to the nucleus
717 (Jensen et al., 1998; Riera et al., 2004). The results of Riera et al. (2004) have
718 revealed that Rab17 phosphorylation delayed seed germination in salinity stress
719 conditions. The subcellular localization of acidic dehydrins SK₂ and SK₃ is
720 controversial. ERD10 and ERD14 have been described as cytosolic (Rorat, 2006;
721 Candat et al., 2014; Cedeno et al., 2017) even though several programs predicted
722 their cytosolic/nuclear localization (Candat et al., 2014). Dehydrin DHN24 from
723 *Solanum sogarandinum*, which is similar to ERD14, has also been considered an
724 exclusively cytoplasmic protein (Rorat, 2006). Only recently has it been shown that
725 DHN24 is present not only in the cytoplasm but also in the nucleus and the
726 microsomal fraction (Szabala et al., 2014). Our results suggest that phosphorylation
727 of S79, the first serine of the serine stretch in the S-segment, plays a role in the
728 transport of ERD14 from the cytoplasm to the nucleus. We found that the
729 phosphorylation of S79 is catalyzed by SnRK2.10 (or other ABA-non-activated
730 SnRK2s), but we do not exclude its phosphorylation by protein kinases belonging to
731 other families or that phosphorylation by SnRK2s enhances phosphorylation by other
732 kinases. Moreover, we do not exclude that phosphorylation of other serines in the S-
733 segment might also be involved in the regulation of the subcellular localization of
734 ERD14. Candat et al. (2014) observed an exclusively cytoplasmic localization of LEA
735 proteins (including ERD10 and ERD14), but their experiments were performed under
736 normal osmolarity conditions only. Cedeno et al. (2017) analyzed ERD10 and EDR14
737 localization in control, cold, and mild osmotic stress conditions; however, the osmotic
738 stress applied was too weak to cause efficient SnRK2 activation. Moreover, they
739 used a heterologous expression system for the production of Arabidopsis proteins:
740 transient expression in *N. benthamiana* leaves. We also did not observe the
741 translocation of EGFP-ERD14 to the nucleus expressed in *N. benthamiana* leaves
742 upon salt application. Furthermore, even when EGFP-ERD14 was transiently
743 expressed in Arabidopsis protoplasts or stably expressed in Arabidopsis plants, we
744 did not observe nuclear localization in response to salt in every protoplast/cell
745 monitored but only in some of them. In the transgenic plants expressing EGFP-
746 ERD14, we did not observe the nuclear localization of EGFP-ERD14 in the highly
747 proliferating cells of the root tip either before or after the salt treatment. It should be

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

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

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

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

783

784 **Acknowledgements**

785

786 We are grateful to Dr. J. Fronk for critically reading the manuscript. We thank Prof.
787 Christa Testerink for sharing the *snrk2.4-1* (SALK_080588), *snrk2.4-2*
788 (SALK_146522), *snrk2.10-1* (*WiscDsLox233E9*), and *snrk2.4-1/2.10-1* knockout
789 mutants and the Arabidopsis lines expressing GFP-SnRK2.10 with us. We also thank
790 the Salk Institute Genomic Analysis Laboratory and NASC for providing the
791 sequence-indexed Arabidopsis TDNA insertion mutants. We are grateful to Adrian
792 Kasztelan for sharing with us plant material and all members of our laboratory for
793 stimulating discussions. This work was supported by the National Science Centre
794 (grants: 2011/03/B/NZ3/00297, 2014/13/D/NZ3/03101, 2014/12/S/NZ3/00746 and
795 2016/23/B/NZ3/03182).

796

797 **Conflict of Interest**

798

799 The authors claim no conflict of interest.

800

801 **References**

802

- 803 Alonso J.M., Stepanova A.N., Leisse T.J., Kim C.J., Chen H., Shinn P., ... Ecker J.R.
804 (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana.
805 *Science (New York, N.Y.)* 301, 653–657.
- 806 Alsheikh M.K., Heyen B.J. & Randall S.K. (2003) Ion Binding Properties of the
807 Dehydrin ERD14 Are Dependent upon Phosphorylation. *Journal of Biological*
808 *Chemistry* 278, 40882–40889.
- 809 Alsheikh M.K., Svensson J.T. & Randall S.K. (2005) Phosphorylation regulated ion-
810 binding is a property shared by the acidic subclass dehydrins. *Plant, Cell and*
811 *Environment* 28, 1114–1122.
- 812 Bouchabke O., Chang F., Simon M., Voisin R., Pelletier G. & Durand-Tardif M.
813 (2008) Natural variation in Arabidopsis thaliana as a tool for highlighting
814 differential drought responses. *PLoS ONE* 3, e1705.

815 Boudsocq M., Barbier-Brygoo H. & Laurière C. (2004) Identification of nine sucrose
816 nonfermenting 1-related protein kinases 2 activated by hyperosmotic and
817 saline stresses in *Arabidopsis thaliana*. *Journal of Biological Chemistry* 279,
818 41758–41766.

819 Brini F., Hanin M., Lumbreras V., Irar S., Pages M., Masmoudi K. (2007) Functional
820 characterization of DHN-5, a dehydrin showing a differential phosphorylation
821 pattern in two Tunisian durum wheat (*Triticum durum* Desf.) varieties with
822 marked differences in salt and drought tolerance. *Plant Science* 172, 20-28.

823 Bucholc M., Ciesielski A., Goch G., Anielska-Mazur A., Kulik A., Krzywińska E. &
824 Dobrowolska G. (2011) SNF1-related protein kinases 2 are negatively
825 regulated by a plant-specific calcium sensor. *The Journal of Biological*
826 *Chemistry* 286, 3429–3441.

827 Burza A.M., Pekala I., Sikora J., Siedlecki P., Malagocki P., Bucholc M., ...
828 Dobrowolska G. (2006) *Nicotiana tabacum* osmotic stress-activated kinase is
829 regulated by phosphorylation on Ser-154 and Ser-158 in the kinase
830 activation loop. *The Journal of Biological Chemistry* 281, 34299–34311.

831 Candat A., Paszkiewicz G., Neveu M., Gautier R., Logan D.C., Avelange-Macherel
832 M.-H. & Macherel D. (2014) The ubiquitous distribution of late
833 embryogenesis abundant proteins across cell compartments in *Arabidopsis*
834 offers tailored protection against abiotic stress. *The Plant Cell* 26, 3148–3166.

835 Cedeno C., Pauwels K. & Tompa P. (2017) Protein Delivery into Plant Cells: Toward
836 In vivo Structural Biology. *Frontiers in Plant Science* 8, 519.

837 Cho HY, Wen TN, Wang YT, Shih MC. (2016) Quantitative phosphoproteomics of
838 protein kinase SnRK1 regulated protein phosphorylation in *Arabidopsis* under
839 submergence. *Journal of Experimental Botany* 67, 2745-2760.

840 Chou M.F. & Schwartz D. (2011) Biological sequence motif discovery using motif-x.
841 *Current Protocols in Bioinformatics* 35, 13.15.1-13.15.24.

842 Clough S.J. & Bent A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-
843 mediated transformation of *Arabidopsis thaliana*. *Plant Journal* 16, 735-743.

844 Czechowski T., Stitt M., Altmann T., Udvardi M.K. & Scheible W.-R. (2005) Genome-
845 wide identification and testing of superior reference genes for transcript
846 normalization in *Arabidopsis*. *Plant Physiology* 139, 5-17.

847 Ellouzi H., Hamed K. Ben, Cela J., Müller M., Abdely C. & Munné-Bosch S. (2013)
848 Increased sensitivity to salt stress in tocopherol-deficient *Arabidopsis*

849 mutants growing in a hydroponic system. *Plant Signaling & Behavior* 8,
850 e23136.

851 Fujii H., Verslues P.E. & Zhu J.-K. (2007) Identification of two protein kinases
852 required for abscisic acid regulation of seed germination, root growth, and
853 gene expression in Arabidopsis. *The Plant Cell* 19, 485–494.

854 Fujii H. & Zhu J.-K. (2009) Arabidopsis mutant deficient in 3 abscisic acid-activated
855 protein kinases reveals critical roles in growth, reproduction, and stress.
856 *Proceedings of the National Academy of Sciences of the United States of*
857 *America* 106, 8380–8385.

858 Fujii H., Verslues P.E. & Zhu J.-K. (2011) Arabidopsis decuple mutant reveals the
859 importance of SnRK2 kinases in osmotic stress responses in vivo.
860 *Proceedings of the National Academy of Sciences of the United States of*
861 *America* 108, 1717–1722.

862 Fujii H. & Zhu J.-K. (2012) Osmotic stress signaling via protein kinases. *Cellular and*
863 *Molecular Life Sciences* 69, 3165–3173.

864 Fujita Y., Nakashima K., Yoshida T., Katagiri T., Kidokoro S., Kanamori N., ...
865 Yamaguchi-Shinozaki K. (2009) Three SnRK2 protein kinases are the main
866 positive regulators of abscisic acid signaling in response to water stress in
867 arabidopsis. *Plant and Cell Physiology* 50, 2123–2132.

868 Furihata T., Maruyama K., Fujita Y., Umezawa T., Yoshida R., Shinozaki K. &
869 Yamaguchi-Shinozaki K. (2006) Abscisic acid-dependent multisite
870 phosphorylation regulates the activity of a transcription activator AREB1.
871 *Proceedings of the National Academy of Sciences of the United States of*
872 *America* 103, 1988–1993.

873 Geiger D., Scherzer S., Mumm P., Stange A., Marten I., Bauer H., ... Hedrich R.
874 (2009) Activity of guard cell anion channel SLAC1 is controlled by drought-
875 stress signaling kinase-phosphatase pair. *Proceedings of the National*
876 *Academy of Sciences of the United States of America* 106, 21425–30.

877 Goday A., Jensen A. B., Culiáñez-Macià F. A., Mar Albà M., Figueras M., Serratosa J.,
878 ... Pagès M. (1994) The maize abscisic acid-responsive protein Rab17 is
879 located in the nucleus and interacts with nuclear localization signals. *The*
880 *Plant Cell* 6, 351–360.

881 Godoy J.A., Lunar R., Torres-Schumann S., Moreno J., Rodrigo R.M. & Pintor-Toro
882 J.A. (1994) Expression, tissue distribution and subcellular localization of

883 dehydrin TAS14 in salt-stressed tomato plants. *Plant Molecular Biology*
884 26,1921-1934.

885 Graczyk D., Debski J., Muszyńska G., Bretner M., Lefebvre O. & Boguta M. (2011)
886 Casein kinase II-mediated phosphorylation of general repressor Maf1
887 triggers RNA polymerase III activation. *Proceedings of the National Academy*
888 *of Sciences of the United States of America* 108, 4926-4931.

889 Graether S.P. & Boddington K.F. (2014) Disorder and function: a review of the
890 dehydrin protein family. *Frontiers in Plant Science* 5, 1–12.

891 Grondin a, Rodrigues O., Verdoucq L., Merlot S., Leonhardt N. & Maurel C. (2015)
892 Aquaporins Contribute to ABA-Triggered Stomatal Closure through OST1-
893 Mediated Phosphorylation. *The Plant Cell* 27, 1945–1954.

894 Hanin M., Brini F., Ebel C., Toda Y., Takeda S. & Masmoudi K. (2011) Plant
895 dehydrins and stress tolerance: versatile proteins for complex mechanisms.
896 *Plant Signaling & Behavior* 6, 1503–1509.

897 Hara M., Shinoda Y., Tanaka Y., Kuboi T. (2009) DNA binding of citrus dehydrin
898 promoted by zinc ion. *Plan, Cell and Environment* 32, 532-541.

899 Hara M. (2010) The multifunctionality of dehydrins: an overview. *Plant Signaling &*
900 *Behavior* 5, 503-508.

901 Harrison S.J., Mott E.K., Parsley K., Aspinall S., Gray J.C., Cottage A. (2006) A rapid
902 and robust method of identifying transformed *Arabidopsis thaliana* seedlings
903 following floral dip transformation. *Plant Methods* 2, 19

904 He P., Shan L. & Sheen J. (2007) The use of protoplasts to study innate immune
905 responses. *Methods in Molecular Biology* 354, 1–9.

906 Heyen B.J., Alsheikh M.K., Smith E.A., Torvik C.F., Seals D.F. & Randall S.K. (2002)
907 The calcium-binding activity of a vacuole-associated, dehydrin-like protein is
908 regulated by phosphorylation. *Plant Physiology* 130, 675–687.

909 Jensen A.B., Goday A., Figueras M., Jessop A.C. & Pagés M. (1998)
910 Phosphorylation mediates the nuclear targeting of the maize Rab17 protein.
911 *Plant Journal* 13, 691–697.

912 Kalembe E.M. & Pukacka S. (2007). Possible roles of LEA proteins and sHSPs in
913 seed protection: a short review. *Biology Letters*. 44, 3–16

914 Kelner A., Pekala I., Kaczanowski S., Muszynska G., Hardie D.G. & Dobrowolska G.
915 (2004) Biochemical characterization of the tobacco 42-kD protein kinase
916 activated by osmotic stress. *Plant Physiology* 136, 3255-3265.

- 917 Kim M.J., Park M.-J., Seo P.J., Song J.-S., Kim H.-J. & Park C.-M. (2012) Controlled
918 nuclear import of the transcription factor NTL6 reveals a cytoplasmic role of
919 SnRK2.8 in the drought-stress response. *The Biochemical Journal* 448, 353–
920 363.
- 921 Kobayashi Y., Yamamoto S., Minami H., Kagaya Y. & Hattori T. (2004) Differential
922 activation of the rice sucrose nonfermenting1-related protein kinase2 family
923 by hyperosmotic stress and abscisic acid. *The Plant Cell* 16, 1163–1177.
- 924 Kobayashi Y., Murata M., Minami H., Yamamoto S., Kagaya Y., Hobo T., ... Hattori T.
925 (2005) Abscisic acid-activated SNRK2 protein kinases function in the gene-
926 regulation pathway of ABA signal transduction by phosphorylating ABA
927 response element-binding factors. *Plant Journal* 44, 939–949.
- 928 Kosová K., Vítámvás P. & Prášil I.T. (2014) Wheat and barley dehydrins under cold,
929 drought, and salinity - what can LEA-II proteins tell us about plant stress
930 response? *Frontiers in Plant Science* 5, 343.
- 931 Krzywińska E., Bucholc M., Kulik A., Ciesielski A., Lichočka M., Dębski J.,
932 ...Dobrowolska G. (2016) Phosphatase ABI1 and okadaic acid-sensitive
933 phosphoprotein phosphatases inhibit salt stress-activated SnRK2.4 kinase.
934 *BMC Plant Biology* 16, 136.
- 935 Kulik A., Wawer I., Krzywińska E., Bucholc M. & Dobrowolska G. (2011) SnRK2
936 Protein Kinases—Key Regulators of Plant Response to Abiotic Stresses.
937 *OMICS: A Journal of Integrative Biology* 15, 859–872.
- 938 Kulik A., Anielska-Mazur A., Bucholc M., Koen E., Szymanska K., Zmienko A., ...
939 Dobrowolska G. (2012) SNF1-related protein kinases type 2 are involved in
940 plant responses to cadmium stress. *Plant Physiology* 160, 868–883.
- 941 Lee H.-J., Park Y.-J., Seo P.J., Kim J.-H., Sim H.-J., Kim S.-G. & Park C.-M. (2015)
942 Systemic Immunity Requires SnRK2.8-Mediated Nuclear Import of NPR1 in
943 Arabidopsis. *The Plant Cell* 27, 3425-3438.
- 944 Lee S.C., Lan W., Buchanan B.B. & Luan S. (2009) A protein kinase-phosphatase
945 pair interacts with an ion channel to regulate ABA signaling in plant guard
946 cells. *Proceedings of the National Academy of Sciences of the United States*
947 *of America* 106, 21419–21424.
- 948 Liu Y, Song Q, Li D, Yang X. & Li D. (2017) Multifunctional Roles of Plant Dehydrins
949 in Response to Environmental Stresses. *Frontiers in Plant Science* 8, 1018.
- 950 Martin K., Kopperud K., Chakrabarty R., Banerjee R., Brooks R. & Goodin M.M.

951 (2009) Transient expression in *Nicotiana benthamiana* fluorescent marker
952 lines provides enhanced definition of protein localization, movement and
953 interactions in planta. *The Plant Journal* 59, 150–162.

954 McLoughlin F., Galvan-Ampudia C.S., Julkowska M.M., Caarls L., van der Does D.,
955 Lauriere C., ... Testerink C. (2012) The Snf1-related protein kinases
956 SnRK2.4 and SnRK2.10 are involved in maintenance of root system
957 architecture during salt stress. *The Plant Journal* 72, 436–449.

958 Michalak M., Robert Parker J.M. & Opas M. (2002) Ca²⁺ signaling and calcium
959 binding chaperones of the endoplasmic reticulum. *Cell Calcium* 32, 269-278.

960 Mikula M., Gaj P., Dzwonek K., Rubel T., Karczmarski J., Paziewska A., ... Ostrowski
961 J. (2010) Comprehensive analysis of the palindromic motif TCTCGCGAGA: a
962 regulatory element of the HNRNPK promoter. *DNA Research* 17, 245-260.

963 Mizoguchi M., Umezawa T., Nakashima K., Kidokoro S., Takasaki H., Fujita Y., ...
964 Shinozaki K. (2010) Two closely related subclass II SnRK2 protein kinases
965 cooperatively regulate drought-inducible gene expression. *Plant and Cell*
966 *Physiology* 51, 842–847.

967 Nakashima K., Fujita Y., Kanamori N., Katagiri T., Umezawa T., Kidokoro S., ...
968 Yamaguchi-Shinozaki K. (2009) Three Arabidopsis SnRK2 protein kinases,
969 SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3, involved in
970 ABA signaling are essential for the control of seed development and
971 dormancy. *Plant and Cell Physiology* 50, 345-363.

972 Nigam S.K., Goldberg A.L., Ho S., Rohde M.F., Bush K.T. & MYu S. (1994) A set of
973 endoplasmic reticulum proteins possessing properties of molecular
974 chaperones includes Ca(2+)-binding proteins and members of the
975 thioredoxin superfamily. *The Journal of Biological Chemistry* 269, 1744–1749.

976 Nukarinen E., Nägele T., Pedrotti L., Wurzinger B., Mair A., Landgraf R.,
977 ...Weckwerth W. (2016) Quantitative phosphoproteomics reveals the role of
978 the AMPK plant ortholog SnRK1 as a metabolic master regulator under
979 energy deprivation. *Scientific Reports* 6, 31697.

980 Peirats-Llobet M., Han S.K., Gonzalez-Guzman M., Jeong C.W., Rodriguez L.,
981 Belda-Palazon B., ... Rodriguez P.L. (2016) A Direct Link between Abscisic
982 Acid Sensing and the Chromatin-Remodeling ATPase BRAHMA via Core
983 ABA Signaling Pathway Components. *Molecular Plant* 9, 136–147.

984 Rahman L.N., Smith G.S.T., Bamm V. V, Voyer-Grant J.A.M., Moffatt B.A., Dutcher

985 J.R. & Harauz G. (2011) Phosphorylation of Thellungiella salsauginea
986 dehydrins TsDHN-1 and TsDHN-2 facilitates cation-induced conformational
987 changes and actin assembly. *Biochemistry* 50, 9587–9604.

988 Riera M., Figueras M., López C., Goday A. & Pagès M. (2004) Protein kinase CK2
989 modulates developmental functions of the abscisic acid responsive protein
990 Rab17 from maize. *Proceedings of the National Academy of Sciences of the*
991 *United States of America* 101, 9879–9884.

992 Rorat T. (2006) Plant dehydrins--tissue location, structure and function. *Cellular &*
993 *Molecular Biology Letters* 11, 536–556.

994 Saito C., Ueda T., Abe H., Wada Y., Kuroiwa T., Hisada A., ... Nakano A. (2002) A
995 complex and mobile structure forms a distinct subregion within the
996 continuous vacuolar membrane in young cotyledons of Arabidopsis. *The*
997 *Plant Journal* 29, 245–255.

998 Saito C., Uemura T., Awai C., Tominaga M., Ebine K., Ito J., ... Nakano A. (2011)
999 The occurrence of “bulbs”, a complex configuration of the vacuolar
1000 membrane, is affected by mutations of vacuolar SNARE and phospholipase
1001 in Arabidopsis. *The Plant Journal* 68, 64–73.

1002 Sato A., Sato Y., Fukao Y., Fujiwara M., Umezawa T., Shinozaki K., ... Uozumi N.
1003 (2009) Threonine at position 306 of the KAT1 potassium channel is essential
1004 for channel activity and is a target site for ABA-activated
1005 SnRK2/OST1/SnRK2.6 protein kinase. *The Biochemical Journal* 424, 439–
1006 448.

1007 Schwartz D. & Gygi S.P. (2005) An iterative statistical approach to the identification
1008 of protein phosphorylation motifs from large-scale data sets. *Nature*
1009 *Biotechnology* 23, 1391–1398.

1010 Sessions A., Burke E., Presting G., Aux G., McElver J., Patton D., ... Goff S.A.
1011 (2002) A High-Throughput Arabidopsis Reverse Genetics System. *The Plant*
1012 *Cell* 14, 2985-2994.

1013 Shin R., Alvarez S., Burch A.Y., Jez J.M. & Schachtman D.P. (2007)
1014 Phosphoproteomic identification of targets of the Arabidopsis sucrose
1015 nonfermenting-like kinase SnRK2.8 reveals a connection to metabolic
1016 processes. *Proceedings of the National Academy of Sciences of the United*
1017 *States of America* 104, 6460-6465.

1018 Sirichandra C., Gu D., Hu H.-C., Davanture M., Lee S., Djaoui M., ... Kwak J.M.

1019 (2009) Phosphorylation of the Arabidopsis AtrbohF NADPH oxidase by OST1
1020 protein kinase. *FEBS Letters* 583, 2982–2986.

1021 Soma F., Mogami J., Yoshida T., Abekura M., Takahashi F., Kidokoro S., ...
1022 Yamaguchi-Shinozaki K. (2017) ABA-unresponsive SnRK2 protein kinases
1023 regulate mRNA decay under osmotic stress in plants. *Nature Plants* 3, 16204.

1024 Szabala B.M., Fudali S. & Rorat T. (2014) Accumulation of acidic SK3 dehydrins in
1025 phloem cells of cold- and drought-stressed plants of the Solanaceae. *Planta*
1026 239, 847–863.

1027 Tsugita A. & Kamo M. (1999) 2-D electrophoresis of plant proteins. *Methods in*
1028 *Molecular Biology* 112, 95-97.

1029 Umezawa T., Yoshida R., Maruyama K., Yamaguchi-Shinozaki K. & Shinozaki K.
1030 (2004) SRK2C, a SNF1-related protein kinase 2, improves drought tolerance
1031 by controlling stress-responsive gene expression in *Arabidopsis thaliana*.
1032 *Proceedings of the National Academy of Sciences* 101, 17306–17311.

1033 Umezawa T., Nakashima K., Miyakawa T., Kuromori T., Tanokura M., Shinozaki K. &
1034 Yamaguchi-Shinozaki K. (2010) Molecular basis of the core regulatory
1035 network in ABA responses: Sensing, signaling and transport. *Plant and Cell*
1036 *Physiology* 51, 1821–1839.

1037 Umezawa T., Sugiyama N., Takahashi F., Anderson J.C., Ishihama Y., Peck S.C. &
1038 Shinozaki K. (2013) Genetics and phosphoproteomics reveal a protein
1039 phosphorylation network in the abscisic acid signaling pathway in
1040 *Arabidopsis thaliana*. *Science Signaling* 6, rs8.

1041 Vlad F., Turk B.E., Peynot P., Leung J. & Merlot S. (2008) A versatile strategy to
1042 define the phosphorylation preferences of plant protein kinases and screen
1043 for putative substrates. *The Plant Journal* 55, 104–117.

1044 Wang P., Xue L., Batelli G., Lee S., Hou Y.-J., Van Oosten M.J., ... Zhu J.-K. (2013)
1045 Quantitative phosphoproteomics identifies SnRK2 protein kinase substrates
1046 and reveals the effectors of abscisic acid action. *Proceedings of the National*
1047 *Academy of Sciences of the United States of America* 110, 11205–11210.

1048 Wessel D. & Fuge U.I. (1984) A method for the quantitative recovery of protein in
1049 dilute solution in the presence of detergents and lipids. *Analytical*
1050 *Biochemistry* 138, 141-143.

1051 Woody S.T., Austin-Phillips S., Amasino R.M. & Krysan P.J. (2007) The WiscDsLox
1052 T-DNA collection: an arabidopsis community resource generated by using an

1053 improved high-throughput T-DNA sequencing pipeline. *Journal of Plant*
1054 *Research* 120,157-165.

1055 Yamada H., Koizumi N., Nakamichi N., Kiba T., Yamashino T.& Mizuno
1056 T.(2004)Rapid response of Arabidopsis T87 cultured cells to cytokinin
1057 through His-to-Asp phosphorelay signal transduction. *Bioscience,*
1058 *Biotechnology, and Biochemistry.* 68, 1966-1976.

1059 Yang Z.-B., Eticha D., Fuhrs H., Heintz D., Ayoub D., Van Dorsselaer A., ... Horst
1060 W.J. (2013) Proteomic and phosphoproteomic analysis of polyethylene
1061 glycol-induced osmotic stress in root tips of common bean (*Phaseolus*
1062 *vulgaris* L.). *Journal of Experimental Botany* 64, 5569–5586.

1063 Yoshida T., Fujita Y., Maruyama K., Mogami J., Todaka D., Shinozaki K. &
1064 Yamaguchi-Shinozaki K. (2015) Four Arabidopsis AREB/ABF transcription
1065 factors function predominantly in gene expression downstream of SnRK2
1066 kinases in abscisic acid signalling in response to osmotic stress. *Plant, Cell*
1067 *and Environment* 38, 35–49.

1068 Zhang S. & Klessig D.F. (1997) Salicylic acid activates a 48-kD MAP kinase in
1069 tobacco. *The Plant Cell* 9, 809-824.

1070 Zhang X., Henriques R., Lin S.-S., Niu Q.-W., Chua N.-H. (2006) *Agrobacterium-*
1071 *mediated transformation of Arabidopsis thaliana* using the floral dip method.
1072 *Nature Protocols* 1, 641-646.

1073 Zhu J.K. (2016) Abiotic Stress Signaling and Responses in Plants. *Cell* 167, 313-324.

1074

1075 **Table 1.** The phosphopeptides derived from ERD10 and ERD14 identified by the
1076 phosphoproteomic approach as being potentially phosphorylated by SnRK2.10

1077

1078

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

1079

1080

1081

1082 **Table 2.** The phosphopeptides derived from ERD10 and ERD14 phosphorylated by
 1083 SnRK2.10 *in vitro*
 1084
 1085

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>I</u> LASEFEHK	40 - 57	T49			
	KPEDSQVVNT <u>T</u> PLVETAT <u>P</u> ADIPEEK	204 - 230	S208	T213	T214	T221
ERD14	VATEESSAEV <u>I</u> DR	16 - 28	S21	T26		
	SD <u>S</u> SSSSSSEEEGSDGEK	77 - 95	S79	S78	S79	
	KPEDGSAVAAAPVVPPVVEEAHPVE	131 - 157	S136			

1086
 1087
 1088 The results represent four independent experiments. The phosphorylation sites
 1089 identified are underlined; those identified in all four experiments are bolded.
 1090
 1091
 1092
 1093
 1094
 1095
 1096

1097 **Figure Legends**

1098

1099 **Figure 1.** The phosphorylation motifs identified within putative SnRK2.10 targets
1100 and/or proteins phosphorylated in an SnRK2.10-dependent manner in *Arabidopsis*
1101 *thaliana* roots exposed to salinity stress.

1102 The phosphorylation motifs were identified using the Motif-X algorithm.

1103

1104 **Figure 2.** ERD10 and ERD14 are Phosphorylated by SnRK2.10 *in vitro*

1105 (a) *In vitro* phosphorylation of GST-ERD10 and GST-ERD14 by SnRK2.4,
1106 SnRK2.10, SnRK2.6, or SnRK2.8. The kinases and the dehydrins studied were
1107 produced in *E. coli* and used for *in vitro* phosphorylation assays. Phosphorylation of
1108 the dehydrins (4 µg, each) or MBP (2 µg) (as a universal kinase substrate used as
1109 the kinase activity control) by the recombinant SnRK2s (1 - 2 µg) was monitored by in
1110 solution kinases activity assay described in “Material and Methods”. The reaction
1111 products were separated by SDS-PAGE, and GST-ERD10, GST-ERD14, and MBP
1112 phosphorylation were determined by autoradiography. The representative results
1113 from one of three independent experiments are shown. (b) MS spectra of
1114 phosphopeptides from ERD10 and ERD14 dehydrins phosphorylated *in vitro* by
1115 SnRK2.10. The phosphorylated residues were identified by LC/MS after *in vitro*
1116 phosphorylation of recombinant GST-ERD10 and GST-ERD14 by SnRK2.10 (see
1117 Table 2). The reaction was performed as described above in (a) but without
1118 radioactive ATP. (c) Analysis of phosphorylation of the wild-type and mutated forms
1119 of ERD10 and ERD14 (ERD10S106A, ERD10T49A, and ERD14S79A) by SnRK2.10.
1120 The reaction was performed and analyzed as described in (a). (d) In-gel kinase
1121 activity assay of 2-week-old *Arabidopsis* seedlings of wt, *snrk2.10-1*, *snrk2.4/2.10*,
1122 and *snrk2.1/2.4/2.5/2.10* knockout mutants. The plants were not treated or treated for
1123 10 min with 250 mM NaCl. The extracts were subjected to an in-gel kinase activity
1124 assay using GST-ERD10 or GST-ERD14 as the substrate. The representative results
1125 from one of three independent experiments are shown. Autorad, autoradiograph;
1126 CBB, Coomassie Brilliant Blue.

1127

1128 **Figure 3. SnRK2.10 Impacts Plant Sensitivity to Water Deficit**

1129 (a) The lack of SnRK2.10 enhances water loss from detached *Arabidopsis* rosettes.
1130 The whole rosettes from six-week-old *Arabidopsis* plants were cut off and weighed.

1131 Then, they were incubated in windless conditions at 24°C for 5 h and weighed every
1132 hour. Finally, the rosettes were dried at 70°C overnight and weighed. The cut rosette
1133 water loss (CRWL) was calculated. The representative results from one of four
1134 independent experiments are shown. Eight plants were used for each line per
1135 experiment. For the statistical analysis, a t-test was applied. The asterisks indicate
1136 significant differences from the wild type (*P < 0.05; **P < 0.01, ***P < 0.001). The
1137 average values ± SE are shown. (b) The lack of SnRK2.10 reduces the survival of an
1138 Arabidopsis plant under drought conditions. The Arabidopsis plants were grown in
1139 pots for 17 days under long day conditions and for an additional 2 weeks without
1140 watering. The pictures were taken before watering was stopped (Before drought),
1141 after two weeks without water (Drought), and one day after rewatering (Rewatering).
1142 Ten pots were used for each line per experiment. Representative plants are
1143 presented.

1144

1145 **Figure 4. SnRK2.10 Interacts with ERD10 and ERD14 *in planta***

1146 *N. benthamiana* leaves were co-transformed with pairs of plasmids encoding
1147 nEYFP–SnRK2.10 with cEYFP–ERD10 or cEYFP–ERD14. For the negative control,
1148 nEYFP–SnRK2.10 was co-expressed with cEYFP. BF indicates bright field, bar = 10
1149 µm. The data represent one of three independent experiments showing similar
1150 results.

1151

1152 **Figure 5. Subcellular Localization of EGFP-ERD10 and EGFP-ERD14 and Their**
1153 **Mutated Forms Transiently Expressed in *N. benthamiana* leaves**

1154 (a) Subcellular localization of EGFP-ERD10 and EGFP-ERD14 and their mutated
1155 forms transiently expressed in *N. benthamiana* leaves in control conditions or under
1156 salinity stress. *N. benthamiana* leaves were transformed with plasmids encoding
1157 EGFP-ERD10, EGFP-ERD14 or their mutated forms, EGFP-ERD10S106E, EGFP-
1158 ERD10S106A, EGFP-ERD14S79E or EGFP-ERD14S79A, and their localization was
1159 analyzed.

1160 (b) Subcellular localization of EGFP-ERD14 produced in *Nicotiana benthamiana*
1161 leaves (in control conditions or exposed to salinity stress) in the cortical cytoplasm.
1162 “Bulb-like” structures are marked with white arrows. BF indicates bright field, bar =
1163 10 µm.

1164

1165 **Figure 6. Subcellular Localization of EGFP-ERD14 In Response to Salt Stress**

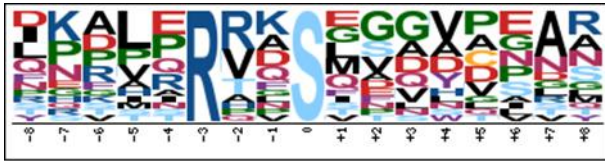
1166 (a) Subcellular localization of EGFP-ERD14 and EGFP-ERD14S79E expressed in
1167 Arabidopsis protoplasts. The protoplasts isolated from T87 cells were transformed
1168 with plasmids encoding EGFP-ERD14 or EGFP-ERD14S79E. The localization of
1169 chimeric proteins was studied before and after exposure to 250 mM NaCl. The
1170 percentage of nuclei containing expressed proteins was calculated from three
1171 independent experiments (approximately 50 protoplasts were analyzed in each
1172 experiment).

1173 (b) Subcellular localization of EGFP-ERD14 and EGFP-ERD14S79E stably
1174 expressed in Arabidopsis. The localization of EGFP-ERD14 and EGFP-ERD14S79E
1175 was monitored in different types of root cells, in the proliferation zone (proliferation),
1176 elongation zone (elongation), and differentiation zone (differentiation), expressing
1177 the proteins studied before and after exposure to 250 mM NaCl (bar = 10 μ m).

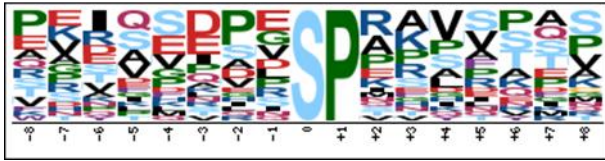
1178 The data represent one of three independent experiments showing similar results.

1179

1180



1181

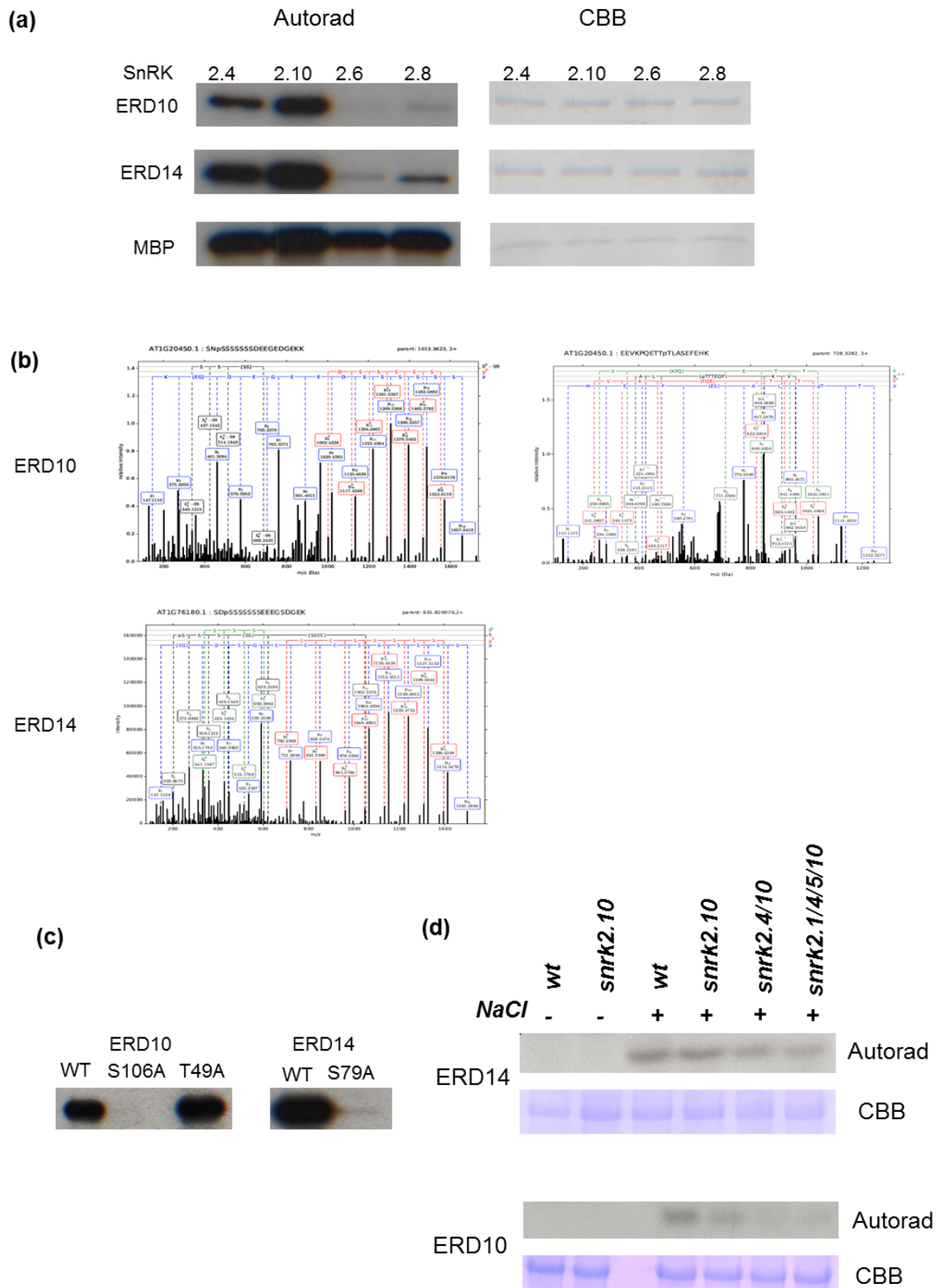


1182

1183 Figure 1

1184

1185

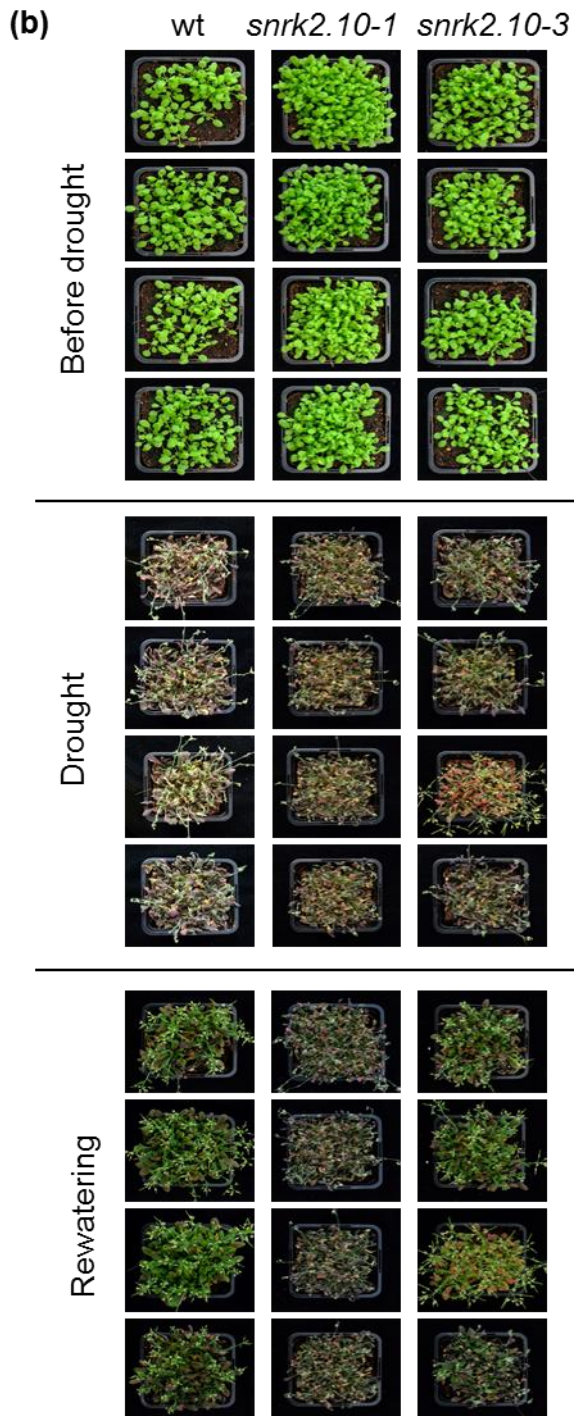
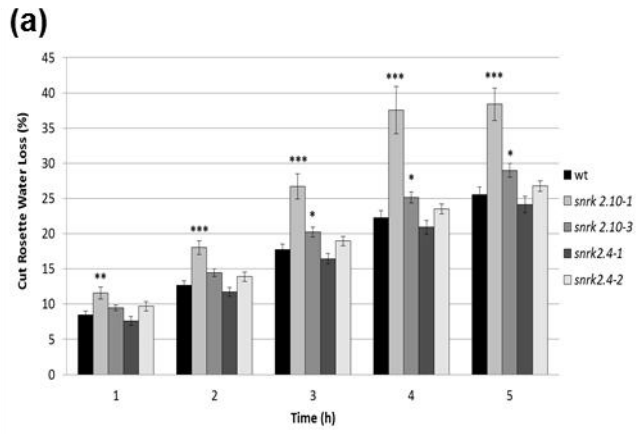


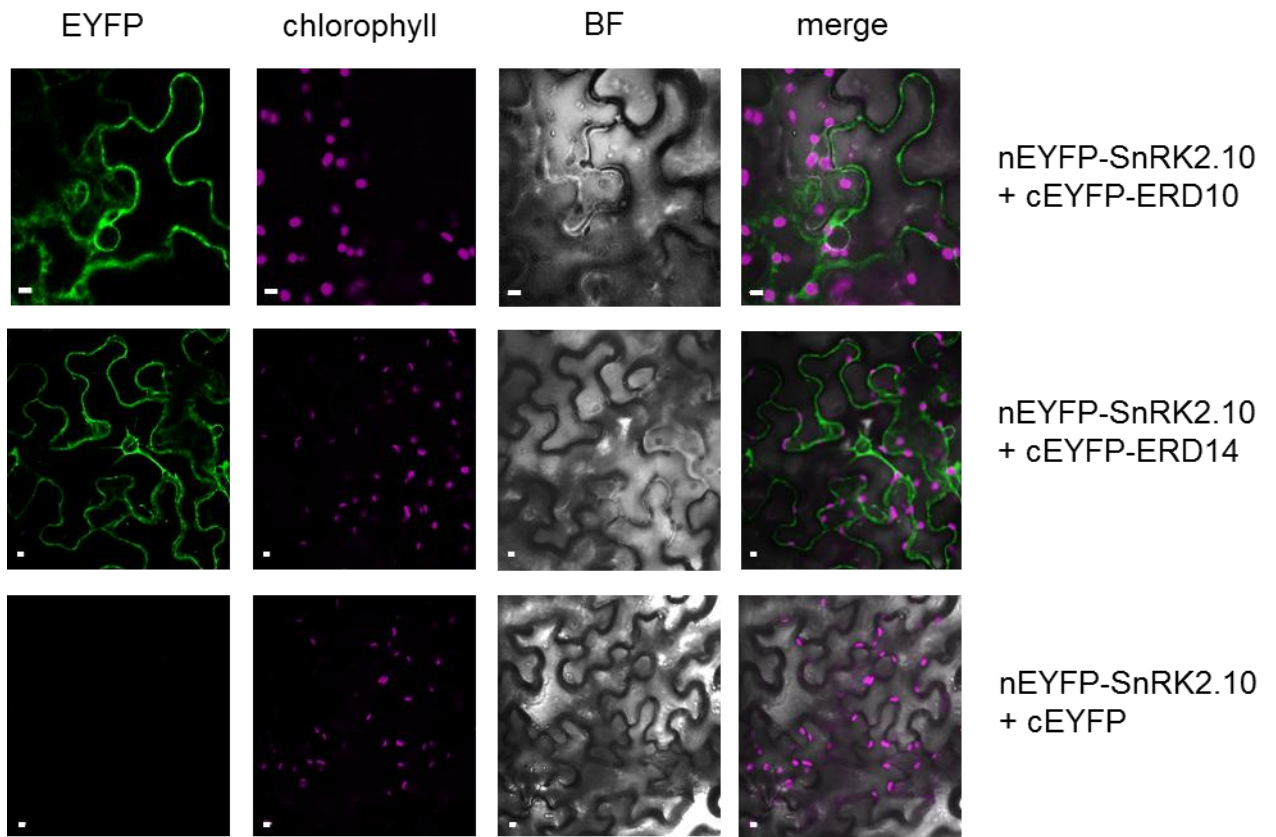
1187

1188

1189

1190 Figure 2



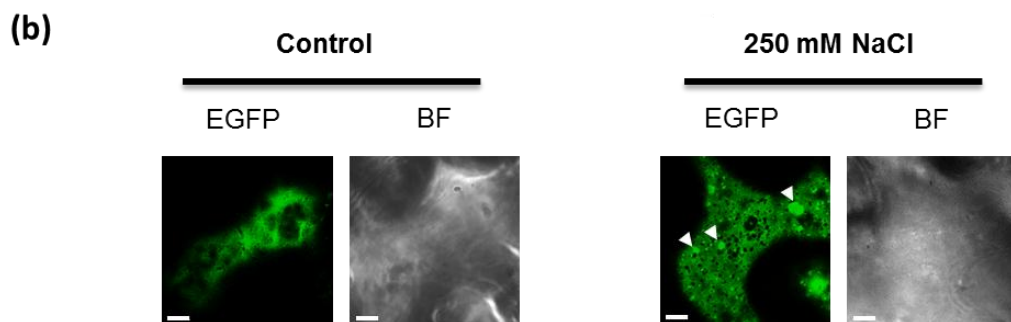
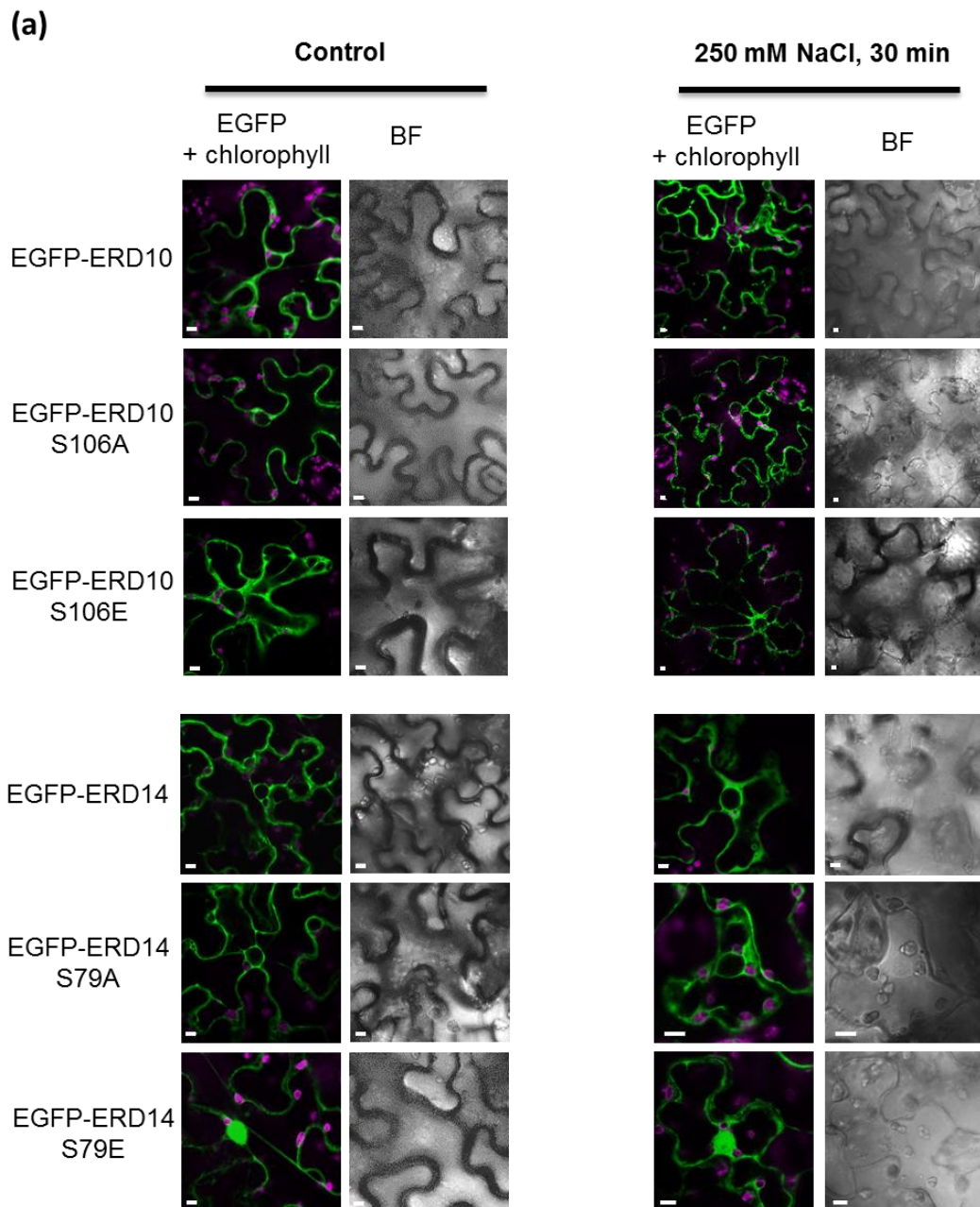


1192

1193

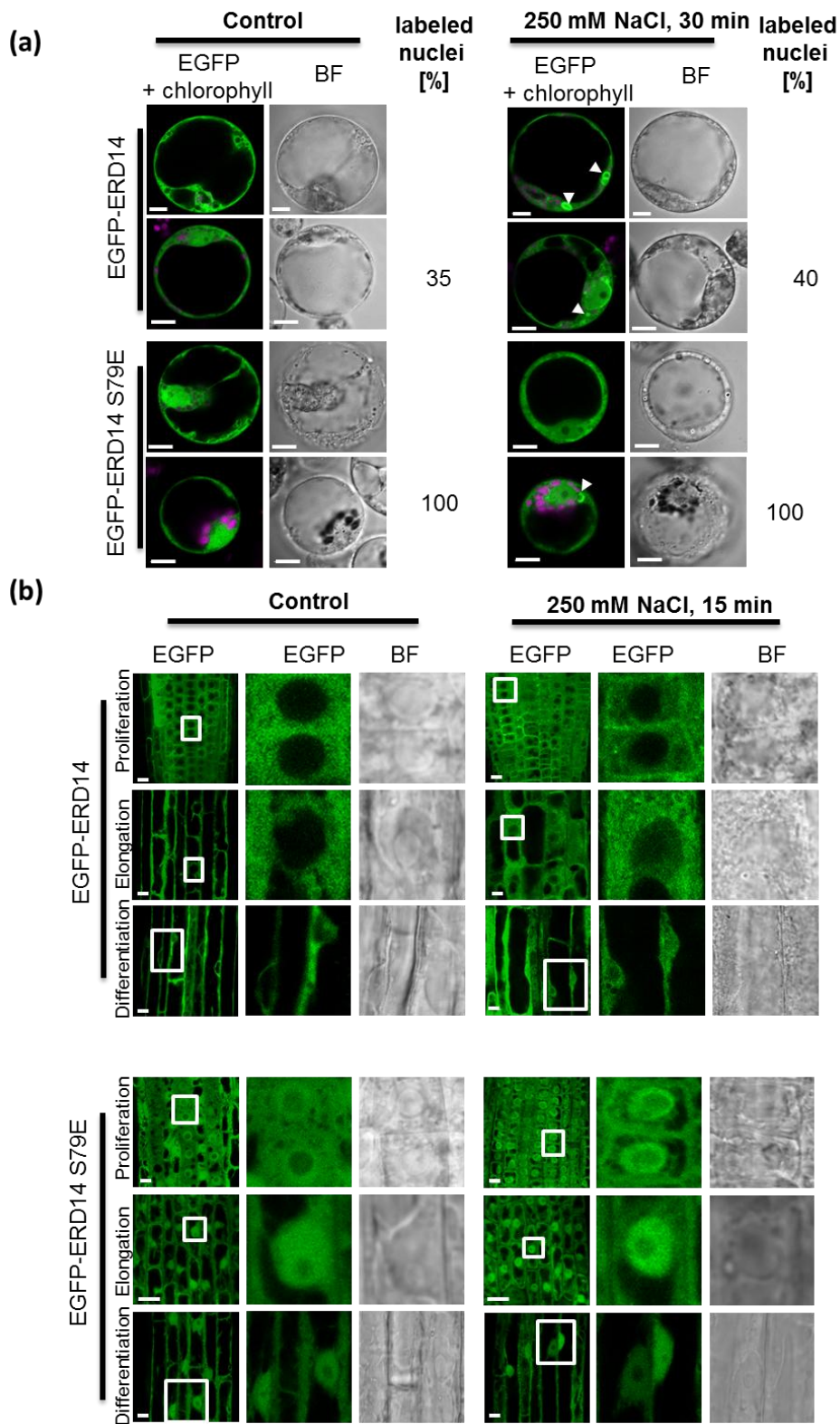
1194 Figure 4

1195



1196
1197
1198
1199

Figure 5



1200

1201

1202 Figure 6