1 **PYL8 ABA receptors of *Phoenix dactylifera* play a crucial role in response**
2 **to abiotic stress and are stabilized by ABA (118)**

3 **Running title:** ABA-induced stabilization of PYL8 ABA receptors in date palm
4 response to abiotic stress (88)

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46 **Date of submission:** 14 September 2020

47 **Number of Figures and tables:** 7

48 **Word count:** 6367

49 **Highlight:** Date palm response to abiotic stress is triggered through PYL8-like
50 ABA receptors that are stabilized by the hormone, which boosts plant
51 adaptation to drought mediated by ABA.

52

53 **Abstract (200 words)**

54 The identification of those prevailing ABA receptors and molecular mechanisms
55 that trigger drought adaptation in crops well adapted to harsh conditions such
56 as date palm (*Phoenix dactylifera*, Pd) sheds light on plant-environment
57 interactions. We reveal that PdPYL8-like receptors are predominantly
58 expressed under abiotic stress, being Pd27 the most expressed receptor in date
59 palm. Therefore, subfamily I PdPYL8-like receptors have been selected for ABA
60 signaling during abiotic stress response in this crop. Biochemical
61 characterization of PdPYL8-like and PdPYL1-like receptors revealed receptor-
62 and ABA-dependent inhibition of PP2Cs, which triggers activation of the
63 *pRD29B-LUC* reporter in response to ABA. PdPYLs efficiently abolish PP2C-
64 mediated repression of ABA signaling, but loss of the Trp lock in the seed-
65 specific AHG1-like phosphatase PdPP2C79 markedly impairs its inhibition by
66 ABA receptors. Characterization of *Arabidopsis* transgenic plants that express
67 PdPYLs shows enhanced ABA signaling in seed, root and guard cells.
68 Specifically, Pd27 overexpressing (OE) plants showed lower ABA content and
69 were more efficient than wild type to lower transpiration at negative soil water
70 potential, leading to enhanced drought tolerance. Finally, PdPYL8-like receptors
71 accumulate after ABA treatment, which suggests that ABA-induced stabilization
72 of these receptors operates in date palm for efficient boosting of ABA signaling
73 in response to abiotic stress.

74 **Keywords:** Absciscic acid, *Phoenix dactylifera*, date palm, PYL8, ABA receptor,
75 AHG1, PP2C

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79 Introduction

80 Among monocotyledons, the palm family plants (Arecaceae or Palmae) is
 81 second in economic importance after the grass family (Poaceae), and among
 82 the palm crops, the top three palm species are African oil palm (*Elaeis*
 83 *guineensis*), date palm (*Phoenix dactylifera*) and coconut (*Cocos nucifera*).
 84 Date palm (*Phoenix dactylifera* L., 2n=36) is thought to be native to the Arabian
 85 Peninsula from where was introduced to northern India, North Africa and
 86 southern Spain (Al-Mssallem et al., 2013). Date palm used to be the main
 87 nutrient for the population of Arabian Peninsula and currently is a major food
 88 crop in the Middle East, northern Africa and other places with suitable climate
 89 (Yin et al., 2012). Additionally, it is a sociocultural symbol in many countries with
 90 long agriculture history, being one of oldest domesticated trees (Al-Mssallem et
 91 al., 2013).

92 Date palm is a plant species adapted for growth under dry conditions and
 93 recent studies indicate that heat and drought stresses only slightly affect
 94 photosynthesis (Arab et al., 2016). Fast guard cell abscisic acid (ABA) signaling
 95 is present in date palm to limit transpirational water loss and the *Phoenix*
 96 *dactylifera* (Pd) SLAC1-type anion channel is regulated by the ABA kinase
 97 PdOST1 (Müller et al., 2017). In the presence of nitrate at the extracellular face
 98 of the anion channel, ABA enhanced and accelerated stomatal closure (Müller
 99 et al., 2017). Additionally, the date palm genome displays an expanded late
 100 embryogenesis abundant (LEA) gene family, whose ABA-induced expression is
 101 important for resistance to abiotic stress, particularly LEA2 genes that are
 102 ubiquitously expressed (Al-Mssallem et al., 2013). Given that ABA plays a key
 103 role for plant adaptation to drought and date palm is well adapted to survive and
 104 produce fruits under harsh environmental conditions, the characterization of
 105 core ABA signaling components in Pd is of great interest. ABA signaling is
 106 initiated by ABA perception through PYRABACTIN RESISTANCE1
 107 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA
 108 RECEPTORS (RCAR) receptors (Park et al., 2009; Ma et al., 2009; Santiago et
 109 al., 2009; Rodriguez et al., 2019), which leads to interaction with and
 110 inactivation of clade A protein phosphatase type 2Cs (PP2Cs), such as ABA
 111 INSENSITIVE 1 (ABI1) and ABI2, HYPERSENSITIVE TO ABA (HAB1) and

112 HAB2, and PROTEIN PHOSPHATASE 2CA/ABA-HYPERSENSITIVE
113 GERMINATION 3 (PP2CA/AHG3), thereby relieving their inhibition on three
114 ABA-activated SNF1-related protein kinases (SnRK2s) termed subclass III
115 SnRK2s, i.e. SnRK2.2/SnRK2D, 2.3/I and 2.6/E/OST1 (Umezawa et al., 2009;
116 Vlad et al., 2009).

117 The discovery of the 14-member gene family of PYR/PYL/RCAR ABA
118 receptors in *Arabidopsis thaliana* (Park et al., 2009; Ma et al., 2009; Santiago et
119 al., 2009) has enabled the identification of the multigene families of ABA
120 receptors in different crops. Functional characterization of crop ABA receptors is
121 required to determine the prevailing receptors for abiotic stress adaptation;
122 however, this knowledge is limited (Gonzalez-Guzman et al., 2014; Miao et al.,
123 2018). Crop PYR/PYL/RCARs belong to multi-gene families, which are
124 composed of 15 members in tomato (Gonzalez-Guzman et al., 2014), 12 in rice
125 (He et al., 2014), 13 in maize (He et al., 2018), 9 in wheat (Mega et al., 2019), 9
126 in barley (Seiler et al., 2014) and 11 in sweet orange (Arbona et al., 2017). The
127 receptors encoded in these genomes are distributed in three distinct subfamilies
128 based on amino acid sequence identity, which are reminiscent of the
129 *Arabidopsis* PYR1/PYL1-like (clade III), PYL4-like (clade II) and PYL8-like
130 subfamilies (clade I) (Rodriguez et al., 2019). However, not all families play
131 similar roles for ABA signaling in different tissues and specific functions for
132 some members of these subfamilies have emerged in the last years (Antoni et
133 al., 2013; Zhao et al., 2016; Dittrich et al., 2019). In particular, PYL8 plays a
134 non-redundant role for ABA signaling in root, and ABA diminishes ubiquitination
135 of this unique receptor, which leads to its stabilization and accumulation in
136 presence of ABA (Belda-Palazon et al., 2018). In contrast to *Arabidopsis*,
137 functional knowledge in crops is scarce and prevents further biotechnological
138 use, for example, to regulate transpiration or root architecture in order to better
139 adapt plants to drought.

140 The genome of date palm (estimated genome length 670 Mb) has been
141 sequenced using different genome assemblies (Al-Dous et al., 2011; Al-
142 Mssallem et al., 2013; Hazzouri et al., 2015 and 2019), and different
143 transcriptomic studies have been published (reviewed by Gros-Balthazard et al.,
144 2018). This race for genomic data has enabled the identification and

145 characterization of the sex determination locus (Torres et al., 2018), pathways
146 responsible for fruit maturation (Al-Mssallem et al., 2013; Hazzouri et al., 2019)
147 and elucidation of the domestication history and diversity (Hazzouri et al., 2015;
148 Gros-Balthazard et al., 2018). RNA-seq expression data are available for the
149 different fruit stages, response to different stress treatments and comparative
150 transcriptome analysis of oil palm and date palm mesocarp (Bourgis et al.,
151 2011; Yin et al., 2012; Yaish et al., 2017; Al-Harrasi et al., 2018; Xiao et al.,
152 2019, Hazzouri et al., 2019). Genomic resources have been used along this
153 work to identify the Pd ABA receptor family and pinpoint the major Pd ABA
154 receptors expressed during ABA treatment, salt, heat, drought or combined
155 heat-drought conditions (Müller et al., 2017; Safronov et al., 2017). As a result,
156 we reveal that the PYL8-like ABA receptors of *Phoenix dactylifera* play a major
157 role in response to abiotic stress and their accumulation is promoted by ABA
158 through a posttranscriptional mechanism. Functional characterization of
159 Phoenix ABA receptors indicates that inhibition of clade A PP2Cs is crucial for
160 drought tolerance of date palm.

161 **Materials and methods**

162 *Plant material and growth conditions*

163 Two-year-old date palm seedlings were cultivated at 22/16°C, 50% RH, 12h
164 light regime. For ABA treatment, single cut pinnae were treated for 2h with
165 CO₂-free air in darkness to fully open stomata, and then illuminated for 4h at a
166 photon flux rate of 620 $\mu\text{mol}/\text{m}^2\text{s}$. Then 25 μM ABA was fed via the petiole. After
167 2h, RNA was extracted from the pinnae. Control was treated equally with ABA-
168 free solution. RNA was extracted from leaves and RNA-seq library generation
169 and sequencing was described by Müller et al., (2017). For heat, drought and
170 heat+drought treatments, plants were maintained under greenhouse conditions
171 and transferred to growth chambers for stress treatments. In growth chambers,
172 plants were first acclimatized for two weeks (16/8 h photoperiod and 20/15°C,
173 70% RH, at a photon flux rate of 200-300 $\mu\text{mol}/\text{m}^2\text{s}$ at leaf level). Experiments
174 were carried out in two batches. In the first batch, plants were irrigated every
175 second day (well-watered) and exposed to different growth temperatures:
176 20/15°C (70% RH) or 35/15°C (60-70% RH) for two weeks, followed by
177 harvesting 6 h after the onset of light. In the second batch, watering was

178 stopped after the acclimation period (drought conditions) and plants grown at
179 35°C were harvested after 4-5 days (drought + heat) whereas plants grown at
180 20°C were collected after 7-8 days (drought). For salt treatment, pots were
181 flooded with salt solution for 1 h every second day starting from day 2. After two
182 weeks, RNA was extracted from plants treated with salt-free solution, 200 mM
183 salt solution or 600 mM salt solution. RNA-seq library generation and
184 sequencing was performed as described by Safronov et al. (2017). Root and
185 fruit expression data for date palm have been reported by Xiao et al., (2019)
186 and Hazzouri et al. (2019), respectively. Briefly, germinated root tips (from 12-
187 week-old seedlings) were sampled for RNA extraction. Total RNA was extracted
188 from 75 mg of plant material. Fruits of the Khalas variety were sampled at 45,
189 75, 105, 120 and 135 days after pollination (DAP). *Arabidopsis thaliana* plants
190 were grown as described by Planes et al. (2015).

191

192 *Transpiration assay*

193 Transpiration was measured in 16 plants per genotype that were grown in
194 individual jiffy peat plugs (Jiffy-7 peat pellets, Semillas Batlle S.A., Barcelona,
195 Spain) under different soil water potential conditions according to de Ollas et al.,
196 (2019). Plants were kept under well-watered conditions for 4 weeks in a growth
197 chamber (Equitec model EGCS 351 3S HR, Getafe, Spain) with a day/night
198 temperature of 23/18 °C, an 8 h light photoperiod ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$), and a
199 relative humidity of 60–65%. To set up the drought conditions plants were
200 randomly divided in 4 groups of 4 plants and differentially watered to cover the
201 soil water content range from well-watered conditions to plant wilting (de Ollas
202 et al., 2019). To characterize transpiration, individual plugs were weighed every
203 24 hours to account for plants transpiration water loss and individually
204 normalized to leaf area. Transpiration was measured two consecutive days
205 before harvesting. Plant tissue of each individual plant was immediately frozen
206 in liquid N₂ and lyophilized to analyze ABA endogenous contend.

207

208 *ABA quantification*

209 ABA analysis was performed according to Durgbanshi, A. et al., (2005) with
210 slight modifications. Briefly, 0.1 g of dry plant material was extracted in 2 ml of
211 distilled H₂O after spiking with 25 μl of a 2 mg l⁻¹ solution of d6-ABA as internal

standard. After centrifugation (10,000g at 4 °C), supernatants were recovered, and the pH was adjusted to 3.0 with 30% acetic acid. The acidified water extract was partitioned twice against 3 ml of di-ethyl ether. The organic layer was recovered and evaporated under vacuum in a centrifuge concentrator (Speed Vac, Jouan, Saint Herblain Cedex, France). The dry residue was then re-suspended in a 9:1 H₂O:MeOH solution by sonication. The resulting solution was filtered and directly injected into a UPLC system (Waters Acquity SDS, Waters Corp., Milford, MA, USA) interfaced to a TQD triple quadrupole (Micromass Ltd, Manchester, UK) mass spectrometer through an orthogonal Z-spray electrospray ion source. Separations were carried out on a Gravity C18 column (50 × 2.1 mm, 1.8 µm, Macherey–Nagel GmbH, Germany) using a linear gradient of MeOH and H₂O supplemented with 0.1% acetic acid at a flow rate of 300 µl min⁻¹. Transitions for ABA/d6-ABA (263 → 153/269 → 159) were monitored in negative ionization mode. Quantitation of plant hormones was achieved by external calibration with known amounts of pure standards using Masslynx v4.1 software.

Generation of *Arabidopsis* transgenic plants

The indicated pAlligator2-35S:*Pd* and pMDC43-35S:*GFP-Pd* constructs were transferred to *Agrobacterium tumefaciens* C58C1 (pGV2260) (Deblaere *et al.*, 1985) by electroporation and used to transform Columbia wild type plants by the floral dip method (Clough and Bent, 1998). T1 transgenic seeds were selected based on seed GFP fluorescence (pAlligator2) or hygromycin resistance (pMDC43), and sowed in soil to obtain the T2 generation. Homozygous T3 progeny was used for further studies and expression of HA-tagged or GFP tagged protein was verified by immunoblot analysis using anti-HA-HRP or anti-GFP antibodies, respectively.

Constructs

The PdPP2C55, PdPP2C79, Pd15, Pd27, Pd32, Pd44, Pd957 coding sequences were obtained as synthetic DNA fragments (Invitrogen), which were amplified using the indicated primers (Supplementary Table S1), verified by sequencing and cloned into pCR8/GW/TOPO. In order to obtain recombinant

244 protein, each coding sequence was excised using an *NcoI-EcoRI* double
245 digestion and cloned into pETM11.

246 *Chemicals*

247 The ABA agonists used in this work were provided by Dr Sean R. Cutler
248 (University of California, Riverside, USA). ABA was obtained from Biosynth
249 (<https://www.biosynth.com/>).

251 *Transient protein expression in Nicotiana benthamiana*

252 *Agrobacterium* infiltration of *N. benthamiana* leaves was performed basically as
253 described by Saez et al. (2008). For transient expression of GFP-PdPP2C55,
254 PdPP2C79 or Pd ABA receptors, the corresponding pMDC43 binary vector was
255 introduced into *A. tumefaciens* C58C1 (pGV2260) by electroporation and
256 transformed cells were selected in LB plates supplemented with kanamycin (50
257 ug/ml). Then, they were grown in liquid LB medium to late exponential phase
258 and cells were harvested by centrifugation and resuspended in 10 mM
259 morpholinoethanesulphonic (MES) acid-KOH pH 5.6 containing 10 mM MgCl₂
260 and 150 mM acetosyringone to an OD_{600 nm} of 1. These cells were mixed with
261 an equal volume of *Agrobacterium* C58C1 (pCH32 35S:p19) expressing the
262 silencing suppressor p19 of tomato bushy stunt virus so that the final density of
263 *Agrobacterium* solution was about 1 (final concentration OD₆₀₀=0.5 each).
264 Bacteria were incubated for 3 h at room temperature and then injected into
265 young fully expanded leaves of 4-week-old *N. benthamiana* plants. Leaves were
266 examined 48-72 h after infiltration using confocal laser scanning microscopy.

268 *Protoplast transfection*

269 We analyzed ABA signaling in *Arabidopsis* protoplasts basically as described by
270 Fujii et al. (2009). Briefly, protoplasts prepared from wild-type Col-0 plants were
271 transfected with the reporter construct pRD29B:LUC, p35S:GUS for
272 normalization, and effector plasmids encoding ABF2, ABF2+HAB1 or
273 ABF2+HAB1+PdPYL proteins. LUC activity was measured in protein extracts
274 prepared from protoplast suspensions 6 h after transfection, either in the
275 absence or presence of 5 μM exogenous ABA that was added 3 h before
276 measuring LUC activity. The activity of the pRD29B:LUC reporter was

277 normalized with p35S:GUS as described by Fujii et al. (2009). To generate the
278 effector plasmids, the coding sequence of ABF2, HAB1, Pd15, Pd27, Pd32,
279 Pd44 or Pd957 was recombined by LR reaction from pCR8 entry vector to
280 pXCS destination vector (Witte et al., 2004). The pRD29B:LUC and 35S:GUS
281 pSK constructs were previously described (Christmann et al., 2005; Moes et al.,
282 2008)

283

284 *Protein extraction, analysis and immunodetection*

285 Protein extracts for immunodetection experiments were prepared from
286 *Arabidopsis* transgenic lines expressing HA-tagged or GFP-tagged ABA
287 receptors or GFP-tagged PP2C/ABA receptors transiently expressed in
288 *Nicotiana benthamiana* as described by Fernandez et al., (2020).

289

290 *Confocal Laser Scanning Microscopy (CLSM)*

291 Confocal imaging was performed using a Zeiss LSM 780 AxioObserver.Z1 laser
292 scanning microscope with C-Apochromat 40x/1.20 W corrective water
293 immersion objective. The GFP fluorophore was excited and fluorescence
294 emission detected at the indicated wavelengths: 488 nm/500-530 nm, and
295 chlorophyll at 561 nm/ 685-760 nm. Post-acquisition image processing was
296 performed using ZEN (ZEISS Efficient Navigation) Lite 2012 imaging software
297 and ImageJ (<http://rsb.info.gov/ij/>).

298

299 *Seed germination and seedling establishment assays*

300 After surface sterilization of the seeds, stratification was conducted in the dark
301 at 4°C for 3 d. Approximately 100 seeds of each genotype were sowed on MS
302 plates supplemented with different ABA concentrations per experiment. To
303 score seed germination, radical emergence was analyzed at 72 h after sowing.
304 Seedling establishment was scored as the percentage of seeds that developed
305 green expanded cotyledons and the first pair of true leaves at 5 or 7 d. Seedling
306 establishment assays in the presence of ABA agonists were performed in 24-
307 well plates, where approximately 25 seeds of the indicated genotype (three
308 independent experiments) were sown on wells lacking or supplemented with the
309 indicated concentration of ABA, QB or AMF4.

310 *Root growth assay*

311 Seedlings were grown on vertically oriented Murashige and Skoog (MS) plates
312 for 4-5 days. Afterwards, 20 plants were transferred to new MS plates lacking or
313 supplemented with 10 μ M ABA. The plates were scanned on a flatbed scanner
314 after 10 days to produce image files suitable for quantitative analysis of root
315 growth using the NIH Image software ImageJ

316

317 *Drought resistance experiments*

318 Three-weeks-old plants (n=10, three independent experiments) were grown
319 under greenhouse conditions (40-50% room humidity) and standard watering
320 for 15 d. Watering was withheld for 20 d (Pd27 OE lines) or 8 d (PdPP2C79 OE
321 lines), and then restored. Survival of the plants was scored 5 d later.

322 *Infrared thermography*

323 Plants were grown in a controlled environment growth chamber at 22°C under a
324 12 h light, 12 h dark photoperiod at 100 μ E m⁻² sec⁻¹ and 40-50% room
325 humidity. Philips bulbs were used (TL-D Super 8036W, white light 840, 4000K
326 light code). Infrared thermography images of rosette leaves were acquired from
327 6 week-old plants with a thermal camera FLIR E95 equipped with a 42° lens.
328 Images were processed and quantified with the FLIR tools software. For
329 quantification, the average temperature of 15 different sections corresponding
330 to 4 leaflets per plant were calculated. 5 plants per genotype were analyzed in
331 each experiment. The mean temperature $\pm$ standard deviation of all the plants
332 for each genotype was reported. Statistical comparisons among genotypes
333 were performed by pairwise t-tests.

334 *PP2C inhibition assays*

335 The expression in bacteria and purification of 6His- Δ NHAB1, PdPP2C55,
336 PdPP2C79, Pd15, Pd27, Pd32, Pd44 and Pd957 was performed as described
337 by Santiago et al. (2009). His-tagged proteins were purified using Ni-NTA
338 affinity chromatography, eluted and analysed by SDS-PAGE, followed by
339 Instant Blue staining. Phosphatase activity of Δ NHAB1 was measured using
340 pNPP (15 mM) as substrate, 1 μ M of the PP2C and 2 μ M of the indicated
341 receptors. Dephosphorylation of pNPP was monitored with a ViktorX5 reader at

405 nm (Antoni et al., 2012). Phosphatase activity of PdPP2C55 and PdPP2C79 was measured using the RRA(phosphoT)VA peptide as substrate, which has a K_m of 0.5-1 μM for eukaryotic PP2Cs. Assays were performed in a 100 μl reaction volume containing 25 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 25 μM peptide substrate and 1 μM of the PP2C and 2 μM of the indicated receptors. After incubation for 60 min at 30°C, the reaction was stopped by addition of 30 μl molybdate dye (Baykov et al., 1988) and the absorbance was read at 630 nm with a 96-well plate reader.

Native gel electrophoresis

Native Red Electrophoresis was carried out as described previously (Ruiz-Partida et al., 2018). Protein samples, each containing 8.5 μg of purified protein in sample buffer: 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 15 % glycerol and 0.02 % Red Ponceau 2S, with 50 mM DTT, were loaded onto 15 % polyacrylamide gels prepared in 375 mM Tris-HCl (pH 8.8), 10 % glycerol, 0.012 % of Ponceau Red S. A solution of Tris-Glycine (pH 8.8) with or without 0.012 % Ponceau Red S was used as the cathode and anode buffer, respectively. Electrophoresis was performed at a constant current of 25 mA for 150 min at 4 °C. Finally, proteins were detected in gel using standard Coomassie Blue staining.

361

Results

The date palm genome encodes 12 putative PYR/PYL/RCAR ABA receptors

We did a BLAST search in Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg-bin/show_organism?org=pda) to identify PYR/PYL/RCAR Pd ABA receptors using as a query the *Arabidopsis* receptors PYL1, PYL4 and PYL8. As a result, we found a family composed of 12 Pd ABA receptors (Fig. 1A). With the exception of 91, they were distributed in three subfamilies, which match the corresponding groups from *Arabidopsis* receptors. This enables a tentative translation to Pd ABA receptors of those biochemical and physiological features that are already known in *Arabidopsis* ABA receptors (Rodriguez et al., 2019). In subfamily III we found three Pd receptors that show sequence similarity with dimeric AtPYL1, whereas in both

374 subfamilies II and I we found four Pd receptors that are related to PYL4 and
 375 PYL8, respectively. Given that the *Arabidopsis* genome contains 14 receptors
 376 and date palm only 12, it is not possible to establish a direct correlation in the
 377 nomenclature with each *Arabidopsis* receptor. Therefore, we have preferred to
 378 maintain the KEGG-NCBI-Gene ID nomenclature to assign unambiguous
 379 names, using the last two or three digits of the full KEGG code to name each
 380 individual receptor (Fig. 1). Tentatively, some features of *Arabidopsis* PYL
 381 receptors can be assigned to Pd receptors based on sequence similarity and
 382 their location in different branches of the family. Amino acid sequence alignment
 383 of Pd ABA receptors (Fig. 1B) revealed the conserved residues of the gate
 384 (SGLPA) and latch (HRL) loops (defined by the b3-b4 and b5-b6 regions),
 385 which are a hallmark to define PYR/PYL/RCAR ABA receptors in different plant
 386 species (Rodriguez et al., 2019).

387 *Transcription profiles of Pd ABA receptors reveal primacy of the PYL8-like* 388 *family in abiotic stress response*

389 Different RNA-Seq gene expression data for the Pd transcriptome have been
 390 published (Bourgis et al., 2011; Yin et al., 2012; Al-Mssallem et al., 2013; Yaish
 391 et al., 2017; Hazzouri et al., 2019; Xiao et al., 2019). We extracted transcription
 392 profiles of Pd ABA receptors from abiotic stress studies performed by Müller et
 393 al. (2017) and Safronov et al. (2017). Additionally, in this study we generated
 394 new RNA-seq data after mock- or salt treatment (200 and 600 mM) of two-year-
 395 old date palms. We screened the ArrayExpress archive (E-MTAB-5261) at
 396 EMBL-EBI (<https://www.ebi.ac.uk/arrayexpress/submit/overview.html>) in order
 397 to obtain gene expression profiles for the 12 Pd ABA receptors after different
 398 treatments (Fig. 2A). In *Arabidopsis*, at least some representative members of
 399 the PYR1/PYL1-like, PYL4-like and PYL8-like subfamilies are expressed to
 400 similar levels (Gonzalez-Guzman et al., 2012), but few analyses have been
 401 reported in crops (Gonzalez-Guzman et al., 2014). Interestingly, in a crop
 402 adapted to harsh conditions as date palm, we found that four PYL8-like and
 403 one/two PYL1-like ABA receptors were preferentially expressed, whereas gene
 404 expression of the rest was low or almost undetectable in leaves under the
 405 following conditions: well-watered, heat, drought, heat+drought, salt or ABA
 406 treatment (Fig. 2A, B). Pd15 and Pd93 are PYL1-like receptors that show high

407 nucleotide sequence identity and some of the 100 bp RNA-seq data were
408 ambiguous reads, therefore we could not unequivocally assign them. Abiotic
409 stress and ABA treatment did not substantially affect transcript expression of
410 ABA receptors (Fig. 2A, B).

411 Given that date palm has a well-designed and specialized root system to
412 optimally manage water uptake, we investigated expression of Pd ABA
413 receptors in root tissue (Xiao et al., 2019). Expression of several receptors was
414 detected in roots and particularly the PYL8-like Pd27 receptor was expressed at
415 5-6 fold higher levels than other receptors (Fig. 2C). PYL8 plays an important
416 and non-redundant role for ABA signaling in *Arabidopsis* root (Antoni et al.,
417 2013; Belda-Palazon et al., 2018). The predominant expression of Pd27 (and
418 other PYL8-like receptors) in root tissue suggests that ABA signaling mediated
419 by PYL8-like receptors might play a key role to maintain primary root growth at
420 low water potential, to regulate root system architecture or for root
421 hydrotropism, as it has been described in other plant species (Sharp et al.,
422 2004; Dietrich et al., 2017; Orman-Ligeza et al., 2018). Expression of Pd15 and
423 Pd93 could be distinguished in 150 bp RNA-seq reads of root tissue (Xiao et al.,
424 2019), and also Pd45 or Pd37 were expressed in roots, although to 10-15 %
425 levels compared to Pd27. Finally, RNA-seq data were also compiled for
426 different fruit stages (at 45, 75, 105, 120 and 135 days after pollination, DAP)
427 and again, PYL8-like ABA receptors showed the highest expression, whereas
428 the rest were expressed at very low levels or undetectable (Fig. 2D).

429 *In vitro* activity of Pd ABA receptors against PP2Cs of *Arabidopsis* and *Phoenix* 430 *dactylifera*

431 Inhibition of clade A PP2Cs by ABA receptors is the key signature of their
432 biochemical function (Park et al., 2009; Ma et al., 2009; Santiago et al., 2009).
433 In order to biochemically characterize Pd ABA receptors we have obtained
434 recombinant protein for five of them. We ordered synthetic genes of Pd15,
435 Pd27, Pd32, Pd44 and Pd957, cloned them in pCR8 and next in pETM11 vector
436 for expression in *E. coli*. Recombinant His-tagged protein was obtained for each
437 receptor (Fig. 3A, top) and in addition to SDS-PAGE we performed native gel
438 electrophoresis in order to test the oligomeric state of each protein (Fig. 3A,
439 bottom). The PYL1-like Pd15 receptor migrated as a dimer, whereas the PYL8-

like Pd27, 32, 44 and 957 receptors migrated mostly as monomers, which shows a good correspondence with the oligomeric state of the *Arabidopsis* relatives (Dupeux et al., 2011; Hao et al., 2011). Next, we measured the capability of Pd receptors to inhibit *Arabidopsis* phosphatase HAB1 using pNPP as a substrate. As a result, all Pd ABA receptors showed ABA-dependent inhibition of HAB1 (Fig. 3B).

In order to identify Pd PP2Cs that might be a target of Pd ABA receptors, we performed a BLAST search in the date palm genome using ABI1 as a query to identify closely related Pd PP2Cs (Supplementary Fig. S1). *Arabidopsis* PP2Cs form a large family composed of 76 members (Schweighofer et al., 2004) and more than 100 genes in date palm show the PP2C signature (https://www.genome.jp/kegg-bin/show_organism?org=pda). We selected the top 10 PP2Cs that showed the highest sequence similarity to the catalytic PP2C domain of ABI1 as putative clade A PP2Cs from *Phoenix dactylifera* and we used the last two digits of the full KEGG code to name them (Supplementary Fig. S1). Further phylogenetic analysis revealed that some of these PP2Cs showed closer relationship to HAB1, AHG1 or PP2CA (Supplementary Fig. S1). Transcription profiles of PdPP2Cs were obtained from RNAseq studies performed by Müller et al., (2017). ABA treatment strongly upregulated expression of PdPP2Cs as occurs in *Arabidopsis* and other plant species (Fig. 2E) (Gonzalez-Guzman et al., 2014; Wang et al., 2019). We ordered synthetic genes for PdPP2C55 (the most expressed PP2C after ABA treatment), and PdPP2C79, which is only expressed during fruit development (Yin et al., 2012; Hazzouri et al., 2019) (Fig. 2F). PP2C79 shows high similarity with the *Arabidopsis* seed-specific PP2C AHG1, and a closely related PP2C is present in the oil palm (*Elaeis guineensis*) as well as other crops (Supplementary Fig. S2A and 2B). AHG1 plays an important role to regulate seed dormancy in *Arabidopsis* via interaction with DOG1; however, AHG1-like PP2Cs in monocot species have not been investigated yet (Née et al., 2017; Nishimura et al., 2018). The corresponding synthetic genes were cloned in pETM11 vector for expression in *E. coli* and recombinant His-tagged protein was obtained for both PP2Cs. PdPP2C55 phosphatase activity was measured using the RRA(phosphoT)VA phosphopeptide as a substrate because pNPP was a poor substrate for this phosphatase in contrast to HAB1. We found that all Pd ABA

receptors showed ABA-dependent inhibition of PdPP2C55 (Fig. 3C). Therefore, Pd ABA receptors are functional in vitro to inhibit both HAB1 and PdPP2C55 in an ABA-dependent manner.

PdPP2C79, as previously reported for AHG1, lacks a conserved Trp residue required for efficient binding to ABA's ketone group and *Arabidopsis* ABA receptors (Dupeux et al., 2011) (Supplementary Fig. S2A). In contrast, this Trp residue is conserved in other clade A PP2Cs (Trp385 in HAB1, Trp300 in ABI1) and establishes a water-mediated hydrogen-bond network (known as Trp lock) with the ketone group of ABA and the gate/latch loops of the receptor (Dupeux et al., 2011). Therefore, mutations in this conserved Trp residue (HAB1^{W385A} and ABI1^{W300A}), or changes in neighboring residues as occurs in *Striga hermonthica* PP2C1, render receptor-insensitive PP2Cs that lead to attenuation of ABA signaling (Dupeux et al., 2011; Fujioka et al., 2019). In general, PP2C79 was quite refractory to inhibition by Pd ABA receptors and in the best case, Pd32 rendered only 30% inhibition of this phosphatase whereas inhibited by 80% PdPP2C55 at 10 μ M ABA (Fig. 3D). Expression of PP2C79 in *Arabidopsis* led to plants that were strongly insensitive to ABA in germination and root growth assays (Supplementary Fig. 2C, D). Additionally, these plants showed higher water loss than wild-type Col-0 and were prone to wilting when watering was withheld (Supplementary Fig. 2E). Ectopic expression of PdPP2C79 in root tissue led to reduced sensitivity to ABA-mediated inhibition of root growth (Supplementary Fig. 2F). Given that in date palm PdPP2C79 is only expressed during fruit development, these results suggest that PdPP2C79 might provide a mechanism to attenuate ABA signaling at that stage in order to prevent developmental arrest induced by ABA accumulation (Xiao et al., 2019).

Sensitivity of Pd ABA receptors to ABA agonists

ABA receptor agonists are a promising tool to control plant transpiration and drought resistance, and several agonists have been found to be active against *Arabidopsis* ABA receptors (Helander et al., 2016; Okamoto and Cutler, 2018). However, few examples have been reported of agonist activity against crop ABA receptors (Gonzalez-Guzman et al., 2014; Vaidya et al., 2019). We used Pd receptors as targets to test the effect of several ABA receptor agonists: quinabactin (QB) (Okamoto, 2013; Cao et al., 2013), AMF4 (Cao et al., 2017),

507 and cyanabactin (CB) (Vaidya et al., 2017) (Fig. 3E). AtPYL1 and SIPYL1 were
 508 sensitive to ABA agonists that target dimeric receptors such as QB, CB and
 509 AMF4, and Pd15 behaved similarly, which further confirms that Pd15 belongs to
 510 the PYL1-like branch. Pd32 and Pd44 were resistant to QB and AMF4, but were
 511 sensitive to CB, which not only targets the dimeric PYR1 and PYL1 receptors in
 512 *Arabidopsis*, but also inhibits PYL5 efficiently (Vaidya et al., 2017). Pd957
 513 sensitivity to QB, CB and AMF4 was low (only 20-30% inhibition of phosphatase
 514 activity) and AtPYL8 was even less sensitive to these agonists (Fig. 3E).
 515 Unexpectedly, Pd27 inhibited by 80% the activity of HAB1 in the presence of 10
 516 μ M of any agonist, which suggests that crop receptors can show different
 517 sensitivity to ABA agonists than *Arabidopsis* receptors. Vaidya et al. (2019)
 518 reported that a single amino acid difference between wheat PYL8 and
 519 *Arabidopsis* PYL8 led to high PP2C inhibitory effect of opabactin with wheat
 520 PYL8 and low effect with AtPYL8. Inspection of wheat subfamily I ligand-binding
 521 pockets revealed that the bulky Leu163 of AtPYL8 was replaced by a smaller
 522 Val179 in TaPYL8 (Vaidya et al. 2019). The Leu of AtPYL8 would cause steric
 523 clash for the binding of opabactin, whereas it seems that the smaller Val of
 524 TaPYL8 avoids it and leads to high agonist potency for PP2C inhibition. Similar
 525 minor changes occur in the sequence of Pd27 (or TaPYL8) compared to
 526 AtPYL8, for example Leu126 of AtPYL8 (close to the conserved latch loop) is
 527 replaced by Val143 in Pd27 and Val142 in TaPYL8 (Fig. 1B). Further structural
 528 work with crop receptors is required for a full understanding of agonist
 529 sensitivity.

530 *In vivo activity of Pd ABA receptors*

531 We tested the in vivo activity of Pd ABA receptors by measuring ABA signaling
 532 (LUC expression driven by the ABA-responsive RD29B promoter) induced in
 533 protoplasts upon ABA perception. To this end we transfected effector DNA
 534 constructs together with an ABA-responsive luciferase reporter in the absence
 535 or presence of exogenous ABA (Fujii et al., 2009). A constitutively expressed
 536 GUS reporter was used for normalization and accordingly the LUC/GUS activity
 537 ratio was used to measure ABA signaling (Fujii et al., 2009). In the presence of
 538 ABA, LUC activity was elicited by the effector ABF2 construct, whereas co-
 539 expression of HAB1 abolished ABA-induced LUC activity (Fig. 4A). Introduction

of the PYL8 receptor together with the ABF2+HAB1 expression cassettes restored ABA signaling as well as introduction of each Pd ABA receptor. In the case of Pd27 and Pd957, in the absence of exogenous ABA addition, some induction of LUC activity was also observed. In order to visualize the subcellular localization of Pd ABA receptors and PdPP2Cs, we performed transient expression in *Nicotiana benthamiana*. In addition to GFP fusion proteins of receptors or phosphatases, we also co-expressed the nucleolar marker fibrillarin-RFP (Belda-Palazon et al., 2019). In the case of PdPP2C55 and PdPP2C79, both PP2Cs were predominantly localized in the nucleus of *N. benthamiana* epidermal leaf cells (Fig. 4B). We found that Pd ABA receptors were localized both in nucleus and cytosol; however, Pd27 and Pd957 showed preferential nuclear localization (Fig. 4C). Expression of the GFP fusion proteins was verified by immunoblot analysis (Fig. 4D).

Overexpression of monomeric ABA receptors confers enhanced response to ABA and drought resistance (Santiago et al., 2009; Gonzalez-Guzman et al., 2014; Yang et al., 2016; Mega et al., 2019), and tomato ABA receptors are functional when expressed in *Arabidopsis* (Gonzalez-Guzman et al., 2014). We were interested in testing whether monocot ABA receptors such as date palm receptors were functional in a dicot species as *Arabidopsis*. We expressed GFP-tagged Pd ABA receptors in *Arabidopsis* as a first step towards analyzing their role in ABA signaling. Expression of the proteins in vegetative tissue was analyzed by immunoblot analysis and since ABA promotes stabilization and accumulation of the *Arabidopsis* PYL8 receptor (Belda-Palazon et al., 2018), we analyzed protein expression in mock- and ABA-treated plants (Fig. 5A). Pd15 protein level did not substantially change in mock- and ABA-treated samples, whereas PYL8-like Pd receptors showed ABA-induced accumulation (Fig. 5A). Therefore, this result reveals that ABA-induced accumulation of PYL8-like receptors also occurs when monocot receptors are expressed in *Arabidopsis* (Belda-Palazon et al., 2018; Fig. 5A). Moreover, analysis of ABA response in transgenic lines overexpressing GFP-Pd receptors, revealed enhanced sensitivity to ABA mediated inhibition of seedling establishment compared to wild-type Col-0, indicating that Pd receptors are functional when expressed in *Arabidopsis* (Fig. 5B). In agreement with the Pd27

sensitivity to ABA agonists (Fig. 3E), we observed enhanced sensitivity to QB and AMF4 in plants overexpressing GFP-Pd27 compared with wild-type Col-0 (Fig. 5C). Finally, we confirmed ABA-induced accumulation of the GFP-Pd27 ABA receptor by CLSM imaging, both in root and shoot tissues (Fig. 5D, E). Likewise, GFP-Pd32, another PYL8-like ABA receptor, also accumulates in both tissues in response to ABA, whereas Pd15, which is a PYL1-like ABA receptor, does not accumulate in response to ABA (Fig. 5D, E).

580

581 *Overexpression of Pd27 confers drought resistance*

We also expressed HA-tagged Pd ABA receptors in *Arabidopsis* and analyzed ABA response of the transgenic lines in seedling establishment and root growth assays (Fig. 6A-C). As GFP-Pd receptor lines, overexpression of HA-Pd ABA receptors conferred enhanced response to ABA compared to wild type. In order to test whether palm receptors can confer enhanced drought resistance, we selected transgenic lines overexpressing Pd27 because this receptor is the most expressed in all palm tissues analyzed (Fig. 2). Plants were grown under normal watering conditions for 2 weeks and then irrigation was stopped for 20 days. Non-transformed Col-0 plants wilted, in contrast to transgenic lines that express Pd27 (Fig. 6D). The survival of the plants was scored 5 days after watering was resumed and enhanced survival was found for transgenic plants expressing Pd27 (Fig. 6E). Transpiration was monitored using infrared thermography and we found that transgenic plants expressing Pd27 showed higher leaf temperature than wild-type Col-0 plants upon spraying with ABA (Fig. 7A). Further physiological analyses were conducted using normalized gravimetric water loss experiments to measure transpiration of wild-type Col-0 and Pd27 OE plants, which was recorded under different soil water potentials, ranging from well-watered (potential near to 0 MPa) to drought conditions (-2.5 MPa) (Fig. 7B). As a result, we found that Pd27 OE plants showed lower transpiration than wild type. To correlate transpiration and ABA levels, we measured endogenous ABA content (ng/g dry weight) by HPLC/MS analysis in the different conditions of soil water potential (SWP) assayed (Fig. 7C). Interestingly, Pd27 OE plants had a lower ABA content than wild type, which was particularly evident under drought conditions. Therefore, the combination of

transpiration data and ABA content reveals that Pd27 OE plants were more efficient to reduce transpiration at each ABA concentration, particularly when ABA levels increase (Fig. 7D).

Discussion

Abiotic stress is challenging plant agriculture and ABA is a key phytohormone to trigger plant adaptation to stressors, particularly under drought conditions (Yang et al., 2019). Date palm is very well adapted to heat and drought conditions, but little is known about the underlying molecular mechanisms (Arab et al., 2016). Guard cell ABA signaling mediated by PdOST1 limits transpiration water loss in date palm, presumably through ABA-dependent activation mediated by ABA receptors (Müller et al., 2017). Recent findings have revealed the role of certain *Arabidopsis* receptors in stomatal closure signal integration, and for instance PYL2 is critical for guard cell ABA-induced responses whereas PYL4 and PYL5 are essential in the responses to CO₂ (Dittrich et al., 2019). In this work we have identified which members of the date palm family of ABA receptors are particularly relevant for abiotic stress response. The phylogenetic analysis revealed that PdPYLs can be divided into three subfamilies, which correspond to PYL1-like, PYL4-like and PYL8-like *Arabidopsis* relatives. Based on RNA seq analyses of PdPYLs, and somehow unexpectedly, we discovered that PYL8-like receptors play a major role for palm response to abiotic stress. This result contrasts with *Arabidopsis* ABA receptors, where members of the three subfamilies are significantly expressed in different tissues and growth conditions, and particularly *Arabidopsis* dimeric receptors play an important role to regulate stomatal aperture and adaptation to drought (Gonzalez-Guzman et al., 2012; Okamoto et al., 2013; Dittrich et al., 2019). In contrast to *Arabidopsis*, four date palm PYL8-like ABA receptors were predominantly expressed under heat, salt, drought or osmotic stress conditions. In *Arabidopsis*, ABA treatment induced their accumulation, which was only previously observed for AtPYL8; therefore, it seems that date palm has expanded this adaptive mechanism to a full branch of ABA receptors for efficient response to abiotic stress.

Specifically, Pd27 was the most expressed receptor in all the conditions analyzed, including root and fruit tissues. Analysis of transgenic palms is not feasible; therefore, we investigated Pd27 in *Arabidopsis* lines. We measured the effect of Pd27 on transpiration under different soil water potentials, which range from well-watered to drought conditions. In all the conditions tested, expression of Pd27 led to lower transpiration than wild type, and interestingly this effect was accompanied by a reduction in ABA levels, particularly at negative soil water potentials (Fig. 7B and 7C). As a result, reduction of transpiration in Pd27 OE plants per ng ABA was more efficient than in wild type (Fig. 7D). Drought leads to 10-40 fold increase of ABA levels (Cutler et al., 2010); therefore we suggest that stabilization of Pd27 (and likely other PYL8-like receptors such as Pd32) induced by the hormone increase might boost ABA signaling, because higher levels of the receptor will accumulate when ABA levels increase. Transcriptional and posttranslational mechanisms limit accumulation of ABA receptors in *Arabidopsis* (Fernandez et al., 2020); but transcriptional downregulation either by ABA or different stressors was not observed in palm receptors. Moreover, ABA treatment in *Arabidopsis* transgenic lines led to higher accumulation of palm receptors. If this feature of the PYL8-like family is conserved in other crops adapted to drought, it might represent a valuable trait to be introduced in crops lacking such adaptation. Pd27 enhanced drought tolerance upon expression in *Arabidopsis* and was able to bind different ABA agonists, whose application together with Pd27 overexpression might enhance even further plant response to ABA. The result of combining Pd27 OE plants with spraying of different agonists when stress occurs might represent a powerful tool for tuning water use dynamically (Cao et al., 2017; Dejonghe et al., 2018; Vaidya et al., 2019). Additionally, the chemical approach benefits from the higher expression of the ABA receptor because lower dosage of the chemical is needed.

This study also reports the ABA- and receptor-resistant activity of the phosphatase PdPP2C79, as well as the extended presence of AHG1-like PP2Cs in different crops, bot monocot and dicot species. Given that AHG1 is a target of a key QTL that regulates seed dormancy such as *Arabidopsis* DOG1 (Nee et al., 2017; Nishimura et al., 2018), these results suggest that AHG1-

670 DOG1 interaction might be evolutionary conserved and deserves further
671 research in crops. Additionally, loss of the Trp lock in AHG1-like PP2Cs seems
672 to have markedly reduced their regulation by ABA and ABA receptors, which
673 likely enables AHG1 activity at plant developmental stages where high ABA
674 concentration accumulates.

675

676 **Supplementary data**

677 Supplementary data is available at JXB online

678 **Supplementary Fig. S1.** Phylogenetic analysis reveals the presence of HAB1-
679 like, AHG1-like and PP2CA-like PP2Cs in date palm

680 **Supplementary Fig. S2.** AHG1-like PP2Cs lacking the Trp lock residue are
681 present both in monocot and dicot plant species.

682 **Supplementary Fig. S3.** Expression of PdPP2C79 in Arabidopsis reduces ABA
683 sensitivity.

684 **Supplementary Table S1.** List of antibodies and oligonucleotides used in this
685 work.

686

687 **Data Availability**

688 All data supporting the findings of this study are available within the article and
689 its supplementary materials published online. RNA seq crude data are available
690 in the Sequence Read Archive.

691 **Acknowledgements**

692 This study was funded by the Distinguished Scientist Fellowship Research
693 Program (DSFP) of King Saud University to P.L.R. The authors JJ., RR-P and
694 IG-M were supported by a research contract funded by the DSFP.

695 **Author contribution**

696 All authors designed and supervised experiments; IGM, AC, JL-J, MM, CO, JJ,
697 RR-P, GP, BB-P performed experimental analyses; PLR devised the project
698 and wrote the manuscript with contributions of CO and MM; and all authors
699 analyzed the data and approved the final text

References

- Al-Dous EK, George B, Al-Mahmoud ME, et al.** 2011. De novo genome sequencing and comparative genomics of date palm (*Phoenix dactylifera*). *Nature Biotechnology* **29**, 521–527.
- Al-Harrasi I, Al-Yahyai R, Yaish MW.** 2018. Differential DNA methylation and transcription profiles in date palm roots exposed to salinity. *PLoS ONE* **13**, e0191492.
- Al-Mssallem IS, Hu S, Zhang X, et al.** 2013. Genome sequence of the date palm *Phoenix dactylifera* L. *Nature Communications* **4**, 2274.
- Antoni R, Gonzalez-Guzman M, Rodriguez L, et al.** 2013. PYRABACTIN RESISTANCE1-LIKE8 plays an important role for the regulation of abscisic acid signaling in root. *Plant Physiology* **161**, 931–941.
- Antoni R, Gonzalez-Guzman M, Rodriguez L, Rodrigues A, Pizzio GA, Rodriguez PL.** 2012. Selective inhibition of Clade A phosphatases type 2C by PYR/PYL/RCAR abscisic acid receptors. *Plant Physiology* **158**, 970–980.
- Arab L, Kreuzwieser J, Kruse J, Zimmer I, Ache P, Alfarraj S, Al-Rasheid KAS, Schnitzler JP, Hedrich R, Rennenberg H.** 2016. Acclimation to heat and drought-Lessons to learn from the date palm (*Phoenix dactylifera*). *Environmental and Experimental Botany* **125**, 20–30.
- Arbona V, Zandalinas SI, Manzi M, González-Guzmán M, Rodríguez PL, Gómez-Cadenas A.** 2017. Depletion of abscisic acid levels in roots of flooded Carrizo citrange (*Poncirus trifoliata* L. Raf. × *Citrus sinensis* L. Osb.) plants is a stress-specific response associated to the differential expression of PYR/PYL/RCAR receptors. *Plant Molecular Biology* **93**, 623–640.
- Baykov AA, Evtushenko OA, Avaeva SM.** 1988. A malachite green procedure for orthophosphate determination and its use in alkaline phosphatase-based enzyme immunoassay. *Analytical Biochemistry* **171**, 266–270.
- Belda-Palazon B, Gonzalez-Garcia MP, Lozano-Juste J, et al.** 2018. PYL8 mediates ABA perception in the root through non-cell-autonomous and ligand-stabilization-based mechanisms. *Proceedings of the National Academy of Sciences of the United States of America* **115**, E11857–E11863.
- Belda-Palazon B, Julian J, Coego A, et al.** 2019. ABA inhibits myristoylation and induces shuttling of the RGLG1 E3 ligase to promote nuclear degradation of PP2CA. *Plant Journal* **98**, 813–825.
- Bourgis F, Kilaru A, Cao X, Ngando-Ebongue GF, Drira N, Ohlrogge JB, Arondel V.** 2011. Comparative transcriptome and metabolite analysis of oil palm and date palm mesocarp that differ dramatically in carbon partitioning. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 12527–12532.
- Cao M, Liu X, Zhang Y, et al.** 2013. An ABA-mimicking ligand that reduces water loss and promotes drought resistance in plants. *Cell Research* **23**, 1043–1054.
- Cao MJ, Zhang YL, Liu X, et al.** 2017. Combining chemical and genetic approaches to increase drought resistance in plants. *Nature Communications* **8**, 1183.
- Christmann A, Hoffmann T, Teplova I, Grill E, Müller A.** 2005. Generation of active pools of abscisic acid revealed by in vivo imaging of water-stressed arabidopsis. *Plant Physiology* **137**, 209–219.

- Clough SJ, Bent AF.** 1998. Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal* **16**, 735–743.
- Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR.** 2010. Absciscic Acid: Emergence of a Core Signaling Network. *Annual Review of Plant Biology* **61**, 651–679.
- Dejonghe W, Okamoto M, Cutler SR.** 2018. Small molecule probes of ABA biosynthesis and signaling. *Plant and Cell Physiology* **59**, 1490–1499.
- De Ollas C, Segarra-Medina C, González-Guzmán M, Puertolas J, Gómez-Cadenas A.** 2019. A customizable method to characterize *Arabidopsis thaliana* transpiration under drought conditions. *Plant Methods* **15**.
- Dietrich D, Pang L, Kobayashi A, et al.** 2017. Root hydrotropism is controlled via a cortex-specific growth mechanism. *Nature Plants* **3**, 17057.
- Dittrich M, Mueller HM, Bauer H, et al.** 2019. The role of *Arabidopsis* ABA receptors from the PYR/PYL/RCAR family in stomatal acclimation and closure signal integration. *Nature Plants* **5**, 1002–1011.
- Dupeux F, Antoni R, Betz K, et al.** 2011. Modulation of abscisic acid signaling in vivo by an engineered receptor-insensitive protein phosphatase type 2C allele. *Plant Physiology* **156**, 106–116.
- Durgbanshi A, Arbona V, Pozo O, Miersch O, Sancho J V., Gómez-Cadenas A.** 2005. Simultaneous determination of multiple phytohormones in plant extracts by liquid chromatography-electrospray tandem mass spectrometry. *Journal of Agricultural and Food Chemistry* **53**, 8437–8442.
- Fernandez MA, Belda-Palazon B, Julian J, Coego A, Lozano-Juste J, Iñigo S, Rodriguez L, Bueso E, Goossens A, Rodriguez PL.** 2020. RBR-type E3 ligases and the ubiquitin-conjugating enzyme UBC26 regulate abscisic acid receptor levels and signaling. *Plant Physiology* **182**, 1723–1742.
- Fujii H, Chinnusamy V, Rodrigues A, Rubio S, Antoni R, Park SY, Cutler SR, Sheen J, Rodriguez PL, Zhu JK.** 2009. In vitro reconstitution of an abscisic acid signalling pathway. *Nature* **462**, 660–664.
- Fujioka H, Samejima H, Suzuki H, Mizutani M, Okamoto M, Sugimoto Y.** 2019. Aberrant protein phosphatase 2C leads to abscisic acid insensitivity and high transpiration in parasitic *Striga*. *Nature Plants* **5**, 258–262.
- Gonzalez-Guzman M, Pizzio GA, Antoni R, et al.** 2012. *Arabidopsis* PYR/PYL/RCAR receptors play a major role in quantitative regulation of stomatal aperture and transcriptional response to abscisic acid. *Plant Cell* **24**, 2483–2496.
- Gonzalez-Guzman M, Rodriguez L, Lorenzo-Orts L, et al.** 2014. Tomato PYR/PYL/RCAR abscisic acid receptors show high expression in root, differential sensitivity to the abscisic acid agonist quinabactin, and the capability to enhance plant drought resistance. *Journal of Experimental Botany* **65**, 4451–4464.
- Gros-Balthazard M, Hazzouri KM, Flowers JM.** 2018. Genomic insights into date palm origins. *Genes* **9**.
- Hazzouri KM, Flowers JM, Visser HJ, et al.** 2015. Whole genome re-sequencing of date palms yields insights into diversification of a fruit tree crop. *Nature Communications* **6**, 8824.

- Hazzouri KM, Gros-Balthazard M, Flowers JM, et al.** 2019. Genome-wide association mapping of date palm fruit traits. *Nature Communications* **10**, 4680.
- He Y, Hao Q, Li W, Yan C, Yan N, Yin P.** 2014. Identification and characterization of ABA receptors in *Oryza sativa*. *PLoS ONE* **9**, e95246.
- He Z, Zhong J, Sun X, Wang B, Terzaghi W, Dai M.** 2018. The maize ABA receptors ZmPYL8, 9, and 12 facilitate plant drought resistance. *Frontiers in Plant Science* **9**, 422.
- Helander JDM, Vaidya AS, Cutler SR.** 2016. Chemical manipulation of plant water use. *Bioorganic and Medicinal Chemistry* **24**, 493–500.
- Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E.** 2009. Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* **324**, 1064–1068.
- Mega R, Abe F, Kim JS, et al.** 2019. Tuning water-use efficiency and drought tolerance in wheat using abscisic acid receptors. *Nature Plants* **5**, 153–159.
- Miao C, Xiao L, Hua K, Zou C, Zhao Y, Bressan RA, Zhu JK.** 2018. Mutations in a subfamily of abscisic acid receptor genes promote rice growth and productivity. *Proceedings of the National Academy of Sciences of the United States of America* **115**, 6058–6063.
- Moes D, Himmelbach A, Korte A, Haberer G, Grill E.** 2008. Nuclear localization of the mutant protein phosphatase *abi1* is required for insensitivity towards ABA responses in *Arabidopsis*. *Plant Journal* **54**, 806–819.
- Müller HM, Schäfer N, Bauer H, et al.** 2017. The desert plant *Phoenix dactylifera* closes stomata via nitrate-regulated SLAC1 anion channel. *New Phytologist* **216**, 150–162.
- Née G, Kramer K, Nakabayashi K, Yuan B, Xiang Y, Miatton E, Finkemeier I, Soppe WJJ.** 2017. DELAY of GERMINATION1 requires PP2C phosphatases of the ABA signalling pathway to control seed dormancy. *Nature Communications* **8**.
- Nishimura N, Tsuchiya W, Moresco JJ, et al.** 2018. Control of seed dormancy and germination by DOG1-AHG1 PP2C phosphatase complex via binding to heme. *Nature Communications* **9**.
- Okamoto M, Cutler SR.** 2018. Chemical control of ABA receptors to enable plant protection against water stress. *Methods in Molecular Biology*. 127–141.
- Okamoto M, Peterson FC, Defries A, Park SY, Endo A, Nambara E, Volkman BF, Cutler SR.** 2013. Activation of dimeric ABA receptors elicits guard cell closure, ABA-regulated gene expression, and drought tolerance. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 12132–12137.
- Orman-Ligeza B, Morris EC, Parizot B, et al.** 2018. The Xerobranched Response Represses Lateral Root Formation When Roots Are Not in Contact with Water. *Current Biology* **28**, 3165–3173.e5.
- Park SY, Fung P, Nishimura N, et al.** 2009. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* **324**, 1068–1071.
- Planes MD, Niñoles R, Rubio L, et al.** 2015. A mechanism of growth inhibition by abscisic acid in germinating seeds of *Arabidopsis thaliana* based on inhibition of plasma membrane H⁺-ATPase and decreased cytosolic pH, K⁺, and anions. *Journal of Experimental Botany* **66**, 813–825.

- Rodriguez PL, Lozano-Juste J, Albert A.** 2019. PYR/PYL/RCAR ABA receptors. In: Seo M, Marion-Poll A, eds. *Advances in Botanical Research*, Academic Press 92, 51-82.
- Ruiz-Partida R, Prado S, Villarroya M, Velázquez-Campoy A, Bravo J, Armengod ME.** 2018. An Alternative Homodimerization Interface of MnmG Reveals a Conformational Dynamics that Is Essential for Its tRNA Modification Function. *Journal of Molecular Biology* **430**, 2822–2842.
- Saez A, Rodrigues A, Santiago J, Rubio S, Rodriguez PL.** 2008. HAB1-SWI3B interaction reveals a link between abscisic acid signaling and putative SWI/SNF chromatin-remodeling complexes in Arabidopsis. *Plant Cell* **20**, 2972–2988.
- Safronov O, Kreuzwieser J, Haberer G, et al.** 2017. Detecting early signs of heat and drought stress in *Phoenix dactylifera* (date palm). *PLoS ONE* **12**, e0177883.
- Santiago J, Rodrigues A, Saez A, Rubio S, Antoni R, Dupeux F, Park SY, Márquez JA, Cutler SR, Rodriguez PL.** 2009. Modulation of drought resistance by the abscisic acid receptor PYL5 through inhibition of clade A PP2Cs. *Plant Journal* **60**, 575–588.
- Schweighofer A, Hirt H, Meskiene I.** 2004. Plant PP2C phosphatases: Emerging functions in stress signaling. *Trends in Plant Science* **9**, 236–243.
- Seiler C, Harshavardhan VT, Reddy PS, et al.** 2014. Abscisic acid flux alterations result in differential abscisic acid signaling responses and impact assimilation efficiency in barley under terminal drought stress. *Plant Physiology* **164**, 1677–1696.
- Sharp RE, Poroyko V, Hejlek LG, Spollen WG, Springer GK, Bohnert HJ, Nguyen HT.** 2004. Root growth maintenance during water deficits: Physiology to functional genomics. *Journal of Experimental Botany* **55**(407), 2343–2351.
- Torres MF, Mathew LS, Ahmed I, Al-Azwani IK, Krueger R, Rivera-Núñez D, Mohamoud YA, Clark AG, Suhre K, Malek JA.** 2018. Genus-wide sequencing supports a two-locus model for sex-determination in *Phoenix*. *Nature Communications* **9**, 3969.
- Umezawa T, Sugiyama N, Mizoguchi M, Hayashi S, Myouga F, Yamaguchi-Shinozaki K, Ishihama Y, Hirayama T, Shinozaki K.** 2009. Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 17588–17593.
- Vaidya AS, Helander JDM, Peterson FC, et al.** 2019. Dynamic control of plant water use using designed ABA receptor agonists. *Science* **366**.
- Vaidya AS, Peterson FC, Yarmolinsky D, et al.** 2017. A Rationally Designed Agonist Defines Subfamily IIIA Abscisic Acid Receptors As Critical Targets for Manipulating Transpiration. *ACS Chemical Biology* **12**, 2842–2848.
- Vlad F, Rubio S, Rodrigues A, Sirichandra C, Belin C, Robert N, Leung J, Rodriguez PL, Laurière C, Merlot S.** 2009. Protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by abscisic acid in Arabidopsis. *Plant Cell* **21**, 3170–3184.
- Wang X, Guo C, Peng J, et al.** 2019. ABRE-BINDING FACTORS play a role in the feedback regulation of ABA signaling by mediating rapid ABA induction of ABA co-receptor genes. *New Phytologist* **221**, 341–355.
- Witte CP, Noël LD, Gielbert J, Parker JE, Romeis T.** 2004. Rapid one-step protein purification from plant material using the eight-amino acid StreptII epitope. *Plant Molecular Biology* **55**, 135–

147.

Xiao TT, Raygoza AA, Pérez JC, et al. 2019. Emergent protective organogenesis in date palms: A morpho-devo-dynamic adaptive strategy during early development. *Plant Cell* **31**, 1751–1766.

Yaish MW, Patankar H V., Assaha DVM, Zheng Y, Al-Yahyai R, Sunkar R. 2017. Genome-wide expression profiling in leaves and roots of date palm (*Phoenix dactylifera* L.) exposed to salinity. *BMC Genomics* **18**, 246.

Yang Z, Liu J, Tischer S V., Christmann A, Windisch W, Schnyder H, Grill E. 2016. Leveraging abscisic acid receptors for efficient water use in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **113**(24), 6791– 6796.

Yang Z, Liu J, Poree F, et al. 2019. Absciscic Acid Receptors and Coreceptors Modulate Plant Water Use Efficiency and Water Productivity. *Plant Physiology* **180**, 1066-1080.

Yin Y, Zhang X, Fang Y, et al. 2012. High-throughput sequencing-based gene profiling on multi-staged fruit development of date palm (*Phoenix dactylifera*, L.). *Plant Molecular Biology* **78**, 617–626.

Zhao Y, Chan Z, Gao J, et al. 2016. ABA receptor PYL9 promotes drought resistance and leaf senescence. *Proceedings of the National Academy of Sciences of the United States of America* **113**, 1949–1954.

Figure legends

Fig. 1. Cladogram and amino acid sequence alignment of date palm PYR/PYL/RCAR ABA receptors. (A) Cladogram of the multiple sequence alignment of date palm and *Arabidopsis* receptors, revealing three major subfamilies and the ungrouped Pd91. Sequences of *Citrus sinensis* (CsPYL1, Cs46151) and *Solanum lycopersicum* (SIPYL1, SI8g076960) PYL1-like receptors were also included. The nomenclature is given from the last two or three digits of KEGG database entries for Pd genes: 103711815, 103704693, 103709545, 103714404, 103708857, 103696437, 103717496, 103706891, 103707144, 103702957, 103698527 and 103708332. The tree was constructed using GeneDoc and ClustalW software. (B) Sequence and secondary structure alignment of date palm ABA receptors and *Arabidopsis*, sweet orange and tomato PYL1 proteins. The predicted secondary structure of the date palm receptors is indicated, taking as a model the crystallographic structure of AtPYL1 (Protein DataBank Code 3JRS) and using the ESPRIPT program (<http://esprpt.ibcp.fr/ESPrpt/ESPrpt/>). Boxes indicate the position of the gate and latch loops. Black asterisks mark residues involved in interactions with

ABA's carboxylate group (K86, E121, Y147, S149 and E171 of AtPYL1), either direct contact for the Lys side chain or hydrogen bonds mediated by internal water molecules for the other residues.

Fig. 2. Relative gene expression of date palm receptors in abiotic stress conditions and different tissues was determined by RNA-Seq analysis. PYL8-like receptors are underlined. TPM (transcripts per million) normalizes transcript expression according to transcript length and sequencing depth. (A) Expression of Pd ABA receptors in leaves under well-watered (WW), heat, drought, heat+drought or salt treatment. (B) Expression of Pd ABA receptors in mock- or ABA-treated leaves. Data were obtained from the ArrayExpress archive (E-MTAB-5261) at EMBL-EBI. (C) Expression of Pd ABA receptors in root. Data were obtained from the Sequence Read Archive (experiment PRJNA497070) (Xiao et al. 2019). (D) Expression of Pd ABA receptors in fruit. Data were obtained from the Sequence Read Archive (experiment SRX122676) and correspond to different stages of fruit development after pollination: 45, 75, 105, 120 and 135 days after pollination (DAP) (Hazzouri et al., 2019). (E) Expression of Pd PP2Cs in mock- or ABA-treated leaves. Data were obtained from the ArrayExpress archive (E-MTAB-5261) at EMBL-EBI. (F) Expression of PdPP2C55 and PdPP2C79 in fruit at the indicated DAP (Hazzouri et al., 2019).

Fig. 3. Expression, purification and analysis of recombinant Pd ABA receptors. (A) SDS-PAGE (top) and native gel electrophoresis (bottom) of Pd15, Pd27, Pd32, Pd44 and Pd957 ABA receptors. Molecular mass markers in the SDS PAGE are indicated in kDa, whereas in native gel we used monomeric PYL10 and dimeric GST as markers. (B, C, D) ABA-dependent PP2C inhibition mediated by Pd ABA receptors. PP2C activity was measured in vitro using the pNPP substrate (for Δ NHAB1) or the RRA(phosphoT)VA phosphopeptide (for PdPP2C55 and PdPP2C79) in the absence (DMSO) or presence of ABA at a 1:2 ratio (phosphatase:receptor). Data are averages $\pm$ SD for three independent experiments. Phosphatase activity in the absence of receptor and ABA was taken as 100 % activity. (E) Inhibition of Δ NHAB1 phosphatase activity in the presence of Pd, AtPYL8, AtPYL1 or SIPYL1 receptors mediated by 10 μ M ABA or different ABA agonists: cyanabactin (CB), quinabactin (QB) and AMF4.

Fig. 4. ABA signaling mediated by Pd ABA receptors in protoplasts and subcellular localization of PdPP2Cs and ABA receptors. (A) Protoplasts of wild-type Col-0 were transfected with plasmids encoding the indicated proteins in absence or presence of ABA. LUC activity was measured in protein extracts prepared from protoplast suspensions 6 h after transfection, either in the absence or presence of 5 μ M exogenous ABA (added 3 h after transfection). The activity of the LUC reporter was normalized with GUS activity and the ratio LUC/GUS is indicated. (B) (C) Subcellular localization of PdPP2Cs and ABA receptors. PdPP2C55 and PdPP2C79 localize predominantly to nucleus, whereas Pd ABA receptors localize to nucleus and cytosol upon transient expression in *N. benthamiana* leaf cells. Confocal images of transiently transformed epidermal cells expressing GFP-PdPP2C55, GFP-PdPP2C79, GFP-Pd15, GFP-Pd27, GFP-Pd32, GFP-Pd44 or GFP-Pd957. The GFP channel shows the subcellular localization of the GFP fusion proteins, whereas the RFP channel shows the nucleolar marker fibrillarin-RFP. Merged indicates the overlap of the GFP and RFP channels. Scale bars correspond to 50 or 20 μ m. (D) Immunoblotting analysis using anti-GFP antibodies was used to verify the expression of the corresponding fusion proteins.

Fig. 5. PdPYL8-like receptors expressed in *Arabidopsis* accumulate higher levels upon ABA-treatment. (A) Immunoblot analysis using anti-GFP antibody shows that ABA treatment enhances protein levels of PdPYL8-like receptors (two independent *Arabidopsis* T3 transgenic lines for each palm receptor). Ponceau staining is shown below. (B) GFP-Pd fusion proteins are functional in *Arabidopsis*. ABA-mediated inhibition of seedling establishment in transgenic lines compared with non-transformed Col-0 plants. * indicates $P < 0.05$ (Student's t test) when comparing data of transgenic lines to non-transformed Col-0 plants in the same assay conditions. Seedlings were scored for the presence of both green cotyledons and the first pair of true leaves 7 days after sowing. (C) Enhanced sensitivity to QB and AMF4 of GFP-Pd27 OE plants compared with wild-type Col-0. Approximately 25 seeds of the indicated genotype (three independent experiments) were sown on MS multi-well plates lacking or supplemented with the indicated concentration of QB or AMF4, and seedling establishment was scored for the presence of both green cotyledons

and the first pair of true leaves after 7 d. Values are averages $\pm$ SD. The asterisk indicates $p < 0.01$ (Student's t test) with respect to wild type assayed in the same conditions. (D, E) GFP-Pd27 and GFP-Pd32 accumulate after ABA treatment, whereas GFP-Pd15 does not. CLSM imaging of *Arabidopsis* root apex (D) or leaf tissue (E) in lines expressing GFP-Pd15, GFP-Pd27 or GFP-Pd32 that were mock- or ABA-treated for 1 h. Two independent lines were analyzed and representative images obtained with one line are shown. Fluorescence was quantified (arbitrary units) in 10 leaves (three independent experiments) of each transgenic line using images acquired by CLSM.

Fig. 6. Enhanced sensitivity to ABA conferred by overexpression of Pd ABA receptors. (A) Enhanced sensitivity to ABA-mediated inhibition of seedling establishment. Approximately 100 seeds of each genotype (three independent experiments) were sown on MS plates lacking or supplemented with 0.5 μ M ABA and scored for the presence of both green cotyledons and the first pair of true leaves after 7 d. Values are averages $\pm$ SD. The asterisk indicates $p < 0.01$ (Student's t test) with respect to wild type assayed in the same conditions. (B) Enhanced sensitivity to ABA-mediated inhibition of root growth. Quantification of ABA-mediated inhibition of root growth in the indicated genotypes compared to wild type. The asterisk indicates $p < 0.01$ (Student's t test) with respect to wild type assayed in the same conditions. (C) The photographs show representative seedlings 10 d after the transfer of 4-d-old seedlings to MS plates lacking or supplemented with 10 μ M ABA. (D) Enhanced drought resistance of Pd27 OE plants compared to Col-0. Irrigation was withdrawn in three-week-old plants for 20 d and after rewatering, survival of the plants was scored after 5 d. Data are means of three independent experiments $\pm$ SD ($n=10$ per experiment). The survival percentage 5 d after rewatering is indicated in histograms.

Fig. 7. Plants OE Pd27 show lower transpiration than wild type. (A) Infrared thermography analysis shows higher leaf temperature of two independent *Arabidopsis* transgenic lines OE Pd27. False-color images of wild-type Col-0 and Pd27 OE plants (lines #27 and #39) representing leaf temperature, which was quantified by infrared thermal imaging. Data are means $\pm$ SD ($n=5$, aprox. 1000 measurements of square pixels from multiple leaves of each plant). (B) Transpiration assay under different soil water potential (SWP) conditions. (C)

ABA quantification under different SWP conditions. (D) ABA lowers transpiration more efficiently in plants OE Pd27 than in wild type. For experiments of B, C and D panels, n=16. In B, C and D differences in transpiration (TN), endogenous ABA and transpiration (TN), respectively, were significant due to genotype ($p=0.001$, $p=0.003$ and $p=0.001$, respectively) after ANOVA using SWP (B, C) and ABA (D) as covariant.

Fig 1

A

B

C

D

Fig 5

A

B

C

D

E

Fig 6