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Isolation and Characterization of Novel Mutant Loci Suppressing the ABA Hypersensitivity of the Arabidopsis *coronatine insensitive 1-16* (*coi1-16*) Mutant During Germination and Seedling Growth

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The phytohormone ABA regulates seed germination and stress responses. The identification of clade A protein phosphatase type 2C (PP2C)-interacting proteins PYRABACTIN RESISTANCE 1 (PYR1)/RCAR (REGULATORY COMPONENT OF ABA RECEPTOR) and PYR1-LIKEs (PYLs) as ABA receptors has been a major advance in understanding this process. Here, our aim was to identify additional ABA response loci by suppressor screening of the jasmonate (JA)-insensitive *coronatine insensitive 1-16* (*coi1-16*) mutant using its ABA-hypersensitive phenotype. The identification and genetic characterization of *Coi1-16 Resistant to ABA* (*CRA*) loci revealed several unknown and three previously known *abi* mutants (*abi1*, *abi3* and *abi4*), thus providing proof-of-concept evidence for this study. The synergistic effect of ABA and JA on seed germination and cotyledon expansion was analyzed in depth and the roles of *cra5 coi1-16*, *cra6 coi1-16*, *cra7 coi1-16* and *cra8 coi1-16* in ABA signaling during seed germination and stress responses were functionally characterized. The *cra5 coi1-16* mutant showed resistance to ABA, paclobutrazol, and abiotic stresses during germination and early developmental stages. Furthermore, the *cra5 coi1-16* mutation was mapped to the short arm of chromosome V and mutants exhibited differential expression of ABA-responsive genes, suggesting that *CRA5* may function as a positive regulator of ABA signaling. Interestingly, *cra6 coi1-16*, *cra7 coi1-16* and *cra8 coi1-16* mutants display similar ABA- and abiotic stress-insensitive phenotypes during seed germination and seedling establishment. Taken together, our results demonstrate a key role for *CRA* genes in regulating the onset of seed germination by ABA, and highlight how *cra* mutants can be used as powerful tools to analyze novel molecular components of ABA signaling in seeds.

Keywords: ABA • Arabidopsis • Jasmonate • Seed • Stress • Suppressor.

Abbreviations: CAPS, cleaved amplified polymorphic sequence; *coi1-16*, *coronatine insensitive 1-16*; EMS, ethylmethane sulfonate; JA, jasmonate; MeJA, methyl jasmonate; MS, Murashige–Skoog medium; ODPA, oxo-phytodienoic acid; PAC, paclobutrazol; PP2C, protein phosphatase type 2C; RT–PCR, reverse transcription–PCR; SSLP, simple sequence length polymorphism.

Introduction

Seed germination comprises a set of morphogenetic and metabolic processes resulting in the transformation of an embryo into a seedling with the potential to become an adult plant. It is well known that germination is a developmental stage heavily influenced by the action of plant hormones. Whereas gibberellins play an essential role in the positive regulation of seed germination in *Arabidopsis thaliana* (Ogawa et al. 2003), there is direct evidence for the involvement of ABA in blocking seed germination (Penfield et al. 2006, Holdsworth et al. 2008). Other essential regulatory molecules, such as jasmonates (JAs), are also key regulators of seed physiology.

The phytohormone ABA has been shown to play an important role in seed formation, establishment and maintenance of dormancy, and in inhibition of germination in seeds (reviewed in Kermode 2005, Bradford and Nonogaki 2007); later in the early seedling, it also leads to growth arrest under unfavorable environmental conditions (Lopez-Molina et al. 2001, González-Guzmán et al. 2002). To gain further insights into the role of ABA during seed germination and early growth, a considerable number of genetic screenings have been performed to isolate ABA-insensitive mutants during these developmental cues. Thus, several ABA-insensitive loci encoding transcriptional regulators (*ABI3*, *ABI4* and *ABI5*) and type 2C protein phosphatases (PP2Cs; *ABI1*, *ABI2*, *AHG1* and *AHG3*) (Giraudat et al. 1992, Leung et al. 1994, Leung et al. 1997,

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Finkelstein et al. 1998, Yoshida et al. 2006, Nishimura et al. 2007) have been described. Our understanding of the ABA signaling pathway has been greatly improved since the identification of the ABA receptors belonging to the PYR1-PYL (PYRABACTIN RESISTANCE 1-PYR-LIKE)/RCAR (REGULATORY COMPONENT OF ABA RECEPTOR) protein family (Ma et al. 2009, Park et al. 2009). Using biochemical and genetic approaches, Park et al. (2009) and Ma et al. (2009) found that ABA promotes the interaction of PYR1/RCAR with clade A PP2Cs leading to the inhibition of PP2C activity.

JAs are lipidic hormones derivated from fatty acids and, among them, jasmonic acid, methyl jasmonate (MeJA), jasmonyl-isoleucine (JA-Ile) and oxo-phytodienoic acid (OPDA) are well characterized. However, only some JAs are biologically active. The JAs known to possess biological activity are (+)-7-iso-jasmonoyl-L-isoleucine, that seems to play a major role in JA signaling in Arabidopsis leaves and flowers (Fonseca et al. 2009); jasmonoyl-L-tryptophan, an auxin signaling inhibitor in Arabidopsis roots (Staswick 2009), and OPDA, with signaling properties independent of JA signaling (Stinzti et al. 2001) or using part of the JA signaling machinery (Ribot et al. 2008).

Among the plethora of functions regulated by JAs, plant responses to environmental stresses and biotic challenges are well characterized. Additionally, they are involved in several developmental processes including seed germination. Seeds from *Brassica napus* and *Linum usitatissimum* exhibit partial inhibition of germination when the embryos are plated on medium supplemented with jasmonic acid or MeJA (Wilen et al. 1991). Interestingly, germination of Arabidopsis wild-type seeds was not affected in the presence of MeJA at concentrations up to 50 μM (Staswick et al. 1992). However, in a recent study by Dave et al. (2011), higher concentrations of JA (up to 500 μM), that most probably do not represent the physiological level for the hormone, were found to be effective in the inhibition of Arabidopsis seed germination. Moreover, it has been described that OPDA is more effective than JA in the inhibition of Arabidopsis seed germination (Dave et al. 2011).

For most developmental processes and stress situations, the precise plant response is not only activated by one hormone, but is the result of a complex network of interactions among different signaling pathways (Lorenzo and Solano 2005). Several examples of cross-talk between ABA and other hormonal transduction pathways, such as gibberellins, ethylene, salicylic acid, auxins or JAs, have been reported (reviewed in Finkelstein et al. 2008). Germination is a clear example of this type of regulation and, consequently, several reports have demonstrated that JAs and ABA might interact during this developmental cue. Thus, the JA-insensitive mutants *coronatine insensitive 1-16* (*coi1-16*) and *ja resistant 1* (*jar1*) show higher sensitivity than the wild type to exogenous ABA (Staswick et al. 1992, Ellis and Turner 2002). Furthermore, a synergistic effect has been observed when ABA and JAs were combined to inhibit germination. ABA inhibition of seed germination in wild-type seeds was enhanced when MeJA was also present (Staswick et al. 1992, Ellis and Turner 2002). Similar germination inhibition was obtained when a combination of ABA and OPDA was present in the germination medium, even at concentrations that had a low inhibitory effect separately (Dave et al. 2011).

To gain deeper insight into the physiological and developmental role of ABA and the ABA–JA interaction during seed germination and early growth, we developed a novel strategy to isolate new mutants affected in ABA responses during these developmental cues. To this end, we took advantage of the ABA hypersensitivity of the JA-insensitive mutant *coi1-16* as a sensitized background for a suppressor screen to identify additional ABA response loci. Consequently, we have isolated several ABA-insensitive mutants named *cra* (*coi1-16 resistant to ABA*). Detailed phenotypical analyses of four of them, *cra5 coi1-16*, *cra6 coi1-16*, *cra7 coi1-16* and *cra8 coi1-16*, reveal their role in ABA signaling during seed germination and under abiotic stress conditions. Further characterization and function of the mutated genes will provide a better understanding of the regulatory mechanisms involved in ABA signaling and in the ABA- and JA-mediated mechanisms controlling seed germination and stress responses.

Results

Cross-interconnections between ABA and JA signaling pathways during seed germination and early seedling growth.

One of the well-characterized physiological effects of ABA is the inhibition of seed germination. The existence of complex cross-talk between phytohormones during regulation of many developmental processes is common knowledge. In this regard, JAs regulate seed germination through positive and negative interactions with ABA.

Further characterization of the effect of both hormones during seed germination, development of green and expanded cotyledons and early seedling growth is shown in **Figs. 1** and **2**, respectively. Firstly, we analyzed the sensitivity to increasing concentrations of ABA in the JA-insensitive *coi1-16* mutant, compared with the wild type [Columbia-0 (Col-0)] and the ABA-insensitive *abi1-1* mutant (**Fig. 1**). Interestingly, a hypersensitive phenotype is observed in the *coi1-16* mutant background after radicle protrusion (**Fig. 1A**). This higher sensitivity of the *coi1-16* mutant is even more pronounced during cotyledon development and under low ABA concentrations (0.5 μM) (**Fig. 1B, C**).

Furthermore, the joint addition of ABA and JA to the medium has an additive effect on the development of green and expanded cotyledons (**Fig. 2A**) and the subsequent seedling development (**Fig. 2B**). This phenotype is observed not only in the wild type (Col-0), but also in the *abi1-1* mutant.

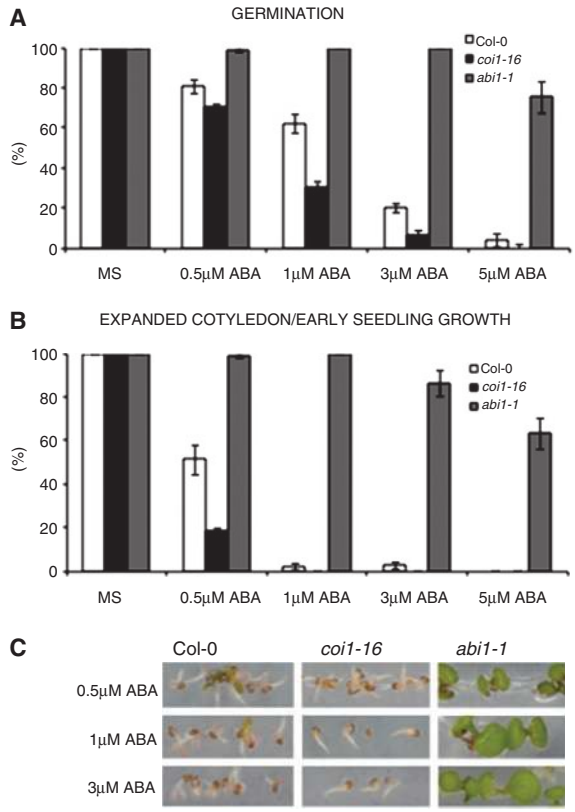

Fig. 1 Sensitivity to ABA of the JA-insensitive *coi1-16* mutant, the wild type (Col-0) and *abi1-1*. (A) Percentage of seed germination sensu stricto and (B) development of green and expanded cotyledons on MS agar plates or supplemented with 0.5, 1, 3 and 5 μM ABA. Seeds were scored 7 d after sowing. Error bars represent ± SD of three independent experiments. (C) ABA responses of JA- and ABA-insensitive mutants during seedling growth. Photographs show 6-day-old seedlings of the wild type (Col-0), and the ABA-insensitive *abi1-1* and JA-insensitive *coi1-16* mutants, in the presence of 0.5, 1 and 3 μM ABA. The JA-insensitive mutant exhibits ABA hypersensitivity.

Identification of *cra* mutants

The ABA hypersensitivity observed in the JA-insensitive mutant supported the idea that alterations in a specific signal transduction pathway may affect plant sensitivity to other hormone signaling pathways (Lorenzo and Solano 2005). Based on this concept, we developed a screening strategy to find mutants affected in ABA responses. We mutagenized *coi1-16* using ethylmethane sulfonate (EMS) and screened 105,000 M_2 seedlings from 17 M_1 EMS-mutagenized parental seed batches for the suppressor of ABA hypersensitivity. For this screening, 0.5 μM ABA, which clearly differentiated seedling growth of the *coi1-16* parental lines and *abi1-1* mutants (**Fig. 1B**), was used. This approach allowed us to identify mutants with phenotypes weaker than the *abi1* phenotype, which were otherwise hardly detectable under other conditions.

The screening yielded 76 new putative mutants (M_2) in which the ABA-hypersensitive seed germination phenotype of *coi1-16* was suppressed. The isolated mutants were named *cra* (*coi1-16 resistant to aba*). M_3 seeds were obtained from 72 putative mutants, in which reduced ABA sensitivity compared with that of *coi1-16* was confirmed. All mutants exhibited phenotypes intermediate between the *abi1-1* and *coi1-16* phenotypes. We were able to distinguish three different phenotypic classes based on the extent of radicle emergence and green cotyledon development, namely weak germination (WG), root (R) and strong germination (SG). A total of 40 WG, 10 R and 15 SG mutants were isolated (**Table 1**, **Fig. 3**) and the number of M_1 parental seed batches represented in each category was 14, 8 and 8, respectively (**Table 1**). There were seven putative mutants, belonging to four different complementation groups (*cra1*–*cra4*), that showed much stronger phenotypes than these three groups and were able to germinate at higher ABA concentrations (1–3 μM). For this reason, they were considered as *abi* mutant-like. Examples of these three different categories will be characterized further in this work, where *cra5 coi1-16* belongs to the SG phenotypic class, *cra6 coi1-16* to the WG class, and both *cra7 coi1-16* and *cra8 coi1-16* are included in the R subgroup, all of them in the *coi1-16* genetic background.

Table 1 *cra* mutants isolated in the screening and different phenotypical categories based on the extent of radicle emergence and green cotyledon development

Mutants selected, $n = 76$	Viable on soil, $n = 72$
WG (weak germination)	40 (14)
R (root)	10 (8)
SG (strong germination)	15 (8)
abi phenotype	7 (4)

The *abi* phenotype belongs to mutants able to germinate at higher ABA concentrations (1–3 μM). The number of M_1 parental seed batches represented in each category is given in parentheses.

Genetic characterization of *cra* mutants

Chromosomal mapping of *cra1 coi1-16*–*cra5 coi1-16* mutants using molecular markers [simple sequence length polymorphisms (SSLPs) and cleaved amplified polymorphic sequences (CAPSs)], together with a test for allelism, uncovered new alleles of the *abi3* and *abi4* mutants (**Fig. 4**). In fact, crosses between the *cra3 coi1-16* and *cra2 coi1-16* mutants and *abi3* and *abi4* rendered F_1 plants ABA resistant, indicating that they represented new alleles of *abi3* and *abi4*, respectively. This notion was further supported by the recessive nature of all five mutants [in backcrosses with the parental *coi1-16* and Landsberg *erecta* (L*er*) wild-type plants] and by their map position near ABI3 and ABI4, respectively (**Supplementary Fig. S1**). In addition, we have identified two dominant mutations, *cra4 coi1-16* belonging to the previously known *abi1-1* mutant (**Fig. 4**; **Supplementary Fig. S1**) and the *cra1 coi1-16* dominant hypermorphic mutation located on the higher arm of chromosome I (**Fig. 4**). In an effort to clone the *CRA5* gene positionally, chromosomal localization of the *cra5 coi1-16* mutant was

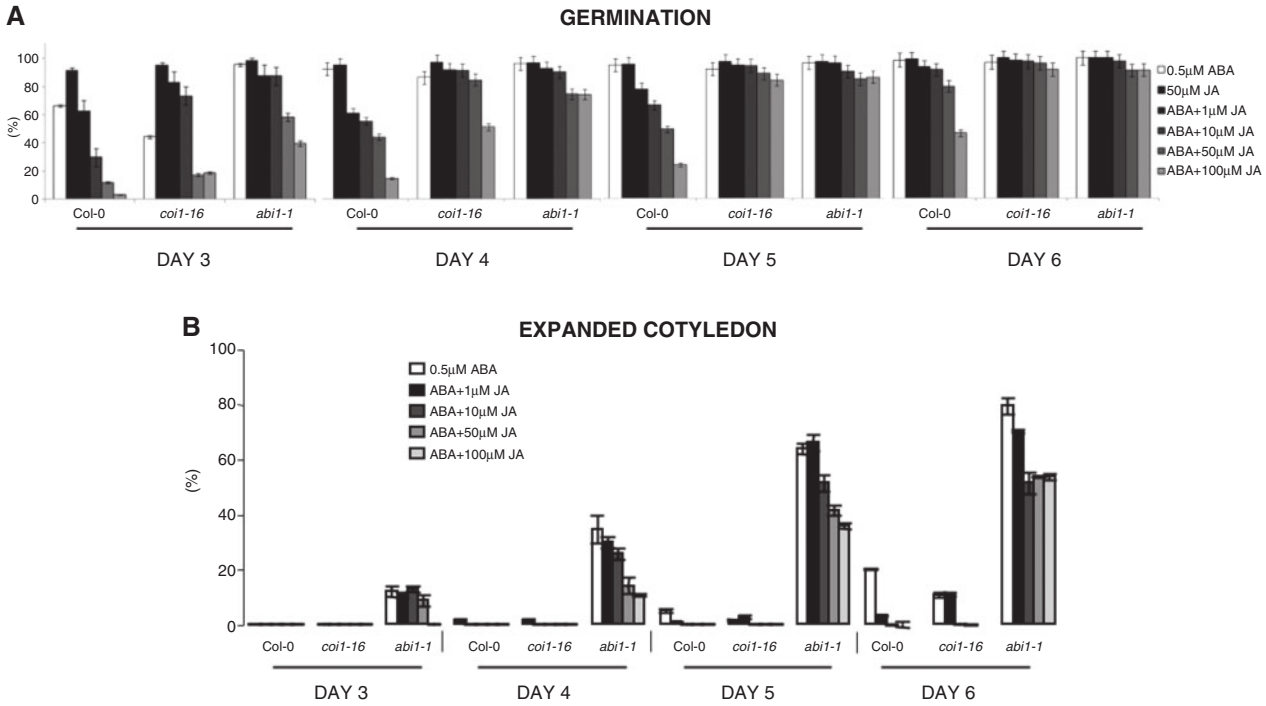

Fig. 2 Effect of ABA and JA on the germination and early seedling growth of the JA-insensitive *coi1-16* mutant, the wild type (Col-0) and *abi1-1*. (A) Graph showing a dose-response assay in *coi1-16*, wild-type (Col-0) and *abi1-1* seeds after 3-6 d in 0.5 μM ABA, 50 μM JA and 0.5 μM ABA plus 1, 10, 50 and 100 μM JA. (B) Percentage of seeds that develop green and expanded cotyledons on agar plates supplemented with the above indicated treatments with time. Error bars represent ± SD of three independent experiments.

performed by outcrossing *cra5 coi1-16* (in a Col-0 genetic background) to L*er* and using molecular markers (SSLPs and CAPs). Linkage analysis of the F_2 mapping populations obtained allowed the IB (Instituto de Bioingeniería)–UMH (Universidad Miguel Hernández) Gene Mapping Facility (Elche, Spain) to delimit this recessive mutation in the higher arm of chromosome V (**Fig. 4**) and more precisely the *cra5 coi1-16* mutation was located in a region between nga151 and AthCTR1 markers, excluding the *AtCTR1* gene. Interestingly, the recessive nature of *cra6 coi1-16*, *cra7 coi1-16* and *cra8 coi1-16* mutants (in backcrosses with the parental *coi1-16* and L*er* wild-type plants) together with allelic analysis and complementation tests by crosses between these mutants and *abi3*, *abi4*, *abi5* and *cra5 coi1-16*, uncovered no alleles of the previously identified *abi* mutants and different complementation groups, respectively.

cra mutations confer ABA insensitivity to Arabidopsis seeds

We analyzed the ABA sensitivity of the *cra5 coi1-16–cra8 coi1-16* mutant seeds in comparison with that of Col-0, *coi1-16* and *abi1-1* mutant seeds. Dose–response analysis using different ABA concentrations (0, 0.5, 0.8 and 1 μM) showed that ABA sensitivity was reduced in all the *cra5-8* mutant seeds (**Fig. 5**). *cra5 coi1-16* mutant seeds germinated and developed expanded cotyledons at up to 0.8 μM ABA (**Fig. 5**; **Supplementary Fig. S2**). However, *cra5 coi1-16* seed

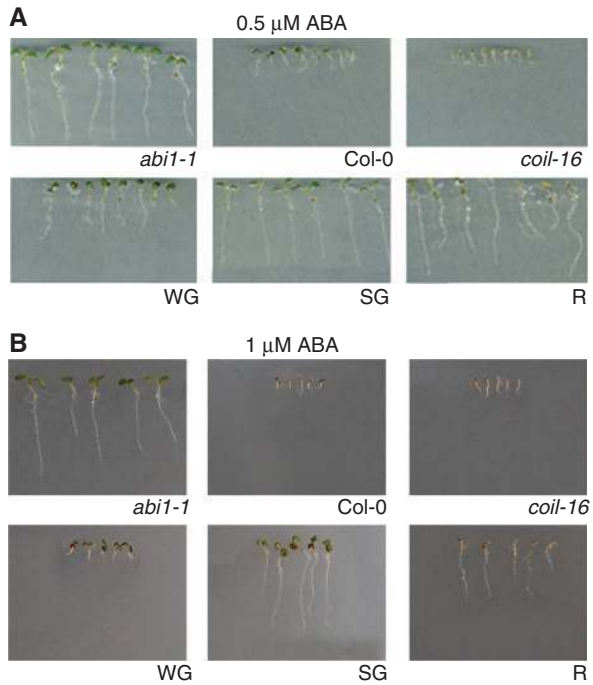

Fig. 3 Phenotypic comparison of the ABA sensitivity of wild-type (Col-0), *coi1-16*, *abi1-1* and the isolated *cra* mutants exhibiting different levels of suppression of ABA sensitivity (WG, weak germination; R, root; and SG, strong germination; see text for the details of the three categories). Seedlings were grown for 7 d on agar plates supplemented with 0.5 μM ABA (A) and 1 μM ABA (B).

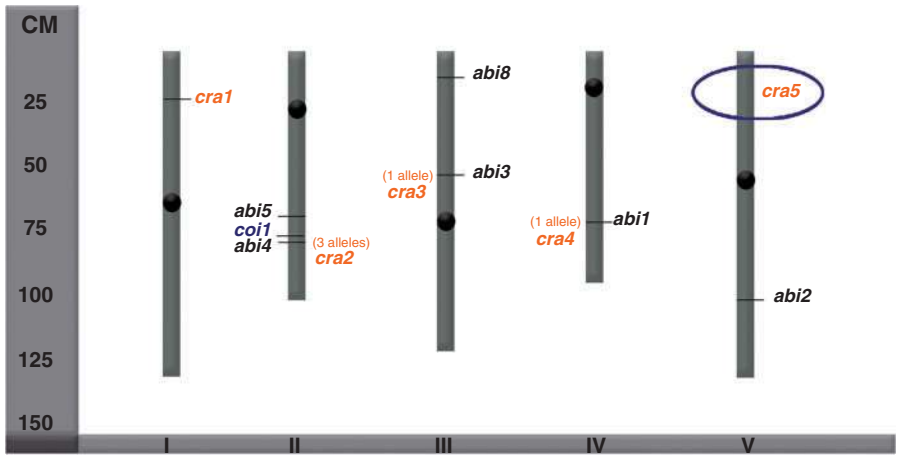

Fig. 4 Chromosomal position of the five identified *cra* loci (*cra1*, *cra2*, *cra3*, *cra4* and *cra5*). The position of *abi* mutants and *coi1* is also shown. Chromosomal localization of the *cra5* mutant using molecular markers (SSLP and CAPS). We have isolated the previously described *abi1-1* mutant (*cra4*) and new alleles of *abi3* (*cra3*) and *abi4* (*cra2*) mutants.

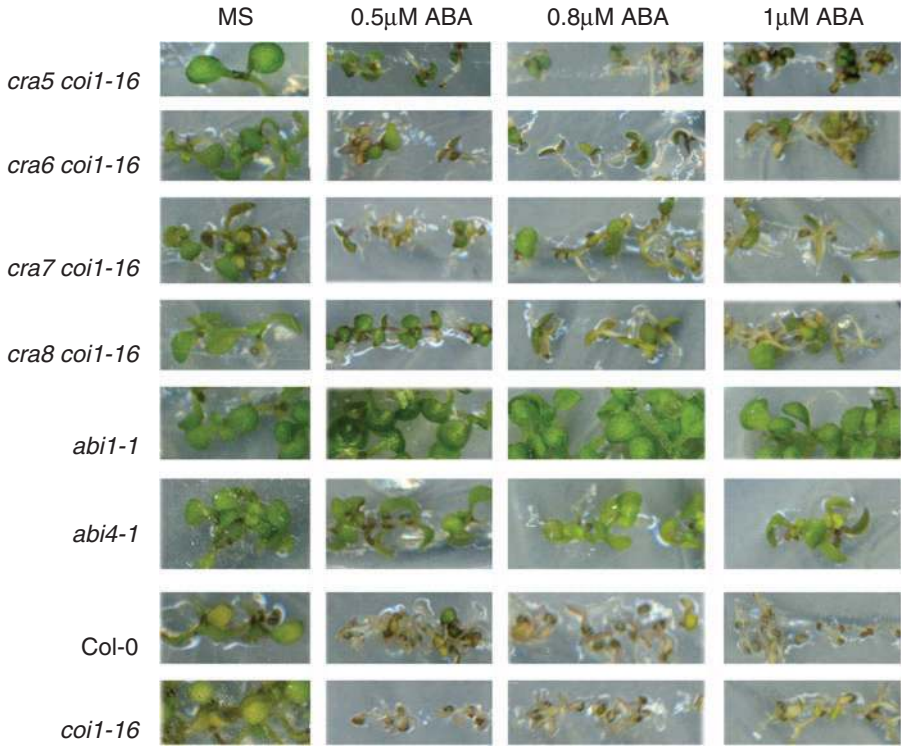

Fig. 5 ABA germination assays in *cra* mutants. Pictures show seed germination in MS medium or supplemented medium (0.5, 0.8 and 1 μM ABA) of *cra5 coi1-16*, *cra6 coi1-16*, *cra7 coi1-16* and *cra8 coi1-16* compared with Col-0, *abi1-1*, *abi4-1* and *coi1-16*. Pictures were taken 7 d after sowing.

germination was still inhibited by higher concentrations (>1 μM) of ABA and was not able to germinate and produce green cotyledons under this condition. *cra6 coi1-16*, *cra7 coi1-16* and *cra8 coi1-16* mutant seeds showed a similar resistance to ABA under 0.5 and 1 μM, even higher than *cra5 coi1-16* (**Fig. 5**; **Supplementary Fig. S3**), indicating that the mutations in the different *cra* mutants, although sufficient to suppress *coi1-16* ABA hypersensitivity, differ in their level of ABA sensitivity. Col-0 germination was greatly inhibited in 1 μM ABA at 7 d post-stratification, while *cra6 coi1-16*, *cra7 coi1-16* and *cra8 coi1-16* mutants showed radicle emergence and developed green cotyledons. The addition of higher ABA concentrations arrested cotyledon greening in *cra5 coi1-16*–*cra8 coi1-16* mutants but not in the strong *abi1-1* mutant.

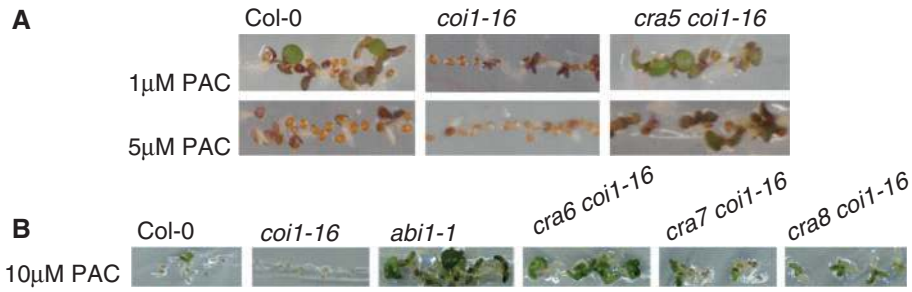

Fig. 6 Seed germination in the presence of the gibberellin biosynthesis inhibitor PAC. Pictures show seed germination in supplemented MS medium (1, 5 and 10 μM PAC) of (A) *cra5 coi1-16* and (B) *cra6 coi1-16*, *cra7 coi1-16* and *cra8 coi1-16* compared with Col-0, *abi1-1* and *coi1-16*. Pictures were taken 7 d after sowing.

To characterize this phenotype further, we analyzed *cra5 coi1-16*, *cra6 coi1-16*, *cra7 coi1-16* and *cra8 coi1-16* germination in response to paclobutrazol (PAC), an inhibitor of gibberellin biosynthesis. It is well known that ABA-insensitive mutants are PAC resistant during germination, showing a lower need for gibberellins to germinate (Koornneef et al. 1998). This requirement was compared in Col-0, *coi1-16*, *abi1-1* and *cra5 coi1-16–cra8 coi1-16* seeds germinated in a medium containing PAC (**Fig. 6**). In contrast to Col-0 and the *coi1-16* mutant, *cra5 coi1-16* seeds germinated and developed green cotyledons in medium supplemented with up to 5 μM PAC, showing about 60 and 20% seed germination and development of expanded cotyledons, respectively (**Fig. 6A**; **Supplementary Fig. S4**). Interestingly, *cra6 coi1-16*, *cra7 coi1-16* and *cra8 coi1-16* mutant seeds were even able to germinate and develop green and expanded cotyledons under a higher concentration of PAC (10 μM), that produced a strong inhibition of germination in Col-0 and the *coi1-16* mutant (**Fig. 6B**).

These data indicated that *cra5 coi1-16–cra8 coi1-16* mutations positively affect germination through a reduction in ABA sensitivity and the requirement for gibberellin to germinate to a different extent.

cra mutations cause different sensitivity to salt and osmotic stresses

Previously, it has been suggested that ABA is also involved in the regulation of different stress responses. In this way, salt and osmotic stresses in seeds and seedlings lead to an increase in ABA biosynthesis and, consequently, to the inhibition of germination and early growth arrest (Lopez-Molina et al. 2001, González-Guzmán et al. 2004).

To analyze if the reduced ABA sensitivity during *cra* seed germination and early seedling growth is also evident in other ABA-mediated stresses that increase ABA levels, we analyzed the germination of *cra* seeds in response to different stresses (i.e. NaCl, mannitol and sucrose) and compared it with that of the wild type, *coi1-16* and *abi1-1* (**Fig. 7**; **Supplementary Figs. S5–S7**). Seed germination under these conditions leads to a severe delay in radicle emergence and further growth arrest in the wild type and *coi1-16*, while *cra5 coi1-16*, *cra6 coi1-16*, *cra7 coi1-16* and *cra8* coi1-16 mutants germinated and developed green cotyledons under such stresses. In particular, we compared the germination of *cra5 coi1-16* with that of Col-0, *coi1-16* and *abi1-1* plants in medium supplemented with NaCl (150 mM) and a low mannitol concentration (250 mM) (**Fig. 7A**). In a similar way, *cra6 coi1-16–cra8 coi1-16* mutants were analyzed under higher NaCl (125 and 200 mM), mannitol (300 and 400 mM) and sucrose (100, 150 and 200 mM) stresses (**Fig. 7B**; **Supplementary Figs. S5–S7**). All the *cra* mutations tested generated both salt- and osmoticum-insensitive phenotypes during these abiotic stresses and, interestingly, differences in the level of insensitivity were observed.

Taken together, these results firmly establish that CRA5, CRA6, CRA7 and CRA8 are positive regulators of the ABA signaling pathway during several physiological responses including seed germination and ABA-mediated abiotic stress responses.

ABA gene expression alterations by *cra5 coi1-16*

To examine if the ABA-insensitive phenotypes in the *cra5 coi1-16* mutant were accompanied by altered expression of ABA-responsive genes, we analyzed RNA levels in two ABA-inducible genes, i.e. *RAB18* (Responsive to ABA 18), a strongly ABA-inducible gene, whose expression is drastically repressed in both *abi1-1* and *abi2-1* mutants (Lang and Palva 1992, Leung et al. 1997), and *AtMYC2*, previously reported to regulate reponses to the phytohormones ABA (Abe et al. 2003) and JA, and with high RNA levels in the *abi1-1* mutant (Lorenzo et al. 2004). With this purpose, RNA isolated from plants treated with 50 μM ABA was analyzed by quantitative reverse transciption–PCR (RT–PCR) in the wild type and in *abi1-1* and *cra5 coi1-16* mutants (**Fig. 8**). Gene expression analysis revealed certain similarities between *cra5 coi1-16* and *abi1-1* mutants, in that diminished induction of *RAB18* but enhanced expression of *AtMYC2* genes was observed upon ABA treatment compared with Col-0 wild-type plants. However, the amount and ratio of transcripts in the presence and absence of ABA are different in each mutant background.

These results indicate that the *cra5 coi1-16* mutation decreases the expression of ABA-responsive genes and is also linked to ABA-related physiological responses such as ABA-insensitive germination and stress-resistant germination.

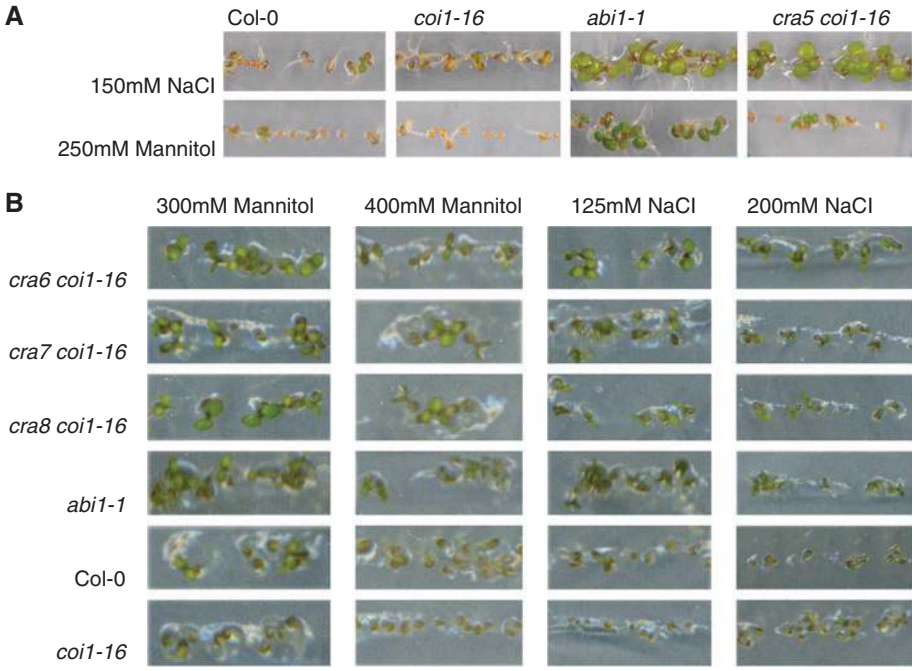

Fig. 7 Stress germination assays. Seed germination and early seedling growth in MS medium supplemented with NaCl (125, 150 and 200 mM) and mannitol (250, 300 and 400 mM) of (A) *cra5 coi1-16* and (B) *cra6 coi1-16*, *cra7 coi1-16* and *cra8 coi1-16* compared with Col-0, *abi1-1* and *coi1-16*. Pictures were taken 4 d after sowing.

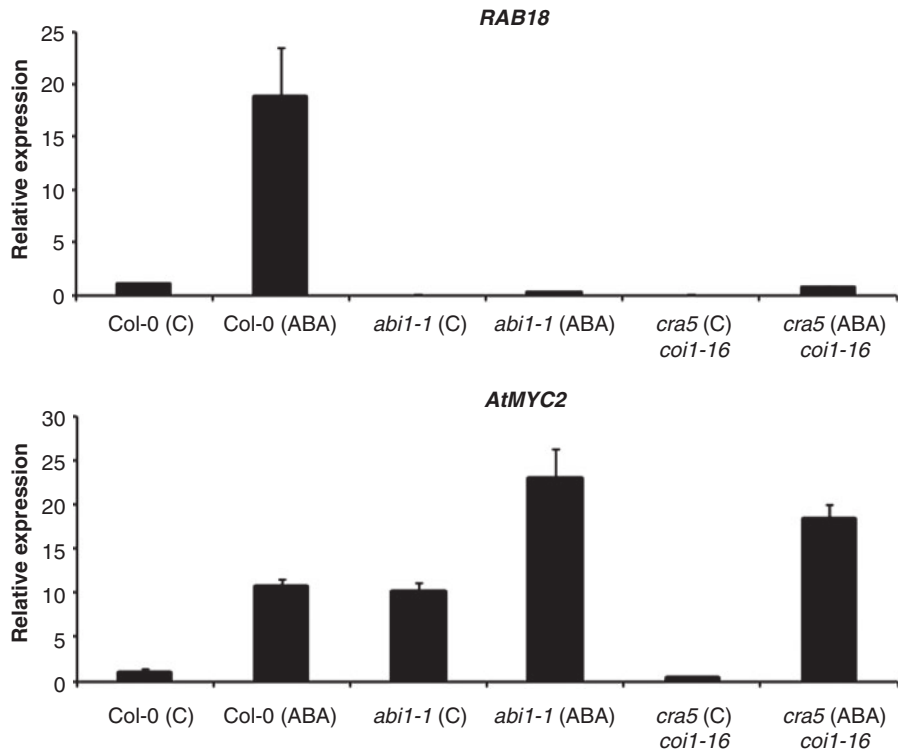

Fig. 8 Expression analysis in the *cra5 coi1-16* mutant by quantitative RT–PCR. Expression of the ABA-responsive genes *RAB18* and *AtMYC2* in *cra5 coi1-16* was compared with that in Col-0 and *abi1-1*. RNA was isolated from mock-treated plants (C) and plants treated with 50 μM ABA (ABA) for 3 h.

Discussion

A seed needs to integrate into the cell the environmental and hormonal signals that provide information about the conditions appropriate to achieve germination. This process is one of the most important and vulnerable parts of a plant life cycle, since the development and formation of new plants could be compromised. Thus, the antagonistic cross-talk between ABA and gibberellins in the regulation of seed germination is widely described. Other essential hormones, such as JAs, are also involved in seed physiology.

The main function of JAs is largely described to be in plant defense responses, but a role for JA in embryogenesis has also been shown (Wilen et al. 1991). In *B. napus*, endogenous JA modulates ABA effects on the expression of genes encoding the seed storage proteins napin and oleosin (Hays et al. 1999). JAs may reduce ABA levels during late stages of seed development (Hays et al. 1999). In tomato, a *COI1* ortholog is required for seed maturation control (Li et al. 2004). It has been demonstrated that JAs also inhibit seed germination in several species and have a synergistic effect with ABA during this process in Arabidopsis (Wilen et al. 1991, Staswick et al. 1992, Ellis and Turner, 2002). This fact is further supported by the results presented in **Fig. 2**, both in the the wild type (Col-0) and in the *abi1-1* mutant, where the joint addition of low ABA and JA concentrations decreases the development of green and expanded cotyledons. On the other hand, ABA enhanced sensitivity of the JA-insensitive mutants *coi1* and *jar1* during seed germination. These data indicate that JA antagonizes ABA-mediated inhibition of germination (Staswick et al. 1992, Ellis and Turner 2002) and fully support the negative cross-talk presented in this work using the conditionally fertile *coi1-16* allele (**Figs. 1**, **2**). Recently, OPDA was found to be 10 times more effective than JA in the inhibition of seed germination (Dave et al. 2011) when seed germination in medium containing OPDA or JA was scored 2 and 7 d after sowing. In this study, the effect of exogenous JA on germination of *coi1-16* seeds was tested, showing a reduction of germination. These results indicate that both OPDA and JA inhibition of germination could be COI1 independent (Dave et al. 2011). In agreement with these results, it was found that OPDA treatments increase ABI5 protein levels. Moreover, ABI5 abundance was enhanced by the combination of OPDA with ABA, suggesting that OPDA levels result in an increase in ABI5 levels and the consequent impairement of seed germination (Dave et al. 2011).

It is known that unfavorable germination conditions result in an increase in ABA levels upon seed imbibition (Seo et al. 2006). Then ABA inhibits germination by stimulating the expression of specific germination repressors such as *ABI3* and *ABI5* genes (Lopez-Molina et al. 2001, Lopez-Molina et al. 2002). Previous reports describe the germination-repressive role of the Arabidopsis seed coat (Lee et al. 2010). It has been described that the endosperm can biosynthesize ABA, and transcriptome analysis has shown that the endosperm expresses genes involved in ABA metabolism (Lefebvre et al. 2006, Penfield et al. 2006, Barrero et al. 2010). Remarkably, the *coi1-16* hypersensitive phenotype is also extended to the requirement for gibberellin to germinate (**Fig. 6**) and other abiotic stresses, such as the presence of high salt and mannitol concentrations (**Fig. 7**). In agreement with the results obtained in this work, it could be feasible that disruption of *COI1* in the *coi1-16* mutant may affect some of these processes, either the elevation of ABA hormone levels, the presence of increased seed germination repressors, structural modifications of the seed layers or a combination of these.

Several pieces of evidence demonstrate that alterations in a concrete signal transduction pathway can affect the sensitivity of the plant to another hormonal signaling pathway (Lorenzo and Solano 2005). Our main objective in this work was the isolation of mutants affected in ABA responses during seed germination and early development by carrying out a new screening strategy using the JA-insensitive and ABA-hypersensitive background *coi1-16*. We report here a screening for suppressors of ABA hypersensitivity observed in the JA-insensitive mutant *coi1-16*. The corresponding isolated mutants were designated as *coi1-16 resistant to aba* (*cra*). Three main phenotypic classes were distinguished, based on the extent of radicle emergence and green cotyledon development under 0.5 μM ABA, and tentatively named weak germination (WG), strong germination (SG) and root (R) (**Fig. 3**), supporting the existence of distinct genes affected by the corresponding mutations. This idea is firstly corroborated by the differences observed in the level of ABA and the stress insensitivity of all the *cra* mutants characterized in this work (i.e. *cra5*, *cra6*, *cra7* and *cra8*) (**Figs. 5–7**). Secondly, allelic analysis and complementation tests by crosses among the four *cra5 coi1-16–cra8 coi1-16* mutants and between these mutants and *abi3*, *abi4* and *abi5* uncovered no alleles of the previously identified *abi* mutants. Consequently, a great deal of evidence that *CRA5–CRA8* are indeed different genes is provided. This notion was further supported by the recessive nature of all four mutants (in backcrosses with the parental *coi1-16* and L*er* wild-type plants) and the ease with which they could be differentiated from the dominant *abi1-1* and *abi2-1* mutations. Interestingly, the *cra5 coi1-16* mutation is located in a region of the genome where no other ABA-insensitive (*abi*) mutant has been reported to date (**Fig. 4**), although we do not discard the possibility that other loci known to affect ABA responses could be present. In spite of this, the ABA-insensitive phenotypes in the *cra5 coi1-16* mutant were accompanied by altered expression of the ABA-responsive genes, *RAB18* and *AtMYC2* (**Fig. 8**), and resemble to some extent that observed in the *abi1-1* mutant bakground.

To summarize, genetic evidence for the role of *CRA5*, *CRA6*, *CRA7* and *CRA8* genes in ABA signaling is presented. All germination phenotypes observed in seeds carrying mutations in *CRA5–CRA8* genes suggest a critical role for the corresponding proteins in ABA-regulated seed development and abiotic stress conditions. In this study, we also gained new insight through the isolation and further characterization of *cra* mutants,

emerging as a powerful tool to analyze ABA and JA interaction in different plant developmental cues and under abiotic or biotic stresses. We conclude that *CRA5*, *CRA6*, *CRA7* and *CRA8* genes may be essential components of ABA signaling during seed germination and seedling establishment under stress responses.

Materials and Methods

Plant materials and treatments

Arabidopsis thaliana ecotype Col-0 is the genetic background used in this work, and the *abi1-1* mutant is in the L*er* background. Seed stocks of *abi* mutants were obtained from the Arabidopsis Biological Resource Center. The *coi1-16* mutant was kindly provided by Dr. John Turner (University of East Anglia, Norwich, UK).

The Arabidopsis plants were grown in a growth chamber or greenhouse under 50–60% humidity, a temperature of 22°C (or 15°C for seed production), and with a 16 h light/8 h dark photoperiod at 80–100 $\mu E\, m^{-2} s^{-1}$ in pots containing a 1 : 3 vermiculite : soil mixture. For in vitro culture, Arabidopsis seeds were surface-sterilized and then sown as described previously (Saavedra et al. 2010). Briefly, seeds were surface-sterilized in 75% (v/v) sodium hypochlorite and 0.01% (v/v) Triton X-100 for 5 min, and washed three times in sterile water before planting. Seeds were stratified for 3 d at 4°C and then sown in Petri dishes (5 cm diameter) containing solid medium composed of Murashige and Skoog (1962) (MS) basal salts and 2% (w/v) glucose, solidified with 0.6% (w/v) agar and then the pH was adjusted to 5.7 with KOH before autoclaving. Plates were sealed and incubated in a controlled environment growth chamber.

ABA, JA and PAC were purchased from Sigma-Aldrich (http://www.sigmaaldrich.com/).

Screening conditions and mutant isolation

EMS-mutagenized M_2 seeds were obtained as follows. Approximately 80,000 *coi1-16* seeds were treated overnight at 4°C with phosphate buffer. Then 0.4% EMS was added for 8 h at room temperature and finally the seeds were washed extensively with water and sown on soil. M_2 generation seeds were separately harvested and formed independent pools.

M_2 *coi1-16* mutagenized seeds were screened to isolate putative mutant seedlings which were able to grow on MS media plates containing 0.8% (w/v) agar, 1% (w/v) sucrose and 0.5 μM ABA (Sigma), at 15°C. Defects in seed germination inhibition by ABA were scored after 5–7 d of growth, and putative mutant seedlings were transferred to soil.

Mapping and positional cloning of the *CRA1–CRA5* loci

Genetic mapping of *cra1 coi1-16–cra5 coi1-16* mutants was performed by crossing the mutants with the Arabidopsis L*er* ecotype, and F_2 progeny were obtained. ABA-insensitive individuals were selected on medium containing 0.5 μM ABA. Recombination events in the F_2 segregants of the mapping population were performed by using SSLP and CAPS markers (Konieczny and Ausubel 1993, Bell and Ecker 1994). Pre-mapping of the *cra5 coi1-16* mutation was performed as described in Ponce et al. (1999, 2006). Briefly, for low-resolution mapping, the DNA of 50 F_2 phenotypically mutant plants was individually extracted and used as a template in multiplex PCR to co-amplify 32 SSLP and In/Del molecular markers using fluorescently labeled oligonucleotides as primers.

Germination assays

To measure ABA and PAC sensitivity, seeds were plated on solid medium composed of MS basal salts, 1% (w/v) sucrose and different concentrations of ABA (0.5, 0.8 and 1 μM) and PAC (1 and 5 μM), respectively. Seed lots to be compared were harvested on the same day from individual plants grown under identical environmental conditions. Each value represents the average germination percentage of 80–100 seeds with the standard deviation of at least three replicates.

To determine germination sensitivity to osmotic stress, the medium was supplemented with mannitol (250, 300 and 400 mM), NaCl (100, 125 and 150 mM) and sucrose (100, 150 and 200 mM). The percentage of seeds with an emerged radicle (germination) or seedlings that germinated and developed green fully expanded cotyledons was determined 7 and 10 d after sowing, respectively.

Quantitative RT–PCR analysis

Seedlings from wild-type (Col-0), *abi1-1* and the *cra5 coi1-16* double mutant were treated after 10 d with 50 μM ABA for 3 h. Plant material was collected and frozen in liquid nitrogen. Quantitative RT–PCR and real-time PCR were performed as described previously (Saavedra et al. 2010). Basically, total RNA was extracted from Arabidopsis 10-day-old frozen seedlings using Tri-reagent as described by the manufacturer (Ambion). Extracted RNAs were then subjected to reverse transcription. A 1 μg aliquot of DNase-treated total RNA was used for reverse transcription with a Superscript Kit (Roche). The reverse transcription product was subjected to quantitative PCR using the primers for *β-actin-8* (At1g49420), forward 5′-AGTGGTCGTACAACCGGTATTGT-3′ and reverse 5′-GAGG ATAGCATGTGGAAGTGAGAA-3′; for *RAB18* (At5g66400), forward 5′-ATGGCGTCTTACCAGAACCGT-3′ and reverse 5′-CCA GATCCGGAGCGGTGAAGC-3′; and for *AtMYC2* (At1g32640), forward 5′-TCATACGACGGTTGCCAGAA-3′ and reverse 5′-A GCAACGTTTACAAGCTTTGATTG-3′, designed with the aid of Primer Express software 1.0 (Applied Biosystems). The actin gene *β-ACT8* was used as a control. Real-time PCR was performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Amplification was carried out with 'Brilliant® SYBR® Green QPCR MasterMix' (Stratagene) according to the manufacturer's instructions. The thermal profile for

SYBR Green real-time PCR was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. To generate the standard curves, cDNA isolated from Arabidopsis seedlings was serially diluted by a factor of 10 and aliquots of the dilutions were used in standard real-time PCRs. Each value determination was repeated three times to ensure the slope of the standard curves and to determine the standard deviation. The concentration of unknown samples was calculated with the ABI-Prism 7000 SDS software, which created threshold cycle values (C_t) and extrapolated relative levels of PCR products from the standard curve. The expression of all genes was normalized against the expression of the endogenous control gene (Actin). All experiments were repeated at least three times and yielded similar results.

Supplementary data

Supplementary data are available at PCP online.

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