Running head: CER9 regulates ABA biosynthesis and signaling

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Summary

A wax locus negatively regulates ABA response within a small time window during seed germination and also affects ABA levels in seeds.
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ABSTRACT

The *ECERIFERUM9* (*CER9*) gene encodes a putative E3 ubiquitin ligase that functions in cuticle biosynthesis and maintenance of plant water status. Here we found that CER9 is also involved in abscisic acid (ABA) signaling in seeds and young seedlings. The germinated embryos of the mutants exhibited enhanced sensitivity to ABA during transition from reversible dormancy to determinate seedling growth. Expression of the *CER9* gene is closely related to ABA levels and displays a similar pattern to that of *ABI5*, which encodes a positive regulator of ABA responses in seeds. *cer9* mutant seeds exhibited delayed germination that is independent of seed coat permeability. Quantitative proteomic analyses showed that *cer9* seeds had a protein profile similar to that of the wild type treated with ABA. Transcriptomics analyses revealed that genes involved in ABA biosynthesis or signaling pathways were differentially regulated in *cer9* seeds. Consistent with this, high levels of ABA were detected in dry seeds of *cer9*. Blocking ABA biosynthesis by fluridone treatment or by combining an ABA-deficient mutation with *cer9* attenuated the phenotypes of *cer9*. Whereas introduction of the *abi1-1, abi3-1* or *abi4-103* mutations could completely eliminate the ABA hypersensitivity of *cer9*, introduction of *abi5* resulted only in partial suppression. These results indicate that CER9 is a novel negative regulator of ABA biosynthesis and the ABA signaling pathway during seed germination.
INTRODUCTION

Seed germination is a critical stage in the life cycle of higher plants, during which the imbibed mature seed must shift rapidly from a state of quiescence to one of active metabolism. The transition from embryo dormancy to germination requires the activation of a series of developmental programs with the simultaneous mobilization of seed storage reserves and the loosening of the seed coat; these processes cumulate in radicle emergence, seedling establishment and subsequent photoautotrophic growth (Bewley, 1997; Finkelstein et al., 2008; Holdsworth et al., 2008; Rajjou et al., 2012). The decision for a seed to germinate depends on certain intrinsic and many environmental factors including water availability, temperature, light, and mineral availability. Among the internal factors, endogenous abscisic acid (ABA) and gibberellic acid (GA) are two important regulators that also mediate interactions with these environmental factors in modulating seed germination. In fact, seed germination or dormancy largely depends on the ratio of these two hormones, which play antagonistic roles in seed germination. ABA inhibits seed germination and mutants defective in ABA biosynthesis or signaling have enhanced germination efficiency and more easily break dormancy under osmotic stress. Conversely, GA promotes seed germination and GA-deficient mutants show delayed seed germination (Finkelstein et al., 2008; Holdsworth et al., 2008; Rajjou et al., 2012).

Protein degradation through the ubiquitination pathway is an important mechanism in regulating hormone biosynthesis and signaling. To date, there are over 1000 predicted E3 ubiquitin ligases in Arabidopsis. Based on the protein structure, these have been classified as, for example, HECT, RING, and U-box (Chen and Hellmann, 2013). Several E3 ligases were found to be involved in ABA signaling pathways and in the regulation of seed germination under various conditions. The RING E3 ligase ABI3-INTERACTING PROTEIN2 (AIP2) was reported to target ABSCISIC ACID-INSENSITIVE3 (ABI3) for degradation. In germination experiments, AIP2
loss-of-function and overexpression increased or decreased the sensitivity to ABA, respectively (Zhang et al., 2005). SALT- AND DROUGHT-INDUCED RING FINGER1 (SDIR1) acts upstream of ABF3, ABF4 and ABI5 in ABA signaling and is a positive regulator of the ABA signaling pathway (Zhang et al., 2007). The RING E3 ligase RING-H2 protein RHA2a regulates ABA-mediated seed germination and early seedling development independently of ABI3, ABI4 and ABI5, these being the main regulators in the ABA signaling pathway in seeds (Bu et al., 2009). KEEP ON GOING (KEG), a multidomain ubiquitin E3 ligase, has been shown to regulate the levels of ABI5 and to act as a negative regulator of ABA signaling. The keg mutation was found to suppress the ABA-insensitive phenotype of ABI5 in a seed germination assay (Stone et al., 2006). In addition, the Arabidopsis PROTEOLYSIS6 (PRT6), a UBR box-containing E3 ligase associated with N-end rule pathway of protein degradation, controls seed ABA sensitivity and seedling establishment (Holman et al., 2009).

The Arabidopsis ECERIFERUM9 (CER9) gene encodes a putative RING domain-containing E3 ubiquitin ligase, which is similar to Doa10 in yeast, and is expected to target its protein substrates for degradation through the ubiquitination pathway (Lu et al., 2012; Doblas et al., 2013). Recent studies revealed a unique association of CER9 with both cuticular wax and cutin synthesis (Lu et al., 2012). Also identified as SUD1, CER9 was suggested to be involved in the regulation of sterol biosynthesis in plants (Doblas et al., 2013). We report here that CER9 deficiency has a dramatic effect on plant ABA sensitivity during seed germination and early seedling growth, indicating that CER9 functions in the ABA response pathways. Genome-wide gene expression and proteomics analyses show that CER9 deficiency affects the abundance of gene transcripts and proteins related to ABA responses. We also show that the hypersensitivity of cer9 seed germination to ABA is due partially to the increased ABA levels found in the cer9 mutant, and that the inhibition of seed germination imposed by the cer9 mutation requires an intact and functional ABA signaling pathway. These studies indicate that CER9 is a new negative regulator of the ABA response in seed germination and early seedling growth.
RESULTS

CER9 deficiency increases ABA sensitivity during seed germination and early seedling establishment

Previous studies showed that the cer9 mutant alters cuticle lipid deposition and whole plant transpiration (Lu et al., 2012). Since ABA is an important regulator of plant water relations, we investigated whether other processes regulated by ABA are affected by cer9 mutations, including the response of cer9 to ABA during seed germination. As shown in Figure 1, both cer9-1 and cer9-2 were hypersensitive to ABA during seed germination and early seedling establishment. In an ABA dose-response assay, seeds of cer9-1 and cer9-2 were planted on ABA-free or ABA-containing medium and the percentage of seed germination at 3 days after the end of stratification was determined. In the absence of ABA, the seed germination percentages of different genotypes were found to be similar. However, in the presence of ABA the germination rate of cer9 seeds was reduced dramatically compared with that of the wild-type (Fig. 1). Furthermore, cotyledon greenness, as scored at 7 days after the end of stratification, was also notably suppressed in cer9 (Fig. 1).

After seed germination (radicle emergence), there is a small time window during which it is determined whether a germinated embryo will remain dormant or make the transition to seedling growth. It is known that ABA also controls this transition. To determine whether CER9 is involved in this germination check-point control, we tested the response of germinating seeds to ABA. As shown in Figure 2A, both the cer9 mutant and the corresponding wild-type seeds were arrested by a higher concentration of ABA (5.0 μM) at 1 day after stratification. Whereas 1.0 μM ABA did not arrest the growth of wild type, it was sufficient to arrest germination in cer9. This concentration of ABA no longer inhibited early seedling growth either for the wild type or cer9 at 2 days after stratification (Fig. 2B). These results indicate that CER9 is involved in the ABA response during the developmental transition from the
embryo stage to the seedling stage.

**CER9 expression is regulated by ABA**

The involvement of CER9 in conditioning ABA sensitivity during seed germination and early seedling establishment prompted us to check whether CER9 is regulated by ABA. RNA was extracted from dry seeds, seeds imbibed for 72 hr, and 1- and 3-day light grown seedlings. The expression level of CER9 was examined using quantitative RT-PCR (Fig. 3A). High levels of CER9 transcripts accumulated in dry seeds but the level fell rapidly after 3 days of stratification and following germination. This pattern of expression coincides with the changes in the level of ABA, which is also high in dry seeds but decreases rapidly after stratification (Ali-Rachedi et al., 2004). To further demonstrate that CER9 expression was indeed induced by ABA, we also compared expression levels in ABA-treated and untreated 1-day light grown seeds. The results revealed that CER9 expression was higher in ABA-treated seeds than in the untreated ones. We also compared the expression of CER9 with that of ABI5 as reported in the public microarray data. ABI5 is a key regulator of the ABA response in seeds and its expression is closely related to relative abundance of ABA (Lopez-Molina et al., 2002). BAR HeatMapper analysis indicated that CER9 expression increases during seed maturation and decreases after imbibition, which is similar to the pattern for ABI5 (Fig. 3B). These results revealed a close relationship between CER9 expression and ABA levels in seeds and young seedlings. It was also showed that negative or positive factors co-exist to fine tune the ABA response in planta.

**cer9 loss-of-function promotes seed dormancy**

Mature seeds require a period of after-ripening to overcome dormancy (Carrera et al., 2008). Two week-old freshly harvested cer9-2 seeds exhibited a higher rate (80%) of dormancy than the wild-type Col-0 (16%) (Fig. 4A). In cer9, increasing the length of dry storage time to 4 weeks decreased the seed dormancy rate to 20%, and 12-week storage led to complete loss of dormancy (Fig. 4A). The high degree of dormancy of
cer9-2 seeds can be fully overcome by a 2-day chilling treatment (stratification) or by applying 100 µM GA (Figs. 4B and 4C). Furthermore, the enhanced dormancy of cer9-2 seeds could also be fully overcome by the introgression of a second mutation, aba2-1, which is an ABA-deficient locus (Fig. 4D).

**cer9 seeds accumulated high levels of ABA**

Since the cer9 mutant has increased seed dormancy that can be reversed by introducing the aba2-1 mutation, it is likely that cer9 seeds have increased ABA levels. We measured ABA levels in dry seeds of the wild type and the mutant using the Phytodeck immunoassay method. It was found that ABA amounts were notably higher in the cer9 mutant compared with the wild type (Fig 5A). To determine whether higher levels of ABA had caused the delay of seed germination in cer9, we used both pharmacological and genetic methods to test the possibility. Fluridone [1-methyl-3-phenyl-5-(3-trifluoromethylphenyl)-4-(1H)-pyridinone] is an inhibitor of phytoenedesaturase, which catalyzes the desaturation step of phytoene to phytofluene in the carotenoid biosynthesis pathway (Bartels and Watson, 1978). Since carotenoids are the main precursors of ABA in plants (Vranova et al., 2013), fluridone can effectively inhibit ABA biosynthesis. To check whether fluridone could improve the seed germination efficiency of cer9, seeds of Col-0 and cer9-2 were imbibed in water or 100 µM fluridone at 4°C for 48 hr before being placed at room temperature under light for germination. As shown in Figure 5B, the fluridone treatment slightly but significantly enhanced germination of cer9-2 seeds compared with the untreated seeds in the presence of 1 or 2 µM ABA. That the fluridone treatment did increase the germination of cer9 seeds but did not fully reverse the ABA suppression of germination suggested that the delayed germination and the hypersensitivity of cer9 seed germination to ABA is at least partially due to the enhancement of ABA biosynthesis in cer9 seeds.

We also investigated whether the introduced aba2 mutation could decrease the sensitivity of cer9 to ABA. ABA2 encodes a short-chain alcohol dehydrogenase,
which catalyzes the conversion of xanthoxin to abscisic aldehyde in the ABA biosynthesis pathway (Gonzalez-Guzman et al., 2002). Overexpression of ABA2 promotes seed dormancy (Lin et al., 2007) whereas its loss-of-function reduces seed ABA levels and seed dormancy (Nambara et al., 1998). Seeds of wild type, cer9-2, aba2-1, and cer9-2 aba2-1 double mutants were planted on culture media containing increasing concentrations of ABA (Fig 6). In the presence of 0.5 µM ABA, green cotyledon expansion in the wild type was moderately inhibited. Whereas germination of aba2 seeds was not affected by the ABA treatment, green cotyledon expansion in cer9 was severely inhibited. For the cer9-2 aba2-1 double mutant, the size of the green cotyledons is larger than that of the cer9 mutant, but still smaller than those of the wild type and aba2-1. This suggests that the aba2-1 mutation partially rescued the cer9-2 mutant phenotypes, likely by decreasing ABA content and/or ABA sensitivity in the double mutant.

The sensitivity of cer9 seed germination to ABA treatment is not associated with seed coat permeability

The seed coat (testa) structure plays important roles in the breaking of dormancy. The seed coat can serve as a barrier to prevent water uptake, gas exchange and inhibitor leakage from the embryo, and can also supply seed germination inhibitors, restrain radical protrusion and filter light. A number of seed coat or testa mutants have maternally inherited reduced seed dormancy (Debeaujon et al., 2000). Removing the seed coat of GA-deficient mutants could release seeds from dormancy (Debeaujon and Koornneef, 2000). Since CER9 is associated with cuticle biosynthesis and its loss of function causes higher levels of cuticle coating on the epidermal cells of stems and leaves (Lu et al., 2012), we investigated whether the cuticle structure of cer9 mutations was altered and whether the alteration might affect seed coat permeability. As shown in Figure 7A, the cer9-2 seed coat cuticle structure is slightly thicker than that of the wild type. To determine whether the altered seed cuticle is responsible for the dormancy observed in cer9 seeds, the seed coats were perforated with a microknife under a dissection microscope and the treated seeds were placed on plates with
various concentrations of ABA for germination assays. Compared with seeds without perforation, the perforated seeds showed enhanced sensitivity to ABA treatment (Fig. 7B). Nonetheless, the perforated cer9 seeds still showed higher sensitivity to ABA compared with the wild type (Fig. 7B). These data suggest that the permeability of the seed coat or seed coat structure may be not associated with the cer9 germination phenotype.

**iTRAQ experiment reveals that CER9 is a negative regulator of ABA biosynthesis and signaling pathway during seed germination**

Since CER9 encodes a putative E3 ligase and its mutant seeds showed hypersensitivity to ABA, we speculated that CER9 might regulate the degradation of proteins associated with ABA biosynthesis or signaling pathways during seed germination. Consequently, proteomics analyses were conducted to examine the abundance of proteins. Seeds of Col-0 and cer9-2 were stratified at 4°C for 3 days before being treated with 1.0 μM ABA at 22°C for 1 day. The germinating seeds were then harvested for the iTRAQ (isobaric tags for relative and absolute quantitation) analysis. As shown in Supplemental Table S1, a total of 1019 proteins were identified by iTRAQ. Using the Col-0 without ABA treatment as a reference, a total of 114 proteins were significantly regulated based on a signal ratio change ≥ 1.5 upon ABA treatment of the Col-0 seeds. Among these significantly regulated proteins, 83 proteins were up regulated and 31 proteins down regulated. Using the same criterion and relative to those of the Col-0, 168 and 284 proteins were found to be up regulated in cer9-2 seeds without ABA and with ABA treatment, respectively. Similarly relative to those of the Col-0, 75 and 81 proteins were down regulated in cer9-2 seeds without and with ABA treatment, respectively.

We compared three sets of protein data using the Draw Venn Diagram software. Interestingly, 48% of the up-regulated proteins in the ABA-treated wild type were also up regulated in cer9-2 without ABA treatment. Furthermore, 201 more proteins were up regulated in ABA treated cer9-2 than in ABA treated Col-0. Notably, 93.5% of the down-regulated proteins in ABA-treated Col-0 were suppressed in cer9-2 (Fig. 8). In
fact, most of these down regulated proteins were suppressed to even greater extents in cer9-2 without ABA treatment (Supplemental Table S1). The high overlapping expression patterns in cer9-2 and the ABA-treated wild type further demonstrated that the impact of cer9 mutation on protein expression resembles that of ABA treatment during seed germination; this suggests that the regulatory role of CER9 in seed germination is closely related to the ABA response. These results indicate that CER9 is a negative regulator of ABA biosynthesis or the ABA signaling pathway during seed germination.

Among the up and down regulated proteins in cer9-2, certain proteins related to ABA response had similar regulations in cer9-2. These proteins include those of putative ABI3 targets (Monke et al., 2012) and those related to ABA biosynthesis and signaling (Tables I and II). We also noted changes in levels for proteins involved in lipid metabolism. Lipid is an important storage reserve, providing energy for radicle protrusion and seedling growth, and lipid mobilization is affected by ABA treatment during seed germination (Penfield et al., 2006). Previous reports indicate that oleosin proteins are involved in seed germination, enlargement of intracellular oil bodies, and total lipid and protein accumulation (Siloto et al., 2006; Shimada et al., 2008). We found that several seed storage proteins including SES1, SES12, SESA3 and two oleosin proteins were down regulated in the cer9-2 mutant (Table II). These proteins are involved in oil body formation and are also ABI3 putative targets (Monke et al., 2012) (Table II).

Genes involved in ABA biosynthesis or signaling are differentially regulated by CER9

To further understand CER9 regulation of global gene expression during seed germination, we carried out comprehensive transcriptome analyses using the RNA-seq technology. The two seed samples used for iTRAQ analysis, Col-0 and cer9-2, were further employed for RNA-seq analysis. We found that a total of 428 genes were differentially regulated in the cer9 mutant compared with the wild type.
Among them, 143 genes were significantly up regulated and 285 were down regulated in the mutant. An analysis of functional categories using the software DAVID (Huang et al., 2009) revealed that these genes were significantly enriched in several biological processes, including response to abiotic and ABA stimulus, seed development, and plant-type cell wall organization (Fig. 9 and Supplemental Table S2).

Given that the germination of cer9 mutants exhibited an enhanced sensitivity to ABA inhibition, we looked into the expression of genes involved in ABA biosynthesis, catabolism, and signaling. As shown in Table III and Figure 10, NCED6 which is involved in ABA biosynthesis was up regulated in the mutants. ABI3, ABI4 and ABI5 are transcription factors belonging to the B3, APETALA2 (AP2), and basic leucine zipper (bZIP) domain families, respectively. They are major positive regulators of the ABA response that largely determine seed germination or maintenance of dormancy. Our results showed that ABI5 was significantly up regulated (Supplemental Table S2). The expression of ABI5 targeted genes EM1 and EM6 was also increased. RNA-seq revealed that ABI3 and ABI4 were slightly up regulated (Supplemental Table S2), whereas real-time PCR results showed that the expression levels of ABI3 and ABI4 were significantly increased (Fig. 10). In contrast, the expression of genes encoding negative regulators of the ABA signaling pathway, such as ABI2, GCR1, and AHG2, were markedly down regulated (Fig. 10 and Supplemental Table S2). These findings further demonstrate that CER9 significantly affects the ABA biosynthesis and/or its signaling pathway.

Genetic analysis of the regulatory roles of CER9 in the ABA signaling pathway

To establish whether the ABA signaling pathway plays any role in CER9-mediated ABA responses, we crossed cer9-1 (Ler-0 background) with abi1-1 and abi3-1 and also crossed cer9-2 (Col-0 background) with abi5-1 and abi4-103 mutants. ABI1 encodes a protein phosphatase type 2C (PP2C) and is a negative regulator of ABA signaling by inhibiting SnRK2 kinases (Weiner et al., 2010). abi1-1 mutants have largely lost responsiveness to ABA. When this mutation was introduced into the cer9
mutant background, the double mutants resembled the *abi1-1* single mutant in terms of seed germination response to ABA (Fig. 11A, B and Supplemental Fig.S1). This result indicates that the dominant mutation of *abi1-1* could completely block ABA signaling in the *cer9* mutant. The ABI3, ABI4 and ABI5 transcription factors positively regulate the expression of certain ABA responsive genes in seeds (Finkelstein and Lynch, 2000; Lopez-Molina et al., 2002; Penfield et al., 2006). ABI3 acts upstream of ABI5 and both are located downstream of ABI1 (Lopez-Molina et al., 2002). When *abi3-1* was introduced into *cer9-1*, both the germination rate and green cotyledon percentage of *abi3-1 cer9-1* double mutant seeds were similar to those of *abi3-1* under different concentrations of ABA (Figs. 11A and 11B). However, after 7 days germination, the *abi3-1 cer9-1* double mutant grew more slowly than *abi3-1*, but much faster than the wild type (Supplemental Fig.S2). Germination of the *abi5-1 cer9-2* double mutant seeds differed in response to different concentrations of ABA. The germination rate and green cotyledons percentage of the *abi5-1 cer9-2* double mutants were higher than those of *cer9-2*, but were similar to those of the wild type Col-0 and Ws, as well as *abi5-1* in the presence of 0.5 and 1.0 µM ABA (Fig. 11C, D). However, the germination rate of the *abi5-1 cer9-2* double mutants was lower than that of the wild type when the seeds were exposed to a higher concentration of ABA (2.0 and 5.0 µM) (Fig. 11C). After 7 days germination, the *abi5-1 cer9-2* double mutants grew better than *cer9-2* but much worse than *abi5-1* in the presence of 1.0 µM ABA, and had an intermediate phenotype between *abi5-1* and *cer9-2* (Supplemental Fig.S3). In the presence of 5 µM ABA, the *abi5-1 cer9-2* double mutants showed increased sensitivity to ABA (Supplemental Fig.S3). When *abi4-103* was introduced into *cer9-2*, both the germination rate and green cotyledon percentage of the *cer9-2 abi4-103* double mutant seeds were similar to those of *abi4-103* under different concentrations of ABA (Figs. 11E and 11F). However, after 7 days germination, the *cer9-2 abi4-103* double mutant grew slightly slower than *abi4-103* but much faster than the wild type (Supplemental Fig. S4).

DISCUSSION
The *CER9* locus plays important roles in cuticle and wax synthesis as well as leaf transpiration in Arabidopsis (Lu et al., 2012). In this study, we found that CER9 also regulates ABA responses during seed germination and early seedling development. Seeds of the *cer9* mutant showed increased dormancy and hypersensitivity to ABA inhibition of germination and seedling growth. One reason for this is that the *cer9* mutant accumulated a higher level of ABA. Application of fluridone or introduction of the *aba2-1* mutation into the *cer9* background suppressed the delayed seed germination of the *cer9* mutant, indicating that CER9 is likely to be a negative regulator of ABA biosynthesis. ABA levels are determined by de novo biosynthesis, catabolism, or hydrolysis of conjugated ABA. The enzyme 9-cis-epoxycarotenoid dioxygenase (NCED) catalyzes a rate-limiting step in the ABA biosynthesis pathway (Nambara and Marion-Poll, 2005). Interestingly, one of the *NCED* genes, *NCED6*, was expressed at a higher level in *cer9-2* than in the wild type (Supplemental Table S2 and Fig. 10). Previous studies indicated that *NCED6* is highly expressed in the testa and endosperm and its overexpression results in increased ABA levels in seeds and enhanced seed dormancy (Lefebvre et al., 2006; Martinez-Andujar et al., 2011). The up regulation of *NCED6* expression in *cer9* mutant seeds may thus contribute to the higher ABA level and increased seed dormancy of the mutants. ABA catabolism also plays an important role in regulating seed germination. ABA 8'-hydroxylases, including CYP707A1 to CYP707A4, are involved in ABA catabolism. Among them, CYP707A1 and CYP707A2 are required for proper control of seed dormancy and germination in Arabidopsis. Their double mutants accumulate high levels of ABA and display hyper dormancy in seeds (Okamoto et al., 2006). The transcripts for *CYP707A1*, -A2, and -A3 were detected in our RNA-seq analyses, yet there was little difference in transcript levels between *cer9-2* and the wild type (Table S2).

The increased levels of ABA observed in the *cer9* mutant could also result from hydrolysis of glucose-conjugated ABA (ABA-GE). This increased amount of ABA could promote ABA biosynthesis through the auto-regulation of ABA biosynthesis.
genes (Xiong and Zhu, 2003). The enzyme β-glucosidase homolog 1 (BG1) can mediate the hydrolysis of ABA-GE and contribute to the increased ABA level (Lee et al., 2006; Xu et al., 2012). Although our iTRAQ analysis failed to detect BG1 and BG2, two β-glucosidases involved in ABA-GE hydrolysis and four other homologous proteins were detected in the proteome profiling. These include BGLU19, 23, 38 and 44. Interestingly, all of these putative β-glucosidases had higher protein levels in the cer9 mutant than in the wild type (Supplemental Table S1). Among these homologs, BGLU19 shares 71% identity with BG1, although it is unclear whether BGLU19 is also involved in ABA-GE hydrolysis. Further investigation into the role of these β-glucosidases in ABA-GE hydrolysis, and whether or not they are the substrates of CER9, is required.

It should be noted that the increased ABA levels in dry seeds could not fully explain the ABA hypersensitivity phenotypes in seed germination and post-germination growth of the cer9 mutant. Neither fluridone treatment nor aba2-1 mutation could fully suppress the cer9-2 ABA-hypersensitive phenotype (Fig. 5 and Fig. 6). Thus, the cer9 mutant is not simply an ABA over-production mutant. Rather, CER9 may represent a negative regulator of ABA response. While it remains unclear which of the ABA signaling components are likely to be direct targets of CER9, the expression of a number of ABA signaling regulators was significantly affected in the cer9 mutant. The transcript levels for several negative regulators of ABA signaling such as ABI2, GCRI, and AHG2 were markedly down regulated (Fig. 10 and Supplemental Table S2), whereas those encoding positive regulators of ABA response were increased, as discussed below.

The transcription factors ABI3, ABI4 and ABI5 are well known positive regulators of the ABA responses during seed germination (Finkelstein and Lynch, 2000; Lopez-Molina et al., 2002; Penfield et al., 2006). Our study showed that the expression levels of ABI3, ABI4, and ABI5 were significantly increased in the cer9 mutant. Double mutant analysis showed that the abi3-1, abi4-103 and abi5-1 mutations suppressed the ABA-hypersensitive phenotypes of cer9 seeds to different
degrees, suggesting that these regulators may act downstream of CER9 in the ABA response pathways. It has been reported that ABI3 interacts with ABI5, and that ABI5 acts downstream of ABI3 (Lopez-Molina et al., 2002). Our results showed that abi3-1 strongly suppressed the sensitivity of cer9 seeds to ABA, whereas abi5-1 only moderately suppressed it. It is likely that ABI5 homologs may play redundant roles in the ABA signaling pathways and that CER9-mediated ABA signaling may be transduced through both ABI5-dependent and ABI5-independent pathways. This mode of action is similar to that of the AHG3 mutant defective in a protein phosphatase 2C (PP2C), a negative regulator of the ABA response during seed germination. The loss-of-function ahg3 mutant exhibits ABA hypersensitivity during seed germination, and this could nearly be reversed by abi3 mutation, but only partially suppressed by abi5-1 (Yoshida et al., 2006). Interestingly, ahg3 shared some other phenotypes similar to cer9. For example, both mutant seeds contain high levels of ABA. In both cases, the mutation specifically affected seed dormancy without effecting mature plant growth. We checked the expression of AHG3 in RNA-seq and iTRAQ data but could not find this gene (Supplemental Table S1 and Supplemental Table S2), and this may be due to the low abundance of this gene under our experimental conditions. The direct involvement of CER9 in ABA responses in seeds was also demonstrated by the observation that the dominant negative mutation abi1-1 was found to completely repress the ABA hypersensitivity of the cer9 mutant. In fact, the increased ABA level in cer9 may have resulted from its enhanced ABA sensitivity since most of the ABA biosynthesis genes are up-regulated by ABA through the same or similar ABA signaling pathways (Xiong and Zhu, 2003).

One interesting observation is that there appears to be little correlation between the seed transcriptome and proteome either for cer9 or the wild type. For example, RNA-seq data showed that cer9 deficiency down regulated the expression of genes associated with response to ABA, plant cell wall structure, and response to light. However, these genes were not represented in down regulated protein groups. This was similarly the case among the genes/proteins in the up-regulated groups. The low
concordance between proteome and transcriptome has also been reported in previous studies (Wegener et al., 2010; Huang et al., 2013). One explanation may be associated with the different sensitivities of the detection technologies. In our experiments, 18,184 transcripts were detected in the RNA-seq experiments, whereas only 1,019 proteins were detected in the proteomics assays. Clearly, RNA-seq is more robust and sensitive in identifying transcript units even at low levels of expression. In contrast, iTRAQ detected only proteins of the highest abundance. Essentially, proteins of low abundance, such as NCED6, ABI3, ABI4 and ABI5, could barely be detected directly by iTRAQ. Secondly, transcript accumulation and translation may not be coordinated in seeds and during seed germination. It has been well documented in several plant species including Arabidopsis that there is a large number of transcripts stored in dry seeds as well as in imbibed seeds (Rajjou et al., 2012). For example, 12,470 transcripts were detected in Arabidopsis dry seeds, this being similar to the number of transcripts detected in 24 h-imbibed seeds (Nakabayashi et al., 2005). These transcripts may be selectively translated during seed imbibition and germination (Rajjou et al., 2012). In addition, there seems to be a time gap between transcription and translation in that major translation may occur relatively later during germination, while stored proteins could function at the early stage of imbibition (Kimura and Nambara, 2010).

In summary, our genetic analysis combined with transcriptome and proteomics data shows that CER9 is an important determinant of seed germination, and that this regulation is closely associated with the negative regulatory roles of CER9 in ABA biosynthesis and signaling. Future work will need to focus on identification of CER9 substrates in order to reveal the precise way in which CER9 regulates ABA accumulation and signaling in seeds and seedlings.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**
*Arabidopsis thaliana* seeds were obtained from the Arabidopsis Biological Resource Center (ABRC), the European Arabidopsis Stock Centre (NASC), or individual researchers. Unless otherwise stated, seeds were stratified for 3 days at 4°C, and then moved to 22°C with a 16-h-light/8-h-dark cycle and a light intensity of 110–120 mol m⁻² sec⁻¹ for germination and growth.

**Germination assay**

Plants of different genotypes were grown in the same conditions, and seeds were collected at the same time and stored similarly before conducting germination experiments. For germination assays, approximately 30-50 seeds were sown on ½ MS medium supplemented with various concentrations of (±)-cis, trans-abscisic acid (ABA, Sigma-Aldrich; St. Louis, MO, USA). Germination (emergence of radicles) and post-germination growth (green cotyledons appearance) were scored daily for 3 and 7 days, respectively.

**Extraction and quantification of ABA**

Dry seeds were ground in liquid nitrogen and ABA was extracted as described (Pena-Cortes et al., 1989). ABA was quantified with the Phytodetek-ABA kit (Idetek, San Bruno, CA, USA) that uses a monoclonal antibody specific to (+)-ABA.

**Protein extraction, iTRAQ labeling and LC-MS analysis**

Seeds of Col-0 and cer9-2 were first incubated in 1/2 MS liquid medium for 3 days at 4°C, and then incubated in the same medium supplemented with or without 1.0 μM ABA by shaking at 22°C under a 16-h-light/8-h-dark cycle with a light intensity of 110–120 mol m⁻² sec⁻¹ for 1 day. The germinating seeds were ground in liquid nitrogen in a pre-chilled mortar with a pestle. Proteins were extracted with the extraction buffer (50 mM Tris, pH 8, 2 M Urea and 0.5% SDS) supplemented with protease inhibitor (Roche Diagnostics). Protein content was measured using 2D-Quant (GE healthcare). Proteins were precipitated in acetone (sample/acetone: 1/5 by volume) and then re-suspended in SDS-PAGE sample buffer. Protein separation by
SDS-PAGE and in gel digestion were performed as described (Meng et al., 2008). The extracted peptides were labeled with 4plexiTRAQ reagents (ABSciex) as follows: wild type plant treated with mock, wild type treated with ABA, mutant treated with mock and mutant treated with ABA were labeled with the iTRAQ reporters 114, 115, 116 and 117, respectively. The combined peptides were fractionated using strong cation exchange chromatography. LC-MS analysis of the resulting 16 fractions as well as MS data processing was carried out following our published procedure (Zhang et al., 2010). Briefly, each fraction was analyzed twice using LTQ-OrbitrapVelos. The MS spectra were recorded in the Orbitrap whereas MS2 spectra were recorded in the c-TRAP for HCD fragmentation and in the LTQ for the CID fragmentation. Both HCD and CID spectra were extracted individually using Proteome Discoverer software and processed by in-house script before Mascot search against an Arabidopsis protein database TAIR10 (total 35624 sequences). The mascot search results were further processed by Scaffold software for validation of protein identification and quantitative assessment. For protein identification, we reported only proteins with a minimal 99% possibility and containing at least one peptide with a possibility greater than 95%. As a result, the scaffold local FDR is 0.1% for protein identification and 0.7% for peptide identification for our dataset. Protein quantitation was processed using Scaffold Q+ based on i-Tracker (Shadforth et al., 2005). The iTRAQ quantitation using HCD is highly accurate, and a greater than 1.45-fold change could be considered as significantly differential expression and was therefore adopted in this study.

**RNA-seq analysis**

Total RNA was extracted from 1-day light-grown seeds using RNeasy Mini kit (Invitrogen) and DNA was cleaned by DNAse I (NEB). About 2-4 μg of cleaned total RNA was used for RNA-seq analysis as described in Tao et al (2013).

**Quantitative RT-PCR**
Dry seeds, seeds incubated in ½ MS medium after a 3-day stratification at 4°C, and germinated seeds of the wild type and cer9-2 mutants in ½ MS medium with or without 1.0 μM ABA for 1 day were harvested and frozen immediately in liquid nitrogen, and stored at -80°C before RNA extraction. For quantitative RT-PCR (qRT-PCR) analysis, total RNA was extracted from the above samples using an RNeasy® plant mini kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. qRT-PCR reactions were performed in 96-well blocks with an Applied Biosystems 7500 Real-Time PCR system using the SYBR Green I master mix in a volume of 20 µL. The reactions were performed in biological triplicates using RNA samples extracted from three independent plant materials and gene-specific primers (Supplemental Table S2). Data were analyzed using the SDS software (Applied Biosystems version 1.0) as described in Lü et al (2012).

**Genetic analysis of double mutants**

The cer9-1 mutant (Ler-0) was crossed with abil-1 (Ler-0). The cer9-2 mutant (Col-0) was crossed with aba2-1 (Col-0) and abi5-1 (Ws). Double mutants were obtained from the F2 progeny by genotyping using the mutation-specific CAPS markers (Table S3).

**Supplemental materials**

**Supplemental Figure S1.** Sensitivity of the cer9-1 abil-1 double mutant to ABA. Ler-0 (wild type), cer9-1, abil-1 and cer9-1 abil-1 seeds were sown onto ½ MS agar medium supplemented with 0, 1.0, or 5.0 µM ABA. Representative pictures show the morphology of seedlings 7 days after being sown on the medium plates.

**Supplemental Figure S2.** Sensitivity of the cer9-1 abi3-1 double mutant to ABA. Ler-0 (wild type), cer9-1, abi3-1 and cer9-1 abi3-1 seeds were sown onto ½ MS agar medium supplemented with 0, 0.5, or 5.0 µM ABA. Representative pictures show the morphology of seedlings 7 days after being sown on the medium plates.
**Supplemental Figure S3.** Sensitivity of the cer9-2 abi5-1 double mutant to ABA. Col-0 (wild type), Ws (wild type), cer9-2, abi5-1 and cer9-2 abi5-1 seeds were sown onto ½ MS agar medium supplemented with 0, 1.0, or 5.0 µM ABA. Representative pictures show the morphology of seedlings 7 days after being sown on the medium plates.

**Supplemental Figure S4.** Sensitivity of the cer9-2 abi4-103 double mutant to ABA. Col-0 (wild type), cer9-2, abi4-103 and cer9-2 abi4-103 seeds were sown onto ½ MS medium supplemented with 0 or 5.0 µM ABA. Representative pictures showed the morphology of seedlings 7 days after being sown on the media plates.

**Supplemental Table S1.** Results of iTRAQ analysis

**Supplemental Table S2.** Results of RNA-seq analysis

**Supplemental Table S3.** Primers used in this study
ACKNOWLEDGEMENTS

We are grateful to Dr Luis Lopez-Molina (University of Geneva, Switzerland) for providing abi3-I seeds.

LITERATURE CITED


conversion of xanthoxin to abscisic aldehyde. Plant Cell 14: 1833-1846


FIGURE LEGENDS

**Figure 1.** The cer9 mutations increase ABA inhibition of seed germination and post germination growth.

Seeds of cer9-1 (Ler-0 background) and cer9-2 (Col-0 background) along with their wild types were sown onto ½ MS agar medium supplemented with 0, 1.0, or 2.0 µM ABA. Representative images show the morphology of seedlings 7 days after imbibition. Germination (radical emergence) percentages were determined 3 days and green cotyledons were scored 7 days after the imbibition. Data are means and SD from four biological replicates each with at least 30 seeds.

**Figure 2.** Seeds of the cer9 mutants are hypersensitive to ABA over a short development window.

(A) Seeds on ½ MS medium were stratified for one day and were then transferred to ½ MS media supplemented with 0, 1.0 or 5.0 µM ABA. Pictures were taken 6 days after the transfer. (B) Seeds on ½ MS medium stratified for two days were transferred to ½ MS medium supplemented with 0, 1.0 or 5.0 µM ABA. Pictures were taken 5 days after the transfer.

**Figure 3.** Transcript abundance of CER9 and ABI5 during various developmental time periods.

(A) Real-time PCR analysis of CER9 in (a) dry seeds and (b) seeds at the end of 3 days stratification, seeds incubated on ½ MS medium for (c) 1 day or (e) 3 days, or seeds incubated on a ½ MS medium containing 1µM ABA for (d) 1 day or (f) 3 days at 22°C after stratification. (B) Expression of CER9 and ABI5 during seed maturation and imbibition. Public microarray data were processed using BAR HeatMapper to visualize gene expression patterns. Higher expression is indicated by more reddish color. Numbers indicate the relative expression level of the mRNA.
Figure 4. *cer9* mutation increases seed dormancy. (A) Germination rates of Col-0 and *cer9*-2 seeds with different after-ripening times assayed on agar medium 4 days after imbibition. (B) Germination potential of 2-week-old seeds imbibed for 4 days on water-agar medium after different stratification times. (C) Germination potential of seeds imbibed for 4 days on agar medium in the absence or presence of 100 µM GA3. (D) Germination potential of 2-week-old seeds of Col-0, *cer9*-2, *aba2*-1 and *cer9*-2 *aba2*-1 imbibed for 4 days on a water-agar medium. Data are means ± SE of three replicates each with at least 30 seeds.

Figure 5. ABA levels in dry seeds and seed germination rates after the fluridone treatment. (A) ABA levels in dry seeds of Col-0 and *cer9*-2 measured using a Phytodetek-ABA kit. (B) Seeds of Col-0 and *cer9*-2 were immersed in water or 100 µM fluridone at 4°C for 48 hr. The treated seeds were washed 3 times with water and planted on ½ MS agar plates supplemented with 0, 1 or 2 µM ABA. Seed germination was scored after 2 days. Seeds were considered germinated when the radicles emerged. * p value < 0.05. Col-0 (+F) and *cer9*-2 (+F) represent Col-0 and *cer9*-2 with fluridone treatment, respectively.

Figure 6. Sensitivity of the *cer9*-2 *aba2*-1 double mutant to ABA during germination. Seeds of Col-0 (wild type), *cer9*-2, *aba2*-1 and *cer9*-2 *aba2*-1 were sown onto ½ MS medium supplemented with the indicated concentrations of ABA. Representative pictures show the morphology of seedlings 7 days after being sown on the medium plates. Germination (radical emergence) percentages were determined 3 days and green cotyledons 7 days after imbibition. Data are means and SD from four biological replicates each with more than 30 seeds.

Figure 7. The cuticle of seed coat and the sensitivity of perforated seeds to ABA during germination.
(A) Transmission electron micrographs of the cuticle layer of Col-0 (upper panel) and cer9-2 (lower panel) seed coats. The cuticular layer is indicated by the black arrows. Bars = 0.2 μm. (B) Germination rates of perforated seeds under ABA treatment. Seeds of Col-0 and cer9-2 mutant were perforated with a surgery stab knife and then sown on 1/2 MS medium supplemented with 0 or 1.0 μM ABA and incubated at 4°C for 3 days before being moved to 22°C. Seed germination was scored 2 days later. Seeds were considered germinated when the radicles emerged. Col-P and cer9-2-P represent perforated Col-0 and cer9-2 seeds, respectively. Data are the means ± SE of three replicates each with at least 30 seeds.

**Figure 8.** Classification of up- and down-regulated proteins in Col-0_ABA (ABA treated), cer9-2 (no ABA), and cer9-2_ABA (ABA-treated) seeds. Total number of up-regulated proteins (A) or down-regulated proteins (B) identified by iTRAQ analyses of Col-0 and cer9-2 germinating seeds in the presence or absence of 1.0 μM ABA. Relative signal intensities of proteins were calculated by comparing with the control experiment (no ABA treated wild type).

**Figure 9.** Functional categories of differentially expressed genes in Col-0 and cer9-2 seeds. RNA-seq analysis was performed in one-day light grown seeds of Col-0 and cer9-2 mutant. Functional annotations were generated by the DAVID software (Huang et al, 2009a; Huang et al, 2009b). Top 30 of functional annotations ordered by the enrichment scores were selected for the 2-D view, which indicates that genes with differential expression were strikingly enriched (colored green) in certain categories.

**Figure 10.** Relative expression levels of selected ABA response and biosynthesis genes in the cer9-2 mutant. RNA was extracted from seedlings grown in 1/2 MS liquid medium for one day after the end of stratification. Relative RNA levels of the shown genes were analyzed using
gene-specific primers by real-time PCR. Data are means and SD of three biological replicates.

**Figure 11.** Genetic analysis between *cer9-1* or *cer9-2* and *abi1-1*, *abi3-1*, *abi5-1*, or *abi4-103* mutants

Seeds of the wild types (Col-0, Ler-0, and Ws), single mutants (*cer9-1*, *cer9-2*, *abi1-1*, *abi3-1*, *abi5-1*, and *abi4-103*), and double mutants (*cer9-1 abi1-1*, *cer9-1 abi3-1*, *cer9-2 abi5-1*, and *cer9-2 abi4-103*) were sown onto ½ MS medium supplemented with 0, 0.5, 1.0, 2.0 or 5.0 µM ABA. Germination (radical emergence) percentages were determined 3 days and green cotyledons were determined 7 days after imbibition. Data are means and SD from four biological replicates each with at least 30 seeds.
Table I. ABA-related proteins up-regulated in *cer9-2* germinating seeds

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Function</th>
<th>Relative fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Putative ABI3 targets</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT3G22490</td>
<td>LEA domain-containing protein</td>
<td>1.65</td>
</tr>
<tr>
<td>AT1G72100</td>
<td>LEA domain-containing protein</td>
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</tr>
<tr>
<td>AT3G05260</td>
<td>Oxidoreductase</td>
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</tr>
<tr>
<td>AT5G01670</td>
<td>Oxidoreductase</td>
<td>1.50</td>
</tr>
<tr>
<td>AT1G54860</td>
<td>Unknown</td>
<td>1.55</td>
</tr>
<tr>
<td>AT5G22470</td>
<td>Ribosyltransferase</td>
<td>1.60</td>
</tr>
<tr>
<td>AT2G28490</td>
<td>Cupins superfamily protein</td>
<td>1.50</td>
</tr>
<tr>
<td>AT3G21370</td>
<td>BGLU19, beta glucosidase 19</td>
<td>1.50</td>
</tr>
<tr>
<td>AT5G45690</td>
<td>Unknown</td>
<td>1.50</td>
</tr>
<tr>
<td>AT4G26740</td>
<td>ATS1, CLO1, ATPXGI</td>
<td>1.70</td>
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<tr>
<td>AT1G05510</td>
<td>Unknown</td>
<td>1.55</td>
</tr>
<tr>
<td>AT3G15670</td>
<td>LEA family protein</td>
<td>1.65</td>
</tr>
<tr>
<td><strong>Abscisic acid signaling pathway</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT4G35790</td>
<td>Phospholipase D delta</td>
<td>1.50</td>
</tr>
<tr>
<td>AT1G74100</td>
<td>ATSOT16, sulfotransferase 16</td>
<td>1.55</td>
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<tr>
<td><strong>Response to abscisic acid stimulus</strong></td>
<td></td>
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<tr>
<td>AT1G35720</td>
<td>ATOXY5, annexin 1</td>
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<tr>
<td>AT5G02240</td>
<td>Oxidoreductase</td>
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<td>AT5G42950</td>
<td>GYF domain-containing protein</td>
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<td>HD2C, histonedeacetylase 2C</td>
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<td>AT3G60240</td>
<td>EIF4G</td>
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<tr>
<td>AT2G05710</td>
<td>ACO3, aconitase 3</td>
<td>1.50</td>
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</table>

Up-regulated proteins in *cer9-2* that are responsive to ABA based on Gene Ontology analysis or literatures are listed. The values are the means of two technical replicates and are shown in relative fold changes. Relative fold change ≥1.5 indicates significant induction.
Table II. ABA related proteins down-regulated in *cer9-2* germinating seeds that are putative ABI3 targets

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Function</th>
<th>Relative fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT5G07190</td>
<td>ATS3, seed gene 3</td>
<td>0.45</td>
</tr>
<tr>
<td>AT4G27160</td>
<td>SESA3, seed storage albumin 3</td>
<td>0.45</td>
</tr>
<tr>
<td>AT4G28520</td>
<td>CRU3, CRC, cruciferin 3</td>
<td>0.4</td>
</tr>
<tr>
<td>AT3G01570</td>
<td>Oleosin family protein</td>
<td>0.4</td>
</tr>
<tr>
<td>AT3G27660</td>
<td>OLEO4, OLE3, oleosin 4</td>
<td>0.35</td>
</tr>
<tr>
<td>AT5G01300</td>
<td>PEBP</td>
<td>0.3</td>
</tr>
<tr>
<td>AT4G25140</td>
<td>OLEO1, OLE1, oleosin 1</td>
<td>0.3</td>
</tr>
<tr>
<td>AT5G54740</td>
<td>SESA5, seed storage albumin 5</td>
<td>0.3</td>
</tr>
<tr>
<td>AT4G16160</td>
<td>ATOEP16-2</td>
<td>0.25</td>
</tr>
<tr>
<td>AT4G27140</td>
<td>SESA1, seed storage albumin 1</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Down-regulated proteins in *cer9-2* that are responsive to ABA based on Gene Ontology analysis or literatures are listed. The values are the means of two technical replicates and are shown in relative fold changes. Relative fold change <0.6 indicates significant repression.
Table III. Genes exhibiting differential expression in the wild type (Col-0) and cer9-2 mutants in response to ABA treatment. Data are log2 fold change listed from largest to smallest.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene name</th>
<th>Description</th>
<th>Change (log2 fold) (cer9-2/Col-0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT3G24220.1</td>
<td>AtNCED6</td>
<td>9-cis-epoxycarotenoid dioxygenase</td>
<td>ND*</td>
</tr>
<tr>
<td>AT2G31320.1</td>
<td>PARP2</td>
<td>A poly(ADP-ribose) polymerase</td>
<td>3.23</td>
</tr>
<tr>
<td>AT5G52300.1</td>
<td>RD29B</td>
<td>Responsive to dehydration stress</td>
<td>2.71</td>
</tr>
<tr>
<td>AT5G62490.1</td>
<td>AtHVA22B</td>
<td>HVA22 homolog B</td>
<td>2.63</td>
</tr>
<tr>
<td>AT3G51810.1</td>
<td>AtEM1</td>
<td>ABA-inducible protein</td>
<td>2.31</td>
</tr>
<tr>
<td>AT2G40170.1</td>
<td>EM6</td>
<td>Group 1 LEA gene</td>
<td>2.63</td>
</tr>
<tr>
<td>AT3G11050.1</td>
<td>AtFER2</td>
<td>Ferritin 2</td>
<td>1.77</td>
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<tr>
<td>AT1G72770.3</td>
<td>HAB1</td>
<td>Protein Phosphatase 2C</td>
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<td>AT2G36270.1</td>
<td>ABI5</td>
<td>Basic leucine zipper transcription factor</td>
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<tr>
<td>AT1G75750.1</td>
<td>GASA1</td>
<td>GA-responsive GAST1 protein homolog</td>
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<td>AT5G44120.3</td>
<td>CRU1</td>
<td>12S seed storage protein</td>
<td>-1.13</td>
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<td>AT4G38620.1</td>
<td>ATMYB4</td>
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<td>AT1G12420.1</td>
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<td>ACT domain repeat 8</td>
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<tr>
<td>AT1G48270.1</td>
<td>GCR1</td>
<td>G-protein-coupled receptor</td>
<td>-2.03</td>
</tr>
<tr>
<td>AT1G48130.1</td>
<td>AtPER1</td>
<td>A protein similar to the 1-cysteine (1-Cys)</td>
<td>-2.20</td>
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<tr>
<td>AT1G55870.1</td>
<td>AHG2</td>
<td>Poly(A)-specific ribonuclease</td>
<td>-2.25</td>
</tr>
<tr>
<td>AT2G26980.2</td>
<td>CIPK3</td>
<td>Serine-threonine protein kinase</td>
<td>-2.93</td>
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<tr>
<td>AT2G47770.1</td>
<td>AtTSPO</td>
<td>Membrane-bound protein</td>
<td>-3.88</td>
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<tr>
<td>AT5G57050.1</td>
<td>ABI2</td>
<td>Protein phosphatase 2C</td>
<td>ND**</td>
</tr>
</tbody>
</table>

ND*, transcripts not detected in Col-0; ND**, transcripts not detected in cer9-2.
corresponding gene-term association positively reported

- response to abiotic stimulus
- response to abscisic acid stimulus
- seed development
- fruit development
- post-embryonic development
- reproductive structure development
- plant-type cell wall organization
- embryonic development ending in seed dormancy
- reproductive developmental process
- response to temperature stimulus
- response to cold
- superoxide metabolic process
- carboxylic acid biosynthetic process
- organic acid biosynthetic process
- cell cycle checkpoint
- response to reactive oxygen species
- response to hormone stimulus
- cell cycle
- hyperosmotic salinity response
- response to radiation
- response to endogenous stimulus
- hyperosmotic response
- fatty acid biosynthetic process
- removal of superoxide radicals
- response to light stimulus
- response to osmotic stress
- root system development
- root development
- response to salt stress
- response to organic substance

corresponding gene-term association not reported yet

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