CHOTTO1, a Double AP2 Domain Protein of Arabidopsis thaliana, Regulates Germination and Seedling Growth Under Excess Supply of Glucose and Nitrate

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Arabidopsis chotto1 (cho1) mutants show resistance to (−)-R-ABA, an ABA analog, during germination and seedling growth. Here, we report cloning and characterization of the CHO1 gene. cho1 mutants showed only subtle resistance to (+)-S-ABA during germination. The cho1 mutation acts as a strong enhancer of the abi5 mutant, whereas the cho1 abi4 double mutant showed ABA resistance similar to the abi4 single mutant. This suggests that CHO1 and ABI4, but not ABI5, act in the same genetic pathway. Map-based cloning revealed that the CHO1 gene encodes a putative transcription factor containing double AP2 domains. The CHO1 gene was expressed predominantly in seed, with the strongest expression in imbibed seed. Induction of CHO1 expression was observed 4h after seed imbibition and reached a maximum level at 24h. Induction of CHO1 expression did not occur in the abi4 mutants, indicating that this is an ABI4-dependent process. Microarray experiments showed that a large number of genes involved in primary metabolism and the stress response were up-regulated in the cho1 mutant. Growth of abi4 and cho1 mutant seedlings was resistant to high concentrations of glucose. In addition, growth of cho1 mutant seedlings was partially resistant to excess nitrate (50 mM), as evident from their expanded green cotyledons. However, their growth was normal under moderate nitrate concentrations (< 10 mM). This nitrate response was specific to the cho1 mutants and was not observed in the abi4 mutants. Taken together, our results indicate that CHO1 regulates nutritional responses downstream of ABI4 during germination and seedling growth.

Keywords: ABA • Arabidopsis • Double AP2 protein • Germination • Nitrate • Sugar.

Abbreviations: ABRE, ABA-responsive element; ANT, ANTEGUMENTA; AP2, APETALA2; BAC, bacterial artificial chromosome; CE1, coupling element1; CHO1, CHOTTO1; GO, gene ontology; MatDB, MIPS Arabidopsis thaliana Database; PLT, PLETHORA; QRT–PCR, quantitative reverse transcription–PCR; TAIR, The Arabidopsis Information Resource; VP1, Viviparous1; WRI1, WRINKLED1.

Introduction

Germination and subsequent seedling growth are regulated by environmental factors such as nutrients, light and temperature, in addition to endogenous signals such as plant hormones (Bewley 1997). In spite of the increasing knowledge of the individual signaling cascades and developmental regulation (Koornneef et al. 2002), the mechanism by which multiple signals coordinate seed physiology remains largely unknown.

ABA is a plant hormone that plays a pivotal role in numerous physiological processes, including seed dormancy and germination (Finkelstein et al. 2002, Nambara and Marion-Poll 2005). Molecular genetic approaches have elucidated that Arabidopsis ABI3, ABI4 and ABI5 genes encode key components of ABA-mediated transcription in seeds (Giraudat et al. 1992, Finkelstein et al. 1998, Finkelstein and Lynch 2000, Lopez-Molina and Chua 2000). The ABI3 gene encodes an ortholog of the maize transcription factor Viviparous1 (VP1),
which contains a B3 DNA-binding domain. VP1/ABI3 binds to the seed-specific enhancer 5Ph/RY element in vitro, and plays a role in linking hormonal and developmental programs (Suzuki et al. 1997). The ABI4 and ABI5 genes encode transcription factors that contain AP2-type and bZIP-type DNA-binding domains, respectively (Finkelstein et al. 1998, Finkelstein and Lynch 2000, Lopez-Molina and Chua 2000). A subset of bZIP transcription factors, including ABI5, bind to typical ABA-responsive elements (ABREs) to activate ABRE-mediated transcription (Jakoby et al. 2002). The maize ABI4 ortholog, ZmABI4, has been shown to bind to the coupling element1 (CE1), a cis-element acting cooperatively with ABRE (Niu et al. 2002). However, physical interaction between ABI4 and other ABIs has not been observed in vitro yeast two-hybrid assays (Nakamura et al. 2001).

Nitrate is a major source of nitrogen (N) in soil and also acts as a signal to regulate plant growth and development (Stitt 1999, Forde 2002). Plants incorporate nitrate through both high-affinity and low-affinity nitrate transporters that adjust to a wide range of external concentrations. Incorporated nitrate is transported through the xylem and assimilated or stored in vacuoles. Exogenous nitrate concentrations affect plant growth and development, such as carbon (C) metabolism, shoot:root growth ratio (Scheible et al. 1997b, Stitt 1999) and seed germination (Hilhorst and Karssen 1989). Nitrate induces a rapid change in the gene expression profiles of C:N metabolism genes (Scheible et al. 2004, Wang et al. 2004). This response occurs even in nitrate reductase-deficient mutants, indicating that nitrate-derived signals include non-assimilated nitrate (Scheible et al. 1997a, Wang et al. 2004). The nitrate-mediated gene expression profiles depend on a range of concentrations, the organ and the period of exposure (Forde 2002). A MADS-box transcription factor, ANR1, regulates lateral root growth positively in response to an optimal concentration of locally applied nitrate (Zhang and Forde 1998). However, ANR1 does not affect lateral root growth under excessively high concentrations of nitrate. Accordingly, plants appear to utilize different regulatory mechanisms in response to low and high concentrations of nitrate for adaptation to a wide range of external nitrate concentrations.

ABA signaling pathway(s) have been reported to interact with nutrient signaling cascades (Gibson 2004). Application of N and C sources in the medium alters ABA sensitivity (GarciaRubio et al. 1997). Isolation of a number of aba and abi4 alleles from sugar-insensitive mutant screening highlights the prominent role of ABI4 in sugar signaling (Finkelstein and Gibson 2001, Gazzarrini and McCourt 2001, León and Sheen 2003). Sugar-insensitive phenotypes are characteristic of abi4 mutants, while other abi mutants show only weak phenotypes. ABI4 has also been shown to regulate lateral root growth negatively under excessively high nitrate conditions (Signora et al. 2001). Regardless of the strong correlation of ABI4 with nutritional responses, the mechanism by which ABI4 influences metabolic signaling remains largely unknown.

To dissect multiple ABA response pathways in seeds genetically, we exploited the properties of (+)-R-ABA, an analog of naturally occurring (+)-S-ABA (Nambara et al. 2002). Our previous study has indicated that (−)-R-ABA partially discriminates the ABI4-dependent ABA response pathway relative to other ABA response pathways. Genetic screening for mutants sensitized to the ABI4 pathway has enabled us to identify novel genetic factors that act on this pathway. In this study, we demonstrate that the CHO1 gene encodes a double AP2 transcription factor and acts downstream of ABI4. We also found that the cho1 mutants alter sugar and nitrate responses during germination and seedling growth. The role of CHO1 in the ABA, sugar and nitrate signaling network will be discussed.

## Results

### Germination phenotypes of cho1 seeds

Previously, we reported the isolation of Arabidopsis thaliana mutants that are able to germinate and initiate seedling growth in the presence of 10 µM (−)-R-ABA (Nambara et al. 2002). The (−)-R-ABA-insensitive mutant collections contained a high proportion of abi4 alleles (11 abi4 alleles among 24 mutants) compared with the (+)-S-ABA-insensitive mutant population (four abi4 alleles among 18 mutants) (data not shown). This finding indicates that (−)-R-ABA screening might discriminate, at least in part, ABA signaling components acting on the ABI4 pathway. In addition, we identified four new (−)-R-ABA-insensitive mutants, which fell into two new loci. Three alleles correspond to the chotto1 (cho1) gene and one allele to the chotto2 (cho2) gene. In this study, we focused on characterizing the cho1 mutants.

The cho1 seeds showed only weak resistance to exogenously applied (+)-S-ABA during seed germination. In the presence of 1 µM (+)-S-ABA, cho1 seeds germinated (radicle emerged) slightly faster than wild-type seeds; however, germination was slower compared with rates for seeds from the abi4 and abi5 mutants (Fig. 1A). The significant effect of the cho1 mutations on ABA responses was observed under abi5 mutant backgrounds. The cho1 mutations act as a strong enhancer of the null abi5 allele, abi5-7 (Fig. 1B, see Materials and Methods). In contrast, abi4 cho1 double mutant seeds germinated at rates comparable to those of the abi4-5 mutant seeds in the presence of exogenous (+)-S-ABA, suggesting that CHO1 acts in the same pathway as ABI4.

Germination of cho1 seeds was faster than that of wild-type seeds when freshly harvested seeds were sown without stratification. Once seeds were stratified or after-ripened, cho1 and wild-type seeds germinated at similar rates under normal conditions (without exogenous ABA), thus indicating that...
cho1 seeds have reduced primary seed dormancy. Nitrate application to the medium accelerates germination of Arabidopsis seeds (Ali-Rachedi et al. 2004, Alboresi et al. 2005). Therefore, we investigated the effect of KNO₃ on the germination rates of the wild type and the cho1-3 mutant (Fig. 1C). As anticipated, concentrations of KNO₃ up to 10 mM accelerated the germination rate of both wild-type and cho1-3 mutant seeds. Conversely, application of KNO₃ at 50 mM retarded germination of wild-type seeds. The inhibitory effect of 50 mM KNO₃ on seed germination was less pronounced in the cho1-3 mutant.

Map-based cloning of CHO1

We performed map-based cloning of CHO1, and fine mapping of the cho1-1 mutation revealed that CHO1 was located within a 58,500 bp region between the markers MJB24 and MUA2 (see Materials and Methods). Southern blot analysis was performed using the fast-neutron allele cho1-3 in order to identify polymorphisms. Out of 14 annotated genes in this region, one polymorphism was found. Sequencing of At5g57390 in the cho1-3 mutant identified the insertion of a 1,399 bp DNA fragment from chromosome 3 (corresponding to the bacterial artificial chromosome (BAC) clone T10K17) with a 42 bp deletion proximal to the insertion (data not shown). We also found point mutations within this gene in the cho1-1 and cho1-2 mutants. From these results we concluded that At5g57390 is the CHO1 gene.

The CHO1 gene encodes a putative transcription factor that contains double AP2 domains (Okamura et al. 1997). This gene is identical to the previously reported AINTEGUMEATA-like 5 (AIL5) (Nole-Wilson et al. 2005). Arabidopsis contains 144 AP2 domain genes, which are divided into five subfamilies (Riechmann and Meyerowitz 1998, Sakuma et al. 2002). The AP2 subfamily contains two AP2 domains connected by a conserved linker sequence (Sakuma et al. 2002). Worth mentioning in this context is that ABI4, which belongs to the EREBP subfamily and contains only a single AP2 domain, is structurally less related to CHO1. Fourteen Arabidopsis genes belong to the AP2 subfamily that includes APETALA2 (AP2) (Jofuku et al. 1994), AINTEGUMENTA

Seeds of the wild type, cho1-1, cho1-2 and cho1-3 did not germinate in this condition. abi4-5, open triangle; abi5-7, open square; cho1-1abi4-5, light gray triangle; cho1-2abi4-5, dark gray triangle; cho1-3abi4-5, filled triangle; cho1-1abi5-7, light gray square; cho1-2abi5-7, dark gray square; cho1-3abi5-7, filled square. (C) The percentage germination of freshly harvested wild-type and cho1-3 mutant seeds in the presence of nitrate. Seeds were sown on 0.8% agar medium (pH 5.7) supplemented with the indicated concentrations of KNO₃ and incubated under continuous light at room temperature. Triplicate experiments were performed, and an average with standard error is shown. The KNO₃ concentration is indicated in parentheses. Open circle, wild type (0 mM); open triangle, wild type (10 mM); open square, wild type (50 mM); filled circle, cho1-1 (0 mM); filled triangle, cho1-2 (10 mM); filled square, cho1-3 (50 mM).
ANT (Klucher et al. 1996), BABY BOOM (AtBBM) (Boutilier et al. 2002) and PLETHORA (PLT) (Aida et al. 2004). WRINKLED1 (WRI1) encodes a double AP2 protein, which plays a key role in primary C metabolism in seeds (Focks and Benning 1998). Interestingly, wri1 mutants are hypersensitive to ABA and sugars during germination and seedling establishment (Masaki et al. 2005, Cernac et al. 2006). The cho1-1 and cho1-2 mutations substituted a conserved alanine residue for threonine at the second and first AP2 domains, respectively (Fig. 2A). The cho1-1 mutation is equivalent in position to the ant-8 mutation that is thought to destabilize folding of the AP2 domain (Krizek 2003).

CHO1 is expressed during seed development and germination

Quantitative reverse transcription–PCR (QRT–PCR) was conducted to investigate whether the CHO1 mRNA levels reflect the phenotypes that are restricted in seed germination and seedling growth. Nole-Wilson et al. (2005) reported that this gene is expressed weakly throughout various organs, including meristems of the inflorescence and flowers. Our expression analysis indicated that the CHO1 gene is strongly induced in late embryogenesis and imbibed seeds. As shown in Fig. 2B, the CHO1 mRNA was most abundantly expressed in imbibed seeds. Kinetic expression analysis showed that the level of CHO1 mRNA began to increase within 4 h after seed imbibition, reached a maximum level after 24 h, and gradually decreased thereafter (Fig. 2C).

We then examined if the expression pattern of CHO1 is altered in other ABA-insensitive mutants. Induction of CHO1 expression was observed similarly in the abi3-8, abi5-7 and cho2-1 mutants (Fig. 2D). Induction of CHO1 expression was ABI4 dependent because the abi4-11 mutant failed to induce CHO1 expression after imbibition (Fig. 2D). Our QRT–PCR expression analysis showed that expression of CHO1 was not remarkably affected by application of exogenous ABA or nitrate (data not shown).

Transcriptome analysis of the cho1 mutant during seed germination

To investigate the role of CHO1 in seed germination and seedling growth, microarray analysis was performed using dry seed, and 24 h- and 48 h-imbibed seeds of the wild type and the cho1-3 mutant. The cho1 phenotype was found in...
Fig. 3 Transcriptome analysis of the cho1-3 mutant. (A) Metabolic gene expression in 24 h-imibed cho1-3 seeds supplemented with 25 mM KNO₃. Two independent microarray experiments were performed. At 24 h after imbibition, up-regulated (top) and down-regulated (bottom) genes whose expression levels changed in the mutant by >2-fold in duplicate experiments are listed. Each block represents the fold change in expression levels during a course of seed germination. Blocks that indicate changes at 24 h after imbibition are highlighted by green.

Up-regulated genes in the mutant were analyzed ontologically by using the MIPS database (http://mips.gsf.de/proj/funcatDB/search_main_frame.html). Up-regulated genes at 24 h (92 genes) were different from those up-regulated at 48 h (57 genes), and only three genes from both time points were shared in common (At1g04660, At1g47400 and At3g25050). These genes were significantly over-represented in GO categories corresponding to ‘cellular rescue and defense’ genes for oxidative stress responses, such as peroxidases, and enzymes involved in programmed cell death. Over-representation was found in the functional categories of cellular rescue and defense (P = 6.19e-07 at 24 h, 1.76e-03 at 48 h), interaction of the organism with the environment (2.69e-04 at 24 h, 1.51e-02 at 48 h), systemic interaction with the environment (3.64e-2 at 24 h, 6.91e-04 at 48 h), C compound and carbohydrate metabolism (2.45e-03 at 24 h, 4.21e-02 at 48 h) and lipid/fatty acid transport (7.51e-03 at 24 h, 1.86e-03 at 48 h). The GO category corresponding to ‘cellular rescue and defense’ genes for cho1-3 at 24 h was enriched with genes mostly involved in oxidative stress responses, such as peroxidases, and enzymes involved in programmed cell death.
for glutathione conjugation. In contrast, the same GO category at 48 h was enriched with abiotic and biotic stress response genes (Supplementary data). Importantly, we found that most of the genes up-regulated in cho1-3 at 24 h were highly induced in wild-type seedlings at 48 h (Fig. 3A), indicating that up-regulation of the above-mentioned GO categories and genes is due to precocious expression of genes involved in seedling establishment in the cho1-3 mutant. In agreement with this finding, metabolic genes up-regulated in the cho1-3 mutant encode key enzymes for sugar and amino acid metabolism (Fig. 3A). The sugar metabolism-related up-regulated genes in the mutant at 24 h included those for vacuolar invertase—Atbfructo4 and GDP-D-mannose-4,6-dehydratase (GMD1); wax-fatty acid elongase (KCS1), CER1 and CER2; and non-specific lipid transfer proteins. At 48 h, up-regulated genes in the cho1-3 mutant included those
involved in the metabolism of organic acids (isocitrate lyase), amino acid metabolism genes including serine O-acetyltransferase (AtSerat2;1) and glycine cleavage system H protein (Fig. 3B). In addition, three cysteine protease genes were up-regulated at 24 h in the cho1-3 mutant. This observation suggests that the cho1-3 mutation alters the flow of sugar and amino acid metabolism.

**cho1 seedlings are able to grow under excess glucose or nitrate conditions**

Previous studies have shown that abi4 mutants are insensitive to high concentrations of sugars during seedling development (Finkelstein and Gibson 2001, Gazzarrini and McCourt 2001, León and Sheen 2003). Therefore, we examined whether the cho1 mutant also displays a similar phenotype to the abi4 mutant. The cho1 mutants were able to grow under a high concentration of glucose (5%) (Fig. 4A). We also found that cho1 mutants were able to grow under excessively high concentrations of KNO₃, which are normally inhibitive to growth (Fig. 4B). The cho1 mutant responded to growth-permitting concentrations of nitrate (≤10 mM) similarly to the wild type (Fig. 4C). The abi4 mutants did not display resistance to excess nitrate in the clear manner that cho1 mutants did; however, the abi4 mutants were also slightly more resistant to excess nitrate than the wild type. The cho1 mutants showed normal growth, even when KNO₃ was substituted by NH₄NO₃ but not when substituted by NH₄Cl or KCl (Fig. 4D). KNO₃ accelerated growth of both wild type and cho1 mutants at concentrations up to 10 mM. Higher concentrations of KNO₃ in the medium caused growth inhibition of the wild type. In contrast, cho1 mutants produced expanded green cotyledons under high nitrate conditions, indicating normal aerial growth (Fig. 4B, C). It is important to note that resistance of cho1 mutants to high nitrate conditions was restricted to cotyledon growth, as growth of the hypocotyls and roots was inhibited similarly to the wild type (Fig. 4B).

**Discussion**

**CHO1 is a growth regulator that regulates germination and seedling establishment**

Genetic analysis showed that the CHO1 gene encodes a putative double AP2 transcription factor (Fig. 2A). The expression of CHO1 was highly restricted in seeds, especially in imbibed seeds (Fig. 2B). The cho1 mutants exhibited defects in seed dormancy, ABA-mediated inhibition of germination (Fig. 1) and also in seedling growth under high sugar and nitrate conditions (Fig. 4A, B). The phenotypes of cho1 mutants resembled those of weak abi4 mutants. However, cho1 and abi4 mutants showed different phenotypes when seedlings were grown under high nitrate conditions. Under these conditions, cho1 mutant seedlings were resistant to high nitrate concentrations. In contrast, abi4 mutants showed inhibited growth that was similar to wild-type growth under the same conditions (Fig. 4B). Taken together, our study indicates that CHO1 is a growth regulator downstream of hormonal and nutritional signaling and plays a role in the control of seed dormancy and germination.

Out of 14 double AP2 transcription factors in Arabidopsis, the majority of those characterized to date play a pivotal role in developmental processes, such as establishment of organ and cell type identity (Jofuku et al. 1994, Klucher et al. 1996, Aida et al. 2004), phase transition (Aukerman and Sakai 2003) and as a developmental switch for primary metabolism (Focks and Benning 1998). AIL5/CHO1 is most closely related to PLT1 and PLT2, which are implicated in establishment and maintenance of the identity of root stem cells, and mitotic activities of the stem cell daughters (Galinha et al. 2007). PLT3 and AtBBM are more distally related to PLT1 and PLT2 than AIL5/CHO1, but have similar functions as master regulators of root stem cells (Galinha et al. 2007). It is also the case that ANT and AP2 are distally related among 14 double AP2 genes, but they have similar functions in establishing the petal identity via negatively regulating the AGAMOUS gene (Krizek et al. 2000). Ectopic expression of AIL5/CHO1 resulted in the production of larger flowers than those of the wild type, similar to the ectopic expression of ANT (Nole-Wilson et al. 2005). It remains unknown if AIL5/CHO1 is involved in the processes regulated by PLTs or by ANT. Nonetheless, highly abundant AIL5/CHO1 mRNAs in imbibed seeds suggest that a major function of this gene is to regulate germination and seedling growth. The mutant phenotypes also support this notion.

**CHO1 acts downstream of ABI4 during seed germination and seedling growth**

ABI4 is known to be involved in both ABA and sugar responses; however, it is yet to be determined how ABI4 (or the ABI4 pathway) coordinates and reflects hormonal and nutritional signals during growth. Several lines of evidence in this study showed that CHO1 acts downstream of ABI4 to regulate rates of germination and seedling growth. This notion is supported by the similarity of the cho1 and abi4 mutant phenotypes (Nambara et al. 2002, Fig. 4A) and double mutant analyses. The ABA insensitivity of the abi4 cho1 double mutants was comparable with that of abi4 mutants, whereas the cho1 mutations act as strong enhancers of the abi5 mutant (Fig. 1B). These results suggest that CHO1 acts in the ABI4 pathway, which is suitably distinct from the ABI5 pathway. Further evidence for this comes from our finding that imbibition-induced CHO1 expression required ABI4 (Fig. 2D).

The maintenance of seed dormancy requires de novo ABA synthesis in imbibed seeds (Debeaujon and Koornneef
However, the involvement of ABA after seed imbibition is poorly described compared with its role in seed development. Previous studies have shown that expression of ABI4 and CHO1 is induced both during seed development and after seed imbibition (Arroyo et al. 2003; Fig. 2B, C), whereas ABI5 induction is not observed after seed imbibition unless exogenous ABA is applied (Lopez-Molina et al. 2001). Brocard-Gifford et al. (2003) reported that the defect of the abi5 mutant was more prominent than that of abi4 mutants in inhibiting precocious germination during seed development. This work also reported that the abi4 mutants showed a defect in germination more prominently than abi5 mutants when mature seeds were analyzed. ABI4 and CHO1 play dual functions both in seed development and after imbibition, and might play a more prominent role in imbibition responses than ABI5. Therefore, it is likely that the key components of ABA responses play distinct roles in these developmental stages. The mechanisms of ABA response have been studied primarily in seed development. Elucidation of CHO1 functions in this study provides a novel insight into the ABA response after imbibition. ABI4-dependent CHO1 induction after imbibition is possibly a previously missing component that might explain how seed alters the ABA responsiveness after imbibition.

Multiple nutritional signaling pathways interact with distinct ABA signaling pathways

Our results indicate that sugar and nitrate signaling pathways interact differentially with the ABA pathway (Fig. 5). ABI4 is involved in glucose signaling, and therefore operates the downstream target CHO1. As a result, both abi4 and cho1 mutants exhibit growth resistance to high concentrations of glucose. In contrast, growth inhibition caused by excess nitrate requires CHO1, but not ABI4 (Fig. 5). Growth of cho1 mutants on ammonium was similar to that of the wild type (Fig. 4D), indicating that CHO1 functions specifically in high nitrate responses.

Plants respond differentially to different concentrations of nitrate. Nitrate up to 10 mM accelerates plant growth, whereas high nitrate concentrations have inhibitory effects. CHO1 regulates germination and seedling growth in response to excess nitrate, but not to growth-promoting concentrations of nitrate. A MADS-box transcription factor, ANR1, is a positive growth regulator of lateral root elongation in response to adequate concentrations of nitrate. Interestingly, ANR1 is not involved in growth inhibition caused by excess KNO3 (Zhang and Forde 1998). Coruzzi and Zhou (2001) argued that C:N sensing in plants exhibits a ‘matrix effect’ in which downstream responses are dependent on developmental and environmental conditions. Based on this notion, CHO1 is probably a component of the ‘matrix effect’ that acts as a node of ABA, sugar and high NO3− responses during germination and seedling growth.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana mutants (Columbia accession) used in this study are described elsewhere (Nambara et al. 2002). The abi4-5 mutant contains a point mutation that substitutes G56 by D, whereas the abi5-7 mutation causes a premature stop codon at W75 in the ABI5 gene (data not shown). Wild-type and mutant plants were grown under continuous light at 22°C. To isolate total RNA, various organs (except for roots) were harvested from 6-week-old plants grown in pots containing a 1:2 mixture of vermiculite:Jiffy mix (Sakatonotane, Yokohama, Japan). To harvest roots, plants were grown for 2 weeks on 0.8% agar plates containing 1/2 MS salts. For gene expression analyses, seeds were sown and imbibed on filter papers moistened with water that contained the indicated supplements.

Germination assay

Surface-sterilized seeds were sown on 0.8% agar medium supplemented with 1/2 MS salts, the indicated concentrations of the hormone and 5 mM MES buffer (pH 5.7) as described previously (Nambara et al. 2002). Seeds were stratified for 3 d and transferred to continuous light conditions at room temperature, unless they were used for dormancy assays where stratification was omitted. Radicle emergence was used as a criterion of seed germination. Germination was scored at the indicated time points using a dissecting
microscope. Germination tests were performed in at least triplicate experiments using > 50 seeds for an individual assay. The average germination percentage ± SE of triplicate experiments was calculated.

Chlorophyll measurement
Surface-sterilized seeds were sown on 1% agarose supplemented with 5 mM MES buffer (pH 5.7) and nutrients as indicated. Seeds were incubated under continuous light at room temperature after stratification for 3 d. Four-day-old seedlings were used for the extraction and measurement of chlorophylls as described by Nakabayashi et al. (1999).

Map-based cloning
Fine mapping was performed using Cereon single nucleotide polymorphisms (SNPs). The CHO1 region was narrowed down between the markers MJB24 (CER437922) and MUA2 (CER440468) after PCR-based genotyping of the 640 F2 lines.

Quantitative reverse transcription–PCR
Total RNA from dry and imbibed seeds was prepared using RNAqueous columns with the Plant RNA isolation aid (Ambion, Austin, TX, USA) according to the manufacturer’s instruction. Procedures for total RNA isolation from siliques were carried out according to Nambara et al. (1992). To isolate total RNA from other tissues, TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) was used. Procedures for the first-strand cDNA synthesis and QRT–PCR with Taq-Man technology were performed as described by Kushiro et al. (2004).

Microarray analysis
RNA extraction, purification and microarray analysis were performed as described previously by Kushiro et al. (2004). Briefly, total RNA was extracted from dry or imbibed seeds using RNAqueous columns (Ambion). Duplicate microarray analysis using independent seed batches was performed using the GeneChip Arabidopsis ATH1 Genome Array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer’s protocol.

Data analysis
Values for signal intensity and detection call were obtained using Microarray Suite 5.0 software (MAS; Affymetrix). The microarray data were further analyzed using Microsoft Excel and GeneSpring software version 4.5 (Silicongenetics, Redwood, CA, USA). First, the genes judged by MAS as ‘Present’ in either the wild type or the mutant were selected for further analysis of up-regulated and down-regulated genes in the mutant. The data sets from wild-type and mutant seed batches harvested at the same time were used to extract genes whose expression levels changed > 2-fold.

Ontological analysis was performed using MIPS Arabidopsis thaliana Database (MATDB, http://mips.gsf.de/proj/thal/db/index.html) as described elsewhere (Nakabayashi et al. 2005). Metabolic genes presented in Fig. 3A and B were obtained from gene sets analyzed using Gene Ontology Annotations (GOA) Arabidopsis version 6.0at The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org/tools/bulk/go/index.jsp). Genes categorized into ‘hydrolase activity’, ‘transferase activity’ and ‘other metabolic activity’ were extracted, and modified manually by omitting those whose substrates are possibly proteins or macromolecules.

Supplementary data
Supplementary data are available at PCP online.

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References


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