Arabidopsis *CPR5* Independently Regulates Seed Germination and Postgermination Arrest of Development through LOX Pathway and ABA Signaling

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Abstract

The phytohormone abscisic acid (ABA) and the lipoxygenases (LOXs) pathway play important roles in seed germination and seedling growth and development. Here, we reported on the functional characterization of *Arabidopsis CPR5* in the ABA signaling and LOX pathways. The *cpr5* mutant was hypersensitive to ABA in the seed germination, cotyledon greening and root growth, whereas transgenic plants overexpressing *CPR5* were insensitive. Genetic analysis demonstrated that *CPR5* gene may be located downstream of the *ABI1* in the ABA signaling pathway. However, the *cpr5* mutant showed an ABA independent drought-resistant phenotype. It was also found that the *cpr5* mutant was hypersensitive to NDGA and NDGA treatment aggravated the ABA-induced delay in the seed germination and cotyledon greening. Taken together, these results suggest that the *CPR5* plays a regulatory role in the regulation of seed germination and early seedling growth through ABA and LOX pathways independently.

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Introduction

Seed germination is the first adaptive decision in the developments of many land plants. Advances in genetics and molecular physiology have taught us much about the control of germination by the phytohormone abscisic acid (ABA) using the model plant *Arabidopsis thaliana*. For example, ABA helps maintain seed dormancy to ensure that seed germinate under favorable conditions. Immediately after germination, ABA may inhibit the establishment and subsequent development of young seedlings, with this post-germinative arrest representing an early developmental checkpoint to slow seedling growth until better conditions arise [1–3].

The phytohormone abscisic acid (ABA) is well known for its regulatory roles in integrating environmental constraints with the developmental programs of plant [2–10]. ABA-regulated processes of plant development are generally divided into two broad and overlapping categories: ABA signaling in seeds (maintenance of seed dormancy and control of early seedling development) and ABA signaling in guard cells of more mature plants [11]. Molecular genetics studies have significantly advanced our understanding on the molecular basis of ABA signaling in seeds and seedlings. Notably, through the characterizations of a series of ABA-insensitive mutants, which are resistant to ABA mediated inhibition of germination and/or post-germinative growth, several important components regulating ABA signaling in seeds and/or guard cells have been identified in *Arabidopsis thaliana* [2,3,7,12]. Among them, ABI1 [13] and ABI2 [14] are protein phosphatases that negatively regulate ABA signaling during seed dormancy and germination. These phosphatases were also shown to be involved in ABA-mediated guard cell signaling as well [15]. In contrast, the ABI transcription factors including ABI3, ABI4 and ABI5 act positively to regulate ABA signaling in seeds [2,16,17,18].

In plants, products of the lipoxygenases (LOXs) pathway have showed diverse functions involved in abiotic stress [19]. However, some results have suggested that LOXs play important roles in seed germination and seedling growth and development [20,21]. Lipoxygenases are non-heme iron-containing dioxygenases widely distributed in plants and animals. LOX catalyzes the addition of molecular oxygen to polyunsaturated fatty acids containing a \( \text{Z,Z} \)-1,4-pentadiene system to produce an unsaturated fatty acid hydroperoxide, and initiates the synthesis of a group of acyclic or cyclic compounds collectively called oxylipins, which are products of fatty acid oxidation, with diverse functions in the cell. Feussner and Kindl reported that LOX is the main lipid body protein in cucumber cotyledons during the early stages of seed germination [20]. New LOXs are synthesized in the seedling and the cotyledon during germination. Maximal accumulation of LOXs proteins and the corresponding mRNAs lasts from a few hours to a few days after germination. However, the LOX mRNAs synthesized during
germination also could be found in the mature plant, and their expression levels were increased by the application of abscisic acid [22–24].

Nordihydroguaiaretic acid (NDGA) is a phenolic compound that is present in high concentration in the leaves of the evergreen desert shrub Creosote bush, Larrea tridentate, which grows in some desert areas of southwest United States and northern Mexico [25]. Many studies have shown that NDGA is a potent in vitro scavenger of peroxynitrite anion, singlet oxygen, hydroxyl radical, and hypochlorous acid [26]. The antioxidant properties of NDGA are attributed to its activity as a non-selective inhibitor of lipooxygenases, reducing the iron atom in the iron-enzyme complex to the inactive ferrous state [27]. The oxidative cleavage of certain xanthophylls that also occurs during ABA biosynthesis is mediated by a nonheme oxygenase with lipooxygenase-like properties [28], which further was demonstrated that NDGA is an inhibitor of the NCED enzymes in maize [29]. Thus, NDGA is an ideal chemical to study the relationship between LOX pathway and ABA signaling in regulating the seed germination and post-germination growth.

The CPR5 gene has recently been isolated and shown to encode a protein with 5 potential transmembrane regions at the carboxy terminus, a bipartite nuclear localization signal at the amino terminus, and no sequence similarity to other known proteins [30,31]. CPR5 appears to act just downstream of pathogen recognition and upstream of salicylic acid in a resistance pathway dependent on NPR1 (NONEXPRESSOR OF PATHOGENESIS RELATED GENES 1) [32]. Both and coworkers showed that CPR5 activates the PR gene expression in the RPS2-mediated pathway [33]. However, CPR5 appears to play important roles in plant growth and development as well, because cpr5 mutants exhibit defects in cell proliferation and expansion [30], and the gene also functions in cell wall biogenesis [34]. In addition, and co-workers show that cpr5 (hys1) mutants are hyperresponsive to glucose and sucrose and prematurely accumulate senescence upregulated transcripts [31]. All cpr5 alleles isolated so far exhibit early cotyledon senescence, have areas of localized cell death on the rosette leaves, and have trichomes that are glassy and reduced in size and branching [30–33]. Thus, and propose that CPR5 is a master regulator of cellular ROS status and/or signaling [35], which has close and complex interactions with other signaling networks to control cell proliferation, endoreduplication and trichome development, responses to biotic and abiotic stress [35]. In this report, we provide new evidence that CPR5 also plays important roles in the pathway controlling postgermination arrest of development through LOX pathway and ABA signaling pathway.

Materials and Methods

Plant Materials and Growth Conditions

The Arabidopsis thaliana ecotypes Columbia, Landsberg erecta were used throughout this study. The cpr5 mutant allele used in this paper was cpr5-1 [32]. Seeds were surface-sterilized for 2 min in 75% ethanol, followed by 5 min in 1% NaClO solution and washed five times in sterile distilled water, plated on growth medium (MS medium, 1.5% sucrose, 0.8% agar and pH 5.7). Plates were routinely kept for 2 days in the dark at 4°C to break dormancy (stratification) and transferred to a tissue culture room with a 16-h-light/8-h-dark cycle (light intensity of 120 mol m−2 s−1). After one week, seedlings were potted in soil and placed in a growth room at 22°C. The ABA-insensitive mutant aba1-1 was used to generate double mutants with cpr5-1. The double mutant lines were created by cross-pollination between the relevant mutants, and putative double mutant plants were screened from the phenotype segregation ratio of the F2 progeny.

Transformation Vectors and Construction of Transgenic Plants

Transgenic plants carrying constitutively expressing transgenes were generated. To produce 35S-CPR5 plants, a 1695 bp 5′-Spel fragment containing the CPR5 (The Arabidopsis Information Resource locus At5g64930) DNA was cloned into the vector pCanG vector and verified by sequencing, in which transgene expression is under the control of the CaMV 35S promoter. For the CPR5 promoter and GUS fusion construct, a 741 bp promoter region just upstream of the ATG start codon of CPR5 was amplified from genomic DNA by PCR. The PCR fragment was cloned into the SacI–BamHI site of binary vector pBI101.1 to obtain a transcriptional fusion of the CPR5 promoter and the GUS coding sequence. For the functional analysis of the transmembrane domains predicted in CPR5, a truncated form, CPR5TM, with last transmembrane domains deleted (residues 525 to564) (ATM) was also cloned into the vector pCanG. To prepare the 35S-CPR5-GFP fusion construct, the entire coding region of CPR5 was inserted directly upstream of the EGFP coding region in pBEGFP (pBEGFP is reconstructed based on pBin19). Plants were transformed with Agrobacterium tumefaciens by the floral dipping method [36]. Transgenic seeds were germinated on MS plates containing 50 mg/L kanamycin for pBI101.1, pCanG and pBEGFP, and the resistant plants were transferred to soil to obtain homozygous seeds. Two independent lines of homozygous plants containing a single insertion of each construct were used for detailed analysis.

Histochemical Analysis and Confocal Microscopic Observation

The plants include the CPR5: GUS was assayed for the GUS color reactivity. The plant material was immersed in GUS staining solution (50 mM Na-Phosphate buffer, pH 7.0, 1 mM EDTA, 0.1% Triton X-100, 100 μg/ml chloramphenicol, 1 mg/ml X-Gluc, 2 mM Ferri cyanide, 2 mM Ferro cyanide) and incubated overnight at 37°C. The material was washed repeatedly in 95% ethanol until the tissue was bleached. The stained tissue was then observed and photographed using an Olympus BX51 Microscope (Olympus Corporation, Japan).

35S-CPR5-GFP plants were used for GFP subcellular localization analysis. Roots of 7-day-old transgenic seedlings were used for the green fluorescence analysis (GFP localization) by a Carl Zeiss laser scanning system LSM 510 (http://www.zeiss.com).

ABA, NDGA Treatments and Seed Germination, Cotyledon Greening and Primary Root Length Measurements

Plants of different genotypes were grown in the same conditions and seeds were collected at the same time. For each comparison, seeds were planted in the same plate containing MS medium (0.5×MS salts, 1% sucrose, and 0.8% agar) without or with different concentrations of ABA, NDGA and tea polyphenols. Plates were chilled at 4°C in the dark for 2 d (stratified) and moved to 22°C with a 16-h light/8-h dark cycle. The percentage of seed germination was scored at indicated times. Germination was defined as an obvious emergence of the radicle through the seed coat. Cotyledon greening is defined as obvious cotyledon expansion and turning green. As primary root length measurements, plates were placed vertically in growth chamber. At indicated times, the plates were scanned by an Epson perfection

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V200 photo scanner, and the primary root length was measured by the tool DIGIMIZER 3.2.1.0 (http://www.digimizer.com).

**Drought Treatment and Measurement of Transpiration Rate**

For the soil-grown plant drought tolerance test, one-week-old seedlings were transplanted to the soil for two weeks under standard growth conditions, and then plants were subjected to progressive drought by withholding water for specified times. To minimize experimental variations, the same numbers of plants were grown on the same tray. The entire test was repeated a minimum of three times. To measure the transpiration rate, detached fresh leaves were placed abaxial side up on open petri dishes and weighed at different time intervals at room temperature. Leaves of similar developmental stages (the third to the fifth rosette leaves) from 3-week-old soil-grown plants were used.

**Stomatal Aperture Measurements**

Epidermal peels were stripped from fully expanded leaves of two week-old plants, and were floated in a solution of 30 mM KCl and 10 mM 2-(N-morpholino)-ethanesulphonic acid (MES-KOH; pH 6.15) in Petri dishes. ABA was added to the solution, and stomatal apertures were recorded under a light microscope (BX51; Olympus, http://www.olympus-global.com). Measurements were performed as described by Ichida et al [37] using the free software DIGIMIZER 3.2.1.0 (http://www.digimizer.com).

**Gene Expression Analysis**

Real-time PCR was performed using the ABI Prism 7300 Fast Real-time PCR system (Applied Biosystems Inc.) with SYBR Premix Ex Taq (Takara Bio, Inc.). Total RNA was extracted as described above. cDNAs were synthesized from 0.5 μg of total RNA using PrimeScript™ RT reagent Kit (Perfect Real Time) (Takara Bio, Inc.). Each PCR reaction contained 1× SYBR Premix Ex Taq, 0.2 μM of each primer, and 2 μl of a 1:10 dilution of the cDNA in a final volume of 20 μl. The following PCR program was used: initial denaturation, 95°C, 15 s; 40 cycles of 95°C for 4 s, 60°C for 15 s and 72°C for 31 s. In melting curve analysis, PCR reactions were denatured at 95°C, re-annealed at 60°C, then a monitored release of intercalator from PCR products or primer dimmers by an increase to 95°C. cDNA quantities were calculated by ABI Prism 7300 SDS Software Ver.1.3 (Applied Biosystems Inc.), and transcript data were normalized using UBQ10 gene as an internal control. Relative expression levels of

![Image of expression patterns of CPRS](http://www.plosone.org/figs/100umaba.png)

**Figure 1. Expression patterns of CPRS.** A. Expression patterns of CPRS gene transcripts in response to 100 μM ABA and dehydration treatment. Numbers indicate the time of treatment (hours) B. RT-PCR analysis of CPRS transcripts in different tissues of Arabidopsis plants. Total RNA was isolated from various tissues (root, leaf, stem, flower and silique) of wild-type plants grown under long-day conditions. C. Growth phenotype of cpr5 and wild type plants on MS medium containing 0 μMABA and 0.6 μMABA. D. Histochemical (β-glucuronidase) GUS analysis of CPRS promoter-GUS transgenic plant. (a) four-day old seedling; (b) rosette leaf; (c) flower; (d) stem; (e) silique.

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target genes in WT, cpr5, and CPR5 over-expression plants with or without ABA treatments, were calculated based on corresponding levels in wild-type plants without ABA treatment. Error bars were presented to indicate the standard error of the mean. All experiments were performed with three replicates. The primers used in this paper for Quantitative RT-PCR are listed in Supplemental Table S1.

Results

Expression Pattern of CPR5

To gain further insights into possible functions of CPR5, the expression pattern of this gene was examined. RT-PCR analysis detected CPR5 in all tissues of Arabidopsis, including root, leaf, siliques and seedling (Figure 1B). To determine the expression pattern in detail, the expression of the CPR5 promoter-β-glucuronidase (GUS) fusion was analyzed. Histochemical staining revealed GUS activity mainly at the cotyledons in seedlings (Figure 1D). Interestingly, strong GUS staining was specifically observed in the true leaf emergence site (Figure 1D).

To investigate whether CPR5 is involved in abiotic stress, two-weeks-old Arabidopsis seedlings were subjected to different stress treatments and RNA was extracted for gene expression analysis. Semi-quantitative RT-PCR showed that CPR5 transcript accumulation was decreased after exogenous application of 100 µM ABA or dehydration stress (Figure 1A). To elucidate the possible role of CPR5 in response to ABA, cpr5-1 and wide type seeds were germinated on MS medium with or without ABA, the growth of cpr5-1 plants was arrested compared with that of the wild type (Figure 1C). This indicated an enhanced susceptibility of cpr5-1 plants to ABA.

ABA Response of cpr5-1 and 3SS-CPR5 Plants

ABA plays an important role in regulating plant responses to different stresses [2]. Inhibitory experiments of seed germination have provided useful insights into components of ABA signaling [38]. Since CPR5 is an ABA repressed gene and cpr5-1 was sensitive to exogenous ABA (Figure 1A, C), it is possible that CPR5 plays a role in plant responses to ABA. To confirm whether the phenotypes of the cpr5-1 mutants above were indeed due to the CPR5 function loss, the CPR5 cDNA under the control of the cauliflower mosaic virus (CaMV) 3SS promoter was used to transform the cpr5-1 mutant and wild type plants. The complementation and overexpression lines had clearly increased expression of CPR5 confirmed by RT-PCR (Figure 2A); In addition, the leaf mimic and ABA hypersensitivity were rescued in the complementation lines compared to that of the cpr5-1 mutant (Figure 2B, C). These results indicated that the ABA hypersensitivity of cpr5-1 mutant was due to the CPR5 function loss. Thus, cpr5-1 mutant and CPR5 overexpression lines were used for all further studies.

To determine the role of CPR5 in ABA signaling, the seeds of wild-type, cpr5-1 mutant and CPR5 overexpression lines were germinated on MS medium containing 0 µM, 0.2 µM, 0.4 µM, 0.6 µM, 0.8 µM ABA, 1.0 µM, 1.2 µM ABA, and compared for the differences in germination and postgerminative growth. In the absence of ABA, there was no significant difference among wild-type, cpr5-1 and CPR5 overexpression lines. In the presence of ABA, the ABA-sensitive response of cpr5-1 occurred at concentrations as low as 0.4 µM ABA, cpr5-1 germinated later than wild type (Figure 3C, D). In addition, the cpr5-1 mutant and CPR5 overexpression plants were also assessed for their response to ABA during early seedling development. The cotyledon greening of cpr5-1 was severely inhibited on MS medium with 0.6 µM ABA (Figure 3A), 96% of 3SS-CPR5, 95% of wild-type but only 40% of

![Figure 2. Growth phenotypes of cpr5-1 and CPR5 transgenic plants.](image-url)

- A: WT cpr5-1 cpr5-1/CPR5 3SS-CPR5
- B: Actin
- C: MS
- 0.6 µM ABA

It has been shown that fluridone, an inhibitor ABA biosynthesis, can effectively prevent prevent the biosynthesis of ABA and reduced the ABA sensitivity of different genotypes [11,39–41]. Nondihydroguaiaretic acid (NDGA) also acts as an inhibitor of the NCED enzymes regard to its permeation speed and ability to block ABA biosynthesis [29]. To challenge the possibility that CPR5 may regulate ABA response through ABA biosynthesis, we examined the influence of NDGA and fluridone on ABA sensitivity of cpr5-1 and 3SS-CPR5 plants. Given that NDGA and fluridone are inhibitors of the ABA biosynthesis, we reasoned that if CPR5 regulated ABA response through ABA biosynthesis, the inhibition of exogenous ABA should be partly alleviated by NDGA or fluridone treatment. When seeds were pretreated with 100 mM of fluridone, no difference was observed in the inhibition of exogenous ABA on seed germination and the cotyledon greening (Figure S1). Interestingly, it was found that NDGA treatment aggravated the ABA-induced delay in the seed germination.
germination and cotyledon greening assays (Figure 4). After 7 days, the cotyledon green rate of *cpr5-1* seedlings is 86 and 87% on MS medium with 0.4 μM ABA and 15 μM NDGA respectively, but only 2% of *cpr5* could germinate and develop to green seedlings when grown on MS medium with both 15 μM NDGA and 0.4 μM ABA (Figure 4C). It was found that, similar to the

Figure 3. Responses to ABA of *cpr5-1* and 3SS-CPR5 plants. A. Growth of different genotypes of plants on MS medium containing 0.6 μM ABA. Seeds were germinated for 6 days after stratification. B. Primary root growth of wild-type, *cpr5-1* and 3SS-CPR5 seedlings on MS medium containing a range of concentrations (0 μM, 0.2 μM, 0.4 μM, 0.6 μM, 1.0 μM and 3.0 μM) of ABA. Seeds were germinated for 7 days after stratification. C. Quantification of seed germination. Seed germination percentage of three genotypes grown on different concentrations of ABA was recorded at 3 days after stratification. D. Seed germination time course of three genotypes grown on medium containing 0.6 μM ABA. E. Quantification of cotyledon greening. Cotyledon-greening percentage of three genotypes grown on medium containing different concentrations of ABA was recorded at 6 days after stratification. F. Cotyledon greening time course of three genotypes grown on medium containing 0.6 μM ABA. G. Root growth measurements. Seedling root length of three genotypes grown on different concentrations of ABA was measured at 7 days after stratification. H. Primary root growth time course of three genotypes grown on medium containing 0.6 μM ABA. For A to H, at least three independent experiments were conducted and similar results were obtained.

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situation of cotyledon greening assay, cpr5 displayed much stronger ABA and NDGA hypersensitivity than single ABA or NDGA in the ABA inhibition of the seed germination assay (Figure 4B).

These data suggested that CPR5 affects ABA signaling rather than ABA biosynthesis, and also might involved in NDGA-mediated seed germination and postgermination arrest of development.
Drought Response of cpr5 and 35S-CPR5 Plants

Because the CPR5 gene was down-regulated by dehydration, it is expected that cpr5-1 mutant may have altered response to water deficit. To test this, one-week old wild type, cpr5-1 and 35S-CPR5 plants were transplanted to grow on soil for an additional two weeks. Thereafter, plants were challenged with drought by withholding water for 10 days. The plants were then rewatered and photographed after two days (Figure 5A), and the survived plants were measured. The cpr5-1 mutant exhibited a high survival rate (76%), whereas the corresponding survival rate was 47% for wild type, and 27% for the 35S-CPR5 plants (Figure 5B). The altered drought tolerance of cpr5 mutant plants could be attributed, at least in part, to changes in transpiration rate. To test this, leaf transpiration rate of the wild type, cpr5-1 and 35S-CPR5 plants was compared. After detached for 5 hours, the leaf water loss in cpr5-1 plants was less than 5%, as opposed to 9% and 12% for wild type and 35S-CPR5 transgenic plants, respectively (Figure 5C). These results suggested that CPR5 plays an important role in plant drought response.

The rate of plant water loss during drought is largely determined by stomatal aperture/closure, and stomatal closure is a key ABA-controlled process that determines the rate of transpiration under water deficit conditions [6]. To investigate whether CPR5 is involved in ABA-related stomatal closure, we treated leaves of three genotypes with ABA to analyze stomatal aperture. Interestingly, stomatal aperture of cpr5-1 was the smallest both in light-induced stomatal opening and ABA inhibition of light-induced stomatal opening (Figure 5D). However, no differences were observed in three genotypes in ABA inhibition assay of light-induced stomatal opening. The results suggested that CPR5 may play a crucial role in guard cell control, but may be an ABA independent pathway.

Expression of Stress-Responsive Genes in cpr5-1 and 35S-CPR5 plants

The enhanced tolerance of cpr5-1 plants to drought, along with the CPR5 involved in ABA signaling during germination and postgermination growth and the inhibitory effect of ABA on CPR5 gene expression in wild type plants, prompted us to evaluate whether the expression of ABA-responsive genes in cpr5-1 mutant plants were affected. In order to address this question, the expression pattern of some stress marker genes was monitored. This analysis includes the widely researched ABI1, ABI2, ABI5, AtPP2C, ABF3, ABF4, RD29A, RD29B and RD22 genes. As shown in Figure 6, ABA treatment induced the expression changes of these marker genes in varying degrees, and no much difference were observed in these stress marker genes except ABI1 and RAB18 among the wild type, cpr5-1 and 35S-CPR5 plants. This indicated that the enhanced susceptibility to ABA exhibited by cpr5 plants was at least partially independent of the above mentioned marker genes.

The cpr5-1 plants are hypersensitive to ABA; this phenotype is opposite to abi1-1 mutant which is a dominant-negative type mutation. To test the genetic relationship between CPR5 and ABI1, double mutant cpr5-1 abi1-1 was generated by crossing cpr5 with abi1-1, and its response to ABA was compared with that of the two single mutants. Given that cpr5-1 is sensitive to ABA while abi1-1 is

Figure 5. Responses to drought of cpr5-1 and 35S-CPR5 plants. A. Drought tolerance of different genotype plants were grown on soil in the same container for 2 weeks, withheld from water for 18 days, and then rewatered for 2 days. B. Comparison of plants survival rates under the conditions described in A. C. Transpiration rates. Water loss during a 5-h period was measured using detached leaves from 3-weeks-old plants. D. Effects of ABA on stomatal aperture in wild-type, 35S-CPR5, and cpr5-1 plants. Data are mean ratios of width to length.

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insensitive to ABA, we reasoned that if CPR5 act in the same pathway to ABI1, the double mutant should be exhibited either the cpr5-1 or the abi1 sensitivity to ABA. In our ABA response assays, double mutant cpr5-1 abi1-1 plants were also sensitive to ABA (Figure 7). These results suggested that CPR5 gene may be located downstream of the ABI1 in the ABA signaling pathway.

NDGA and Tea Polyphenols Response of cpr5 and 35S-CPR5 Plants

Because cpr5 was found to be NDGA-hypersensitive, and NDGA is a general inhibitor of the LOX activity, it is possible that CPR5 may mediate the LOX pathway. As in NDGA response assays, CPR5 was down regulated by exogenous NDGA.
In an NDGA dose-response assay, seeds were germinated and growth on MS medium containing 0 μM, 20 μM and 30 μM NDGA. The germination of the *cpr5-1* seed was slightly delayed; at 3 days, about 80% of the *cpr5-1*, but nearly 100% of the wild type and 35S-CPR5 seeds germinated on medium containing 15 μM NDGA (Figure 8d). In fact, the cotyledon greening and expansion (Figure 8B), as well as the root growth of the *cpr5-1* mutant (Figure 8C, F), were also inhibited at the postgermination stage, and at 7 days, only 40% of the *cpr5* plant had small green cotyledons (Figure 8E). These results indicated that CPR5 is involved in the LOX pathway. In order to further confirm that, it was necessary to examine whether *cpr5* mutants were also sensitive to other inhibitors of the LOX pathway. As expected, *cpr5-1* mutants were also hypersensitive to tea polyphenols (another inhibitor of the LOX pathway) observed in germination assay scored by radicle emergence as well as in assays measuring the emergence of green cotyledons (Figure 9). These results demonstrated that CPR5 mediated the LOX pathway.

**Figure 8. Responses to NDGA of *cpr5-1* and 35S-CPR5 plants.** A. Expression patterns of CPR5 gene transcripts in response to 100 μM NDGA. Numbers indicate the period of treatment (hours). B. Growth of different genotypes plants on MS medium containing 0 μM NDGA and 15 μM NDGA. Seeds were germinated and grown for 7 days. C. Primary root growth of different genotypes plants grown vertically on MS medium containing a range of concentrations (0 μM, 20 μM, and 30 μM) of NDGA. Seeds were germinated and grown for 8 days after stratification. D Quantification of seed germination. Seed germination was recorded at 3 days after stratification. E Quantification of cotyledon-greening. Cotyledon-greening was recorded at 7 days after stratification. F Root growth measurements. Seedling root length was measured at 8 days after stratification. All data show the mean ± SD of three experiment replicates.

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pathway during seed germination and postgermination arrest of development.

Functional importance of the Transmembrane Domains of CPR5

CPR5 was predicted to encode a protein containing an amino terminus bipartite nuclear localization signal and five transmembrane domains at the carboxy terminus. To determine whether transmembrane domains were indispensable to its function, the CPR5 protein with a truncated form, CPR5_DTM, with the last transmembrane domains deleted were constructed and expressed in cpr5 mutant. As shown in Figure 10, the transformants CPR5_DTM also exhibited cpr5-1 phenotype. For example, at maturity, the transformants were much smaller than the wild type and their leaf trichomes were reduced in size and branching (Figure 10A). Furthermore, in root growth assays, the transformants were also hypersensitive to ABA and NDGA as compared with the wild type (Figure 10B, C). All together, the results of our deletion analysis show that the transmembrane domains of CPR5 are important to maintain the function and its capacity to respond to ABA and NDGA.

The CPR5 protein is localized to the Cytoplasm

So far, more cpr5 alleles were identified and pleiotropy functions were found, however, the subcellular localization of the CPR5 protein remains unknown. To verify its sub-cellular localization, CPR5 was fused (C-terminal) with EGFP and permanently expressed in cpr5-1 mutant under the control of the CaMV 35S promoter. The 35S-CPR5-EGFP fusion transgenic plant can complement the phenotype of cpr5-1 mutant (Figure S3), demonstrating that CPR5 fused to EGFP functioned similarly to CPR5 alone. The cytoplasm signal was visualized in 35S-CPR5-EGFP complement root cells by confocal microscopy (Figure 11). Thus, our results demonstrated a cytoplasmic localisation of the CPR5 protein in this assay.

Discussion

CPR5 encode a protein with five potential transmembrane regions at the carboxy terminus which is involved in several processes including cell death, cell cycle, pathogen response, dark-induced leaf senescence, and also severely affected in the ploidy levels of trichomes and exhibit a marked reduction in cell number [30,31]. In this study we provide evidence that CPR5 controls a drought response. We propose that CPR5 is an early negative regulator of ABA signal transduction related to this process. In addition, we also found that CPR5 negatively regulates germination and early seedling growth through LOX pathway. Our results also suggested that CPR5 independently regulates seed germination and postgermination arrest of development through LOX pathway and ABA signaling.

CPR5 is a Cytoplasm Localization protein with Transmembrane domain

CPR5 is predicted to be a Type IIIa membrane protein with five transmembrane helices at the C terminus and a cytoplasmatic N terminus with a putative nuclear localization sequence(NLS). It
has also been proposed that the protein may be involved in a signaling cascade in which the cytoplasmic domain is proteolytically cleaved and transported into the nucleus [30]. However, our results demonstrated that CPR5 is a cytoplasmic protein not nuclear localization protein in Arabidopsis suggesting that the predicted NLS in the cytoplasmatic N terminus maybe not a real functional NLS (Figure 11). Furthermore, our results had also demonstrated that its transmembrane domains are important to maintain its function (Figure 10). CPR5 is involved in several processes including pathogen responses, cell proliferation, cell expansion, dark-induced leaf senescence, and also severely affected in the ploidy levels of trichomes and exhibit a marked reduction in cell number, a possible model to explain the extreme pleiotropy of the cpr5 mutant phenotype that is the CPR5 protein may be required directly for the function of multiple transcription factors involved in a wide range of distinct processes [34]. In fact, some recent work demonstrated that some transcription factors are localized to the nucleus and cytoplasm, which interact with cytosolic protein [42]. Thus, we proposed that CPR5 protein may interact with multiple cytosolic-nucleus localization proteins including transcriptional factor or other cytosolic proteins for its pleiotropy functions.

**CPR5** is a negative Regulator of the ABA-mediated Seed Germination, Postgermination Growth and Drought Response

ABA is an essential mediator in triggering plant responses to most of the common abiotic stresses, including drought, salinity, high temperature, oxidative stress, and cold [2]. Nevertheless, high levels of ABA inhibit plant growth by affecting cell division and elongation [2]. Our data suggest that increase in endogenous ABA (provoked by dehydration treatment), as well as the exogenous application of ABA, resulted in decreased levels of CPR5 transcript (Fig. 1. A). The loss of function of CPR5 resulted in increased sensitivity to ABA, as demonstrated by comparison of the ABA-mediated plant growth inhibition in cpr5-1 and wild type plants (Figure 3).

On the other hand, cpr5 mutant plants showed an increased tolerance to drought (Figure 5A,B). Measurements of water loss in detached leaves from irrigated plants showed faster water loss in cpr5-1 mutant (Figure 5C). The ability of guard cells to respond to environmental changes and close when water available, is one of the major mechanisms that govern water loss in plants [43,44]. Since ABA regulates stomatal activity [45], the stomatal response to ABA was also examined in three genotypes. Interestingly, stomatal aperture of cpr5-1 was the smallest both in light-induced stomatal opening and ABA inhibition of light-induced stomatal opening (Figure 5D). However, these changes were of a similar magnitude in three genotypes in ABA inhibition assay of light-induced stomatal opening. The results suggested that CPR5 may play a crucial role in drought tolerance response by regulating stomatal opening, but may be an ABA independent pathway.

In addition, our findings that mutation or overexpression of CPR5 did not significantly affect ABA-induced marker gene expression support that the ABA sensitivity and drought tolerance exhibited by cpr5-1 plants was not conferred by an elevated or faster induction of the expression of these marker genes except ABI1 (Figure 6), which is similar with the results of Bu et al [46] and Ramirez et al [47] who found rha2a and cipk3 mutants are hypersensitive to ABA, while ABA induced marker genes were unaltered in both mutants.

Furthermore, molecular genetics studies have significantly advanced our understanding on the molecular basis of ABA signaling in seeds and seedlings. The cpr5-1 plants are hypersensitive to ABA; this phenotype is opposite to abi1-1 mutant which is a dominant-negative type mutation. To test the genetic relationship between CPR5 and ABI1, double mutant cpr5-1 abi1-1 was generated by crossing cpr5-1 with abi1-1, and its response to ABA was compared with that of the two single mutants. Given that cpr5-1 is insensitive to ABA while abi1-1 is insensitive to ABA, we reasoned that if CPR5 act in the same pathway to ABI1, the double mutant should be exhibited either the cpr5-1 or the abi1 sensitivity to ABA. In our ABA response assays, double mutant cpr5-1 abi1-1 plants were also sensitive to ABA (Figure 7). Taken together, these results support the view that CPR5 gene may be located downstream of the ABI1 in the ABA signaling pathway.

All these results demonstrated the conclusion that CPR5 is involved in ABA mediated seeds germination, postgermination growth and drought tolerance.

**CPR5 independently Regulates Seed Germination and Postgermination Growth through LOX Pathway and ABA Signaling**

NDGA is an ideal inhibitor of lipoxygenase and is attributed to its activity as a non-selective inhibitor of lipoxygenases [48]; however, Ren et al [49,29] reported that Nordihydroguaiaretic

![Figure 10. Functional importance of the CPR5 transmembrane domains.](image1)

![Figure 11. Subcellular localization of the CPR5.](image2)
acid (NDGA) is an ideal inhibitor of the NCED enzymes, 100 μM NDGA could totally blocked ABA accumulation, and was used to study the relationship of glucose-induced delay of seed germination in rice and ABA biosynthesis [50]. Lipoxygenases (LOXs, linoleate:oxidoreductase; EC 1.13.11.12) catalyze the regio- and stereo-specific oxygenation of polyunsaturated fatty acids to their corresponding hydroperoxy derivatives [51]. Oxidation of fatty acid is essential if lipids are to be used as a carbon source during early stages of seed germination [52].

Increases in lipoxygenase activity during germination have been reported for a number of plant species, including soybean [53]. It has been shown that fluridone, an inhibitor ABA biosynthesis, can effectively reduce endogenous ABA levels. Pretreatment with fluridone generally reduced the ABA sensitivity of different genotypes [11,39–41]. Given that NDGA is also an inhibitor of the ABA biosynthesis, the inhibition of exogenous ABA should be partly alleviated by NDGA treatment. However, this study found that NDGA treatment aggravated the ABA-induced delay in the seed germination and cotyledon greening assays (Figure 7). The finding that NDGA treatment did not diminish the difference in ABA-mediated inhibition of seed germination and cotyledon greening among genotypes suggested that CPR5 does not involved in ABA biosynthesis. In the other hand, NDGA is also an ideal inhibitor of lipoxygenase, the NDGA and tea polyphenols sensitivity of cpr5-1 mutant (Figure 8 and Figure 9) indicates that CPR5 may have a role in LOX pathways responses.

The formation of oxylipins starts with the conversion of polyunsaturated fatty acids (PUFAs). Initial conversion of PUFAs by lipoxygenases (LOXs) [57] or by a-dioxigenase (α-DOX) [58]. Subsequent conversion of hydroperoxides can occur by various alternative pathways, including those initiated by allene oxide synthase (AOS), divinyl ether synthase (DES), hydroperoxide lyases (HPL), peroxygenases (POX), or epoxy alcohol synthase (EAS). The resulting oxygenated derivatives include the phytohormone JA, as well as oxylipins with characteristic reactive epoxide, α, β-unsaturated carbonyl, or aldehyde functionalities. Jasmonic acid (JA) and some of its precursors and derivatives are signal molecules that function as essential mediators of the plant’s wound, anti-herbivore and anti-pathogen responses, as well as in growth and development [38,59,60]. In our MeJA response assays, no obvious differences were observed in wild type, cpr5-1 and 35S-CPR5 transgenic lines (Figure S2), which suggested that the NDGA sensitivity cpr5 displayed may be independent the AOS branch in the LOX pathway.

In summary, our observations indicate that CPR5 plays a regulatory role in regulation of seed germination and early seedling growth through the ABA and LOX pathways, both pathways appear to work independently, but are both regulated by CPR5.

Supporting Information

Figure S1 The influence of fluridone on ABA sensitivity of cpr5-1 and 35S-CPR5 plants. Matched seed lots were pretreated with deionized water or 100 mM fluridone for 24 h at 4°C before being placed at 22°C for germination. Seeds were germinated on MS and MS medium containing 0.4 μM ABA, and grown for 5 days. (TIF)

Figure S2 MeJA response analysis of cpr5-1 and 35S-CPR5 plants. Seeds of wild type, cpr5, 35S-CPR5 plants were germinated and grown for 12 days on MS medium containing 0 μM and 50 μM MeJA. (TIF)

Figure S3 35S-CPR5-EGFP fusion transgenic plant complements the phenotype of cpr5-1 mutant. Two-week-old seedlings of 35S-CPR5-EGFP in cpr5-1 showing the phenotype of the wild-type. (TIF)

Table S1 Primers used in this study. (DOC)

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Author Contributions

Conceived and designed the experiments: GG SZ XY CW JD XS YW CY. Wrote the paper: GG CY.

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