CHOTTO1, a Putative Double APETALA2 Repeat Transcription Factor, Is Involved in Abscisic Acid-Mediated Repression of Gibberellin Biosynthesis during Seed Germination in Arabidopsis

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The phytohormones abscisic acid (ABA) and gibberellins (GAs) are the primary signals that regulate seed dormancy and germination. In this study, we investigated the role of a double APETALA2 repeat transcription factor, CHOTTO1 (CHO1), in seed dormancy, germination, and phytohormone metabolism of Arabidopsis (Arabidopsis thaliana). Wild-type seeds were dormant when freshly harvested seeds were sown, and these seeds were released from dormancy after a particular period of dry storage (after-ripening). The cho1 mutant seeds germinated easily even in a shorter period of storage than wild-type seeds. The cho1 mutants showed reduced responsiveness to ABA, whereas transgenic plants constitutively expressing CHO1 (p35S::CHO1) showed an opposite phenotype. Notably, after-ripening reduced the ABA responsiveness of the wild type, cho1 mutants, and p35S::CHO1 lines. Hormone profiling demonstrated that after-ripening treatment decreased the levels of ABA and salicylic acid and increased GA_{v}, jasmonic acid, and isopentenyl adenine when wild-type seeds were imbibed. Expression analysis showed that the transcript levels of genes for ABA and GA metabolism were altered in the wild type by after-ripening. Genetic analysis showed that cho1 seeds, with a short period of storage, resembled fully after-ripened wild-type seeds. Genetic analysis showed that the cho1 mutation partially restored delayed seed germination and reduced GA biosynthesis activity in the ABA-overaccumulating cyp707a2-1 mutant background but did not restore seed germination in the GA-deficient ga1-3 mutant background. These results indicate that CHO1 acts downstream of ABA to repress GA biosynthesis during seed germination.

Because of the sessile nature of plants, seed dormancy and germination are tightly regulated by both internal and external cues. Seed dormancy inhibits precocious germination and germination of matured dry seeds (Bewley, 1997; Baskin and Baskin, 1998; Donohue, 2005; Fenner and Thompson, 2005). Seed dormancy confers either inhibition or delay of germination, thereby enabling the seed to germinate under proper conditions for plant survival. Seed dormancy status is initiated during seed maturation and is released by endogenous and environmental factors such as phytohormones, temperature, nutrients, and light (Finkelstein et al., 2008; Holdsworth et al., 2008). Precocious germination and deep seed dormancy after harvest is an agricultural and industrial problem causing huge economic losses; therefore, it is imperative to understand how to control seed dormancy and germination in each plant species (Gubler et al., 2005).

The phytohormones abscisic acid (ABA) and GAs are major endogenous factors that regulate seed dormancy and germination. ABA is essential for the induction and maintenance of seed dormancy and inhibits seed germination, while GA is required for the promotion of seed germination. It has been demonstrated that both biosynthesis and inactivation of ABA and GA are important for regulation of seed dormancy and germination (Nambara and Marion-Poll, 2005; Yamaguchi, 2008). It has been shown that seed dormancy is reduced in ABA-deficient mutants, whereas overexpression of ABA biosynthesis genes promotes ABA accumulation and seed dormancy (Koornneef et al., 1982; Léon-Kloosterziel et al., 1996; Marin et al., 1996; Frey et al., 1999). In maize (Zea mays), viviparous14 mutants have been shown to be defective in 9-cis-epoxycarotenoid dioxygenase (NCED), which catalyzes a regulatory step in the ABA biosynthesis...
seed dormancy release than low-dormancy accessions, accession, requires a longer period of after-ripening for this sense, most of previous studies on seed germination have used after-ripened seeds as experimental conditions (Yamauchi et al., 2007). The after-ripening treatment reduces seed dormancy release in the wild type; and (3) the pattern of phytohormone levels and transcript levels indicate that shortly stored cho1 mutant seeds mimic fully after-ripened wild-type seeds. Genetic analysis using cyp707a2-1 and ga1-3 mutants further suggested that CHO1 acts downstream of ABA to repress GA biosynthesis during seed imbibition. The possible role of CHO1 in the regulation of seed dormancy, germination, and phytohormone metabolism is discussed.

**RESULTS**

Seed Dormancy and Germination in cho1 Mutants and p35S::CHO1 Lines

Two cho1 mutant alleles, cho1-1 and cho1-3, were used to examine the role of CHO1 in seed dormancy and germination. We previously showed that the cho1-1 mutation causes an amino acid substitution in the second AP2 domain, whereas the cho1-3 mutation disrupts the CHO1 gene by a DNA insertion in the fifth exon (Yamagishi et al., 2009). First, to investigate the effect of cho1 mutations on seed dormancy and germination, germination of wild-type and cho1 seeds was compared after a variable period of dry storage. Wild-type Columbia displays seed dormancy in a limited period of dry storage (Penfield et al., 2005). When freshly harvested seeds were imbibed on water-agarose without any nutrients under light conditions,
almost no seed germination was observed in both wild-type and cho1 mutant seeds (Fig. 1A). On the other hand, when 1-week dry-stored seeds were tested, the germination frequency of cho1 seeds was remarkably higher than that of wild-type seeds (Fig. 1A). After additional weeks of dry storage, the germination frequency of the wild-type seeds increased and both wild-type and mutant seeds had fully germinated, although cho1 mutants showed faster seed germination than the wild type (Fig. 1A). Primary dormancy of cho1 seeds was variable, and freshly harvested mutant seeds occasionally germinated more than 60%, while fresh wild-type seeds showed no germination under this condition (data not shown). In general, the less dormant phenotype of cho1 seeds was observed only under a short period of dry storage, and this phenotype was mostly indistinguishable when wild-type seeds were fully after-ripened.

We next investigated the effect of after-ripening on the ABA responsiveness during germination of wild-type and cho1-3 mutant seeds. Seeds were dry stored for 0, 1, 2, 4, and 8 weeks and imbibed without stratification. Germination frequencies in the presence of (+)-S-ABA of both wild-type and cho1-3 seeds were higher when seeds were dry stored for longer periods (Fig. 1B). Interestingly, wild-type and mutant seeds germinated fully after 2 weeks of dry storage, and the ABA responsiveness of both the wild type and mutants decreased further by prolonged dry storage for 8 weeks (Fig. 1B). The ABA responsiveness of cho1 mutants on germination was less than that in the wild type when seeds were sown after 1, 2, and 4 weeks of dry storage, as reported previously (Yamagishi et al., 2009). However, the ABA responsiveness of cho1 mutants was comparable to that of wild-type seeds when these seeds were after-ripened for 8 weeks.

To further investigate the function of CHO1, we made transgenic plants that express CHO1 under the control of the cauliflower mosaic virus 35S promoter, designated as p35S::CHO1 lines. Quantitative reverse transcription (QRT)-PCR analysis indicated that the transcript levels of CHO1 in 24-h imbibed seeds were 4-fold higher in the p35S::CHO1 lines than in the wild type (Fig. 1C). Without applying exogenous ABA, the germination frequencies after 1 week of dry storage were restored to the wild-type level in two p35S::CHO1 lines (Fig. 1B). However, after 2 or more weeks of dry storage, p35S::CHO1 showed full germination frequencies similar to the wild type and cho1 mutants (Fig. 1B), suggesting that seed dormancy was normally released by after-ripening even in the p35S::CHO1 lines. However, p35S::CHO1 seeds showed higher responsiveness to exogenous ABA than wild-type and cho1-3 mutant seeds after a series of dry storage periods (Fig. 1B). Even after 8 weeks of dry storage, germination of p35S::CHO1 lines was effectively suppressed by 0.5 or 1.0 μM exogenous ABA, in which germination of the wild type and cho1-3 was permissive.

Both After-Ripening and cho1 Mutations Alter the Levels of Multiple Hormones

After-ripened seeds reduce the ABA level in response to imbibition more rapidly than freshly harvested seeds (Ali-Rachedi et al., 2004). To examine whether the cho1 mutation alters after-ripening-regulated reduction in ABA levels, endogenous ABA levels were
measured in dry seeds and 24-h imbibed seeds using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). ABA levels of dry seeds were slightly lower in cho1-1 and cho1-3 than in the wild type, and the levels of all genotypes were not altered during dry storage periods of 4 weeks (Fig. 2). On the other hand, in 24-h imbibed seeds of both the wild type and cho1 mutants, after-ripened seeds contained lower ABA levels than those of freshly harvested seeds (Fig. 2). Interestingly, the ABA levels in imbibed seeds were significantly lower in cho1 mutants than in the wild type after 0 and 1 week of dry storage (Fig. 2). However, after 4 weeks of dry storage, ABA levels of the imbibed cho1 seeds were comparable to those of wild-type imbibed seeds.

Next, to investigate the effect of the cho1 mutation on the accumulation of other plant hormones, hormone profiling was performed in 36-h imbibed seeds using LC-ESI-MS/MS. Seeds were dry stored for 1 and 6 weeks before imbibition. Endogenous levels of GA$_4$, indole-3-acetic acid (IAA), JA, trans-zeatin (tZ), iP, and SA were determined. Interestingly, after-ripening treatment caused significant changes in the levels of not only ABA but also GA$_4$, JA, iP, and SA in imbibed wild-type seeds. GA$_4$, JA, and iP levels were increased, but ABA and SA levels were decreased in imbibed seeds with after-ripening treatment for 6 weeks relative to those for 1 week (Table I). Imbibed seeds of cho1-1 and cho1-3 contained lower levels of ABA and SA but higher levels of GA$_4$, JA, and iP than wild-type seeds after 1 week of dry storage (Table I). GA$_4$ levels were 2-fold higher in cho1 mutants than in the wild type, whereas ABA levels were 2-fold lower in cho1 mutants. However, after 6 weeks of dry storage, there was no significant difference in all of these hormone levels between the wild type and cho1 mutants.

**Changes in the Expression Levels of ABA and GA Metabolism Genes in cho1 Mutants**

QRT-PCR analysis was performed in dry seeds and imbibed seeds treated with 1 week of dry storage to investigate the expression pattern of genes involved in biosynthesis and inactivation of ABA and GA. The transcript levels of ABA inactivation genes (CYP707A2 and CYP707A3) were up-regulated in cho1 mutants relative to the wild type, while ABA biosynthesis genes (ZEP and NCED9) were down-regulated in the mutants (Fig. 3A). Up-regulation of CYP707A2 in cho1 mutants appears to be a precocious induction, with a peak at 6 h in contrast to the wild type, with a peak at 12 h, whereas the higher levels of the CYP707A3 mRNA were observed in cho1 mutants at 24 h after imbibition. On the other hand, expression of ZEP and NCED9 in cho1 mutants was down-regulated after 12 h of imbibition. Expression levels of NCED6 and CYP707A1 in cho1 mutants were comparable to those in the wild type (Fig. 3A). Among GA biosynthesis genes, the transcript levels of GA$_3$ox1 and GA$_3$ox2 were significantly increased after imbibition (Fig. 3B). The induction of these genes in cho1 mutants was approximately 10-fold more exaggerated in cho1 mutants than in the wild type after imbibition. By contrast, the transcript levels of GA$_2$ox3, a GA biosynthesis gene, were lower in cho1 mutants than in the wild type, which is the opposite direction in terms of higher accumulation of GA$_4$ in the cho1 mutant than in the wild type (Table I; Fig. 3B). There was no significant difference in the transcript levels of GA$_2$ox1, GA$_2$ox2, and GA$_2$ox6 between the wild type and cho1 mutants (Fig. 3B).

Furthermore, we investigated transcript levels of genes involved in the regulation of germination and hormone metabolism and signaling: seed dormancy and germination (DOG1, FUS3A [FUS3], PHYTOCHROME-INTERACTING FACTOR-LIKE5 [PIL5], SPATULA [SPT]), ABA signaling (ABI3, ABI4, ABI5), ABA response (RD29B), and GA signaling (REPRESSOR OF GA1-3 [RGA], GA-SENSITIVE [GAI], RGA-LIKE2 [RGL2], RGL3; Fig. 3C; Supplemental Fig. S1). Of these genes, expression patterns of FUS3, PIL5, and SPT were altered in the cho1 mutant (Fig. 3C), but the transcript levels of DOG1, ABI, and RGA/GAI genes were not remarkably altered in the mutant (Fig. 3C; Supplemental Fig. S1). The transcript levels of RD29B seem to correlate with the ABA level (Fig. 2; Supplemental Fig. S1). Expression of FUS3 was down-regulated in cho1 mutants relative to the wild type after 12 h of imbibition. PIL5 expression was found to be down-regulated more than 2-fold in cho1 mutants after 24 h of imbibition when compared with the wild type, while SPT expression was up-regulated in cho1 mutants after 24 h of imbibition.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Effects of cho1 mutations on ABA levels in dry seeds and imbibed seeds. ABA levels in dry seeds (top) or 24-h imbibed seeds (bottom) were determined in the wild type (Columbia [Col]) and cho1 mutants. Seeds were dry stored for 0 (fresh), 1, or 4 weeks after harvest and then used for analysis.
To investigate the effect of after-ripening on the expression of ABA and GA metabolism genes, qRT-PCR analysis was performed using seeds that had been dry stored for 0, 1, 4, and 9 weeks prior to imbibition. It has been reported that the transcript levels of ABA and GA metabolism genes, such as ZEP, NCED9, CYP707A2, and GA3ox1, are affected by after-ripening in Arabidopsis seeds (Cadman et al., 2006; Finch-Savage et al., 2007; Carrera et al., 2008). Transcript levels of these hormone metabolism genes were analyzed in 24-h imbibed seeds, except for CYP707A2 in 6-h imbibed seeds, after various periods of dry storage. The wild-type Columbia strain used in this study is a low-dormancy accession of Arabidopsis. However, even in this accession, dry storage treatment resulted in down-regulation of ZEP and NCED9 and up-regulation of GA3ox1, GA3ox2, and CYP707A2 in imbibed seeds, which was dependent on the length of dry storage (Fig. 4). The transcript levels of CYP707A3 after imbibition were up-regulated transiently by the first 4 weeks of dry storage but restored to the levels of freshly harvested seeds thereafter (Fig. 4). Interestingly, in both wild-type and cho1-3 seeds, even after the seeds acquired full germination competence by after-ripening, transcript levels of GA3ox1 and GA3ox2 genes continued to be increased and those of ZEP and NCED9 continued to be down-regulated by prolonged after-ripening treatment (Fig. 4). This indicates that after-ripening progressed in these seeds beyond seed dormancy release. When gene expression was compared between the wild type and cho1-3, the transcript levels of ZEP and NCED9 were lower in cho1-3 than in the wild type after fresh harvest and 1 week of dry storage, whereas those of GA3ox1, GA3ox2, CYP707A2, and CYP707A3 were higher in cho1-3 throughout the course of dry storage from fresh harvest to 9 weeks of dry storage (Fig. 4). After 4 weeks of storage, there was no difference in the transcript levels of ZEP and NCED9 between the wild type and cho1-3.

Effects of cho1 Mutation in ABA-Overaccumulating cyp707a2 and GA-Deficient ga1 Mutants

The results described above indicate that the transcript levels of ABA and GA metabolism genes and levels of ABA and GA in imbibed seeds are affected by cho1 mutations. It is known that ABA regulates GA metabolism and vice versa (Toyomatsu et al., 1994; Seo et al., 2006; Sawada et al., 2008). To investigate the epistatic relationship between CHO1 and hormone metabolism, we made double mutants between cho1-3 and cyp707a2-1 or ga1-3. The cyp707a2-1 mutant accumulates higher levels of ABA than the wild type, thereby exhibiting delayed germination (Okamoto et al., 2006), whereas the ga1-3 mutant shows impaired seed germination due to GA deficiency (Sun and Kamiya, 1994). When seed germination was compared, cyp707a2-1 cho1-3 double mutants showed higher germination frequencies than cyp707a2-1 after 4 weeks of storage (Fig. 5A), indicating that cho1 mutation restores the reduced germination potential of cyp707a2-1. Both seeds showed no seed germination just after harvest and 1 week of dry storage. Next, endogenous levels of ABA and GA4 were compared between 4-week-stored seeds of the wild type, cyp707a2-1, and cyp707a2-1 cho1-3. Both cyp707a2-1 and cyp707a2-1 cho1-3 mutants accumulated higher amounts of ABA than the wild type, and there was no difference in the ABA levels between cyp707a2-1 and cyp707a2-1 cho1-3 during the course of imbibition (Fig. 5B). When GA4 content was compared after 24 h of imbibition, it was 4-fold lower in cyp707a2-1 than in the wild type (Fig. 5C). However, despite no changes in the ABA levels, GA4 content was significantly restored in cyp707a2-1 cho1-3 relative to the cyp707a2-1 single mutant. We then analyzed expression patterns of genes for GA biosynthesis and inactivation (Fig. 5D; Supplemental Fig. S2). Expression patterns of GA3ox1 and GA3ox2, but not other genes, were well correlated with the levels of GA4 in 24-h imbibed seeds of the wild type, cyp707a2-1, and cyp707a2-1 cho1-3 (Fig. 5C and D; Supplemental Fig. S2). On the other hand, in dry seeds, expression patterns of CPS, GA20ox1, GA3ox1, and GA20ox6 were regulated in a similar manner as the GA4 levels, where the effect of the cyp707a2-1 mutation was partially alleviated by the cho1-3 mutation (Fig. 5D; Supplemental Fig. S2). Of GA metabolism genes examined, GA3ox1, but not other genes, showed a consistent expression pattern both in dry seeds and imbibed seeds, with GA4 levels as depicted in Figure 5C.

Table 1. Comprehensive analysis of phytohormones in imbibed seeds

| Sample       | ABA   | GA4  | IAA  | JA   | TZ   | iP   | SA  |
|--------------|-------|------|------|------|------|------|-----|
| One week of storage |       |      |      |      |      |      |     |
| Columbia     | 11.4 ± 1.5 | 0.87 ± 0.05 | 53.3 ± 0.8 | 78.5 ± 8.2 | 0.10 ± 0.01 | 0.13 ± 0.01 | 165.6 ± 8.5 |
| cho1-1       | 6.6 ± 0.8  | 1.93 ± 0.22 | 65.5 ± 5.9  | 93.7 ± 14.1 | 0.11 ± 0.01 | 0.23 ± 0.01 | 57.5 ± 2.4  |
| cho1-3       | 5.5 ± 0.4  | 1.83 ± 0.10 | 48.5 ± 4.9  | 99.4 ± 7.8  | 0.09 ± 0.01 | 0.20 ± 0.01 | 56.2 ± 7.8  |
| Six weeks of storage |       |      |      |      |      |      |     |
| Columbia     | 6.1 ± 0.4  | 1.93 ± 0.17 | 48.2 ± 3.0  | 136.2 ± 7.7 | 0.14 ± 0.02 | 0.45 ± 0.03 | 77.1 ± 9.7  |
| cho1-3       | 7.0 ± 0.6  | 1.73 ± 0.14 | 44.6 ± 1.2  | 138.7 ± 10.8| 0.15 ± 0.01 | 0.58 ± 0.02 | 74.4 ± 5.4  |
Then, seed germination was analyzed in the ga1-3 mutant and the ga1-3 cho1-3 double mutant. Both ga1-3 and ga1-3 cho1-3 mutants did not germinate when 4-week-stored seeds were used for germination assays (Fig. 6A). Exogenous application of GA4 recovered germination frequencies in both ga1-3 and ga1-3 cho1-3 mutants in a dose-dependent manner. There was no difference in the GA response curve between ga1-3 and ga1-3 cho1-3 mutants (Fig. 6A). We then analyzed the expression of GA3ox1 and GA3ox2. When compared with the wild type, GA3ox1 gene expression was transiently up-regulated after 6 h of imbibition in ga1-3 mutants, whereas GA3ox2 gene expression was significantly down-regulated in the ga1-3 mutants during the course of imbibition (Fig. 6B), consistent with the previous study showing that GA3ox1 and GA3ox2 gene expression is regulated by a negative feedback mechanism and a positive feedback mechanism, respectively, in imbibed seeds (Yamaguchi et al., 1998).

We next examined the effect of the cho1-3 mutation on the expression of hormone metabolism genes under GA-deficient conditions. The cho1-3 mutation further enhanced expression of GA3ox1, but not of GA3ox2, when the expression patterns in ga1-3 cho1-3 were compared with those in ga1-3 (Fig. 6B). On the other hand, expression of ABA metabolism genes (ZEP, NCED6, NCED9, CYP707A1-3) and ABA response genes (ABI5, RD29B) was compared between ga1-3 and ga1-3 cho1-3. There was no significant difference in the transcript levels of these genes between these two mutants in both dry seeds and imbibed seeds (Supplemental Fig. S3).

**CHO1 Is Expressed in the Radicle in Imbibed Seeds**

To examine the site of CHO1 expression, histochemical analysis of GUS expression was performed using pCHO1::GUS transgenic seeds. When 16-h imbibed seeds were stained, GUS staining was detected in the radicle of the embryo in pCHO1::GUS seeds (Fig. 7A). The staining was intense at the tip of the radicle but absent in the endosperm or testa. When sections of the GUS-stained seeds were examined, pCHO1::GUS expression was observed in the radicle except for the provascular.

Next, the effect of after-ripening on the expression pattern of CHO1 in imbibed seeds was investigated using freshly harvested seeds and 4-week-stored seeds. In the wild type, CHO1 expression was moderately up-regulated in response to imbibition in freshly harvested seeds, while it was up-regulated after 6 h of imbibition in 4-week-stored wild-type seeds (Fig. 7B), suggesting that after-ripening resulted in precocious up-regulation of CHO1 expression after imbibition.

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**Figure 3.** Gene expression analysis in 1-week-stored seeds. Gene expression was investigated during the course of imbibition in 1-week-stored seeds. A, ABA biosynthesis and catabolism genes. B, GA biosynthesis and catabolism genes. C, Dormancy- and germination-related genes.
Interestingly, this up-regulation of CHO1 expression was observed even in freshly harvested cho1-1 mutant seeds (Fig. 7B). In contrast, this was not observed in the cho1-3 mutation due to the disrupted CHO1 expression as a result of the DNA insertion mutation. CHO1 expression was induced in the cyp707a2-1 mutant at 6 h in a similar manner as in the wild type (Fig. 7C). The mRNA of CHO1 maintained a higher level at 24 h in cyp707a2-1 than in the wild type. This difference does not seem to be the direct effect of ABA overaccumulation, because exogenous ABA does not induce CHO1 expression at this time (Yamagishi et al., 2009). On the other hand, CHO1 expression was not altered by the ga1-3 mutation or by the application of GA (Fig. 7C).

The CHO1 AP2 Domain Binds to the AINTEGUMENTA Recognition Element

It has been shown that CHO1 is a member of AP2-like subfamily, which is composed of at least 18 members in the Arabidopsis genome (Kim et al., 2006). The AP2-like members include AINTEGUMENTA (ANT), whose function has been shown to relate to cell size regulation (Krizek, 1999; Mizukami and Fischer, 2000). The amino acid sequences of CHO1 AP2 domains are similar to that of ANT. Because the DNA element recognized by ANT has been reported previously (Nole-Wilson and Krizek, 2000), a gel mobility shift assay was performed to test the binding between CHO1 and the ANT-recognition DNA element (Fig. 8A). For this purpose, recombinant CHO1

Figure 4. Gene expression analysis during the course of dry storage. Transcript levels of ABA metabolism genes (ZEP, NCED9, CYP707A2 and -3) and GA metabolism genes (GA3ox1 and -2) after 24 h of imbibition were investigated using seeds that had been dry stored (ds) for 0, 1, 4, and 9 weeks after harvest. For the CYP707A2 gene, the transcript levels after 6 h of imbibition are shown.

Figure 5. Effects of cho1 mutation on seed germination, hormone levels, and GA metabolism gene expression in the ABA-overaccumulating cyp707a2 mutant. A, Seed germination of the wild type (Columbia [Col]), cyp707a2-1, and cyp707a2-1 cho1-3. Seeds were dry stored for 0 (fresh) or 4 weeks in a desiccation box after harvest and then used for analysis. Germination frequencies were scored after 7 d of imbibition at 23°C. B, Changes in ABA levels during seed imbibition in 4-week-stored wild-type, cyp707a2-1, and cyp707a2-1 cho1-3 seeds. FW, Fresh weight. C, GA4 content in the imbibed seeds of 4-week-stored wild-type, cyp707a2-1, and cyp707a2-1 cho1-3 plants. D, Transcript levels of GA biosynthesis genes before and after 24 h of imbibition in 4-week-stored wild-type, cyp707a2-1, and cyp707a2-1 cho1-3 seeds.
proteins that include two repeats of AP2 domains and the linker region were prepared from wild-type (CHO1AP2-wt) or cho1-1 (CHO1AP2-m) cDNA. When CHO1AP2-wt was used for analysis, it was found to bind to a BS15 element, a previously reported ANT recognition DNA element (Fig. 8B). Excessive amounts of nonlabeled BS15 element interfered with the binding, while mutated nonlabeled BS15 that carries nucleotide substitutions within the consensus nucleotide bases failed to interfere, indicating that binding between CHO1AP2-wt and BS15 is specific. Binding was not detected between CHO1AP2-m and the BS15 element (Fig. 8B). These results indicated that the cho1-1 mutation abolishes the DNA-binding ability of the CHO1 protein.

**DISCUSSION**

**CHO1 Plays a Role in ABA-Mediated Repression of Seed Germination after Imbibition of Dry Seeds**

Seed dormancy is induced during seed maturation and is released by subsequent dry storage, a process called after-ripening. The dry storage increases the germination potential of seeds, enabling the seeds to germinate upon imbibition (Holdsworth et al., 2008). We note that the timing of induction of primary dormancy, after-ripening, and maintenance of dormancy differs. Induction of primary dormancy occurs in seed development, whereas after-ripening is a process occurring in dry storage. On the other hand, maintenance of dormancy occurs mostly after imbibition of dry seeds. The ability for maintenance of primary dormancy is one of the negative factors of germination potential. The process of after-ripening itself occurs in dry storage, but its action is evaluated by the performance of seeds after imbibion (i.e. either germination potential or expression patterns of after-ripening-regulated genes).

In this study, we characterized the effects of after-ripening in wild-type seeds for its germination potential (Fig. 1, A and B), ABA responsiveness (Fig. 1B), endogenous levels of ABA, GA, iP, JA, and SA (Table I), and transcript levels of genes involved in ABA and GA metabolism (Fig. 4). Characterization of the cho1 mutants using these criteria demonstrates that the shortly stored cho1 seeds resembled fully after-ripened wild-type seeds. The cho1 mutants display the phenotype as if it is hypersensitive to after-ripening. A simple hypothesis to explain this is that this phenotype acquires proper induction of primary dormancy.

![Figure 6](image)

**Figure 6.** Effects of cho1 mutation on seed germination and gene expression in the GA-deficient ga1-3 mutant. A, GA responsiveness of seed germination in 4-week-stored wild-type (Columbia [Col]), cho1-3, ga1-3, and ga1-3 cho1-3 seeds. B, Expression patterns of GA3ox1 and GA3ox2 genes during imbibition in 4-week-stored wild-type, ga1-3, and ga1-3 cho1-3 seeds.

![Figure 7](image)

**Figure 7.** pCHO1::GUS expression in imbibed seeds. A, X-gluc staining of GUS activity in pCHO1::GUS. Seeds were imbibed for 16 h at 23°C before staining. Ten-micrometer sections were prepared and photographed. Bars = 50 μm. B, Effects of dry storage on CHO1 expression during imbibition. Seeds were dry stored for 0 (fresh) and 4 weeks after harvest (AR4w). C, CHO1 gene expression patterns during imbibition in cyp707a2-1 and ga1-3. Seeds were dry stored for 4 weeks after harvest. Col, Columbia wild type.
but has low ability to maintain seed dormancy after imbibition of dry seeds. Nondormant mutants might exhibit reduced dormancy even in freshly harvested seeds, and after-ripening hypersensitive mutants acquire primary dormancy in fresh seeds but are released by after-ripening relatively easily. We previously discussed that the genetic components for ABA sensitivity in seed development are similar, but distinct, from those after imbibition of dry seeds (Yamagishi et al., 2009). ABI4 and CHO1 play prominent roles in repression of germination after imbibition, whereas ABI5 plays a more prominent role in seed development, possibly for the induction of primary dormancy. It is noteworthy that these are not specific to each developmental stage, but the phenotypes of these mutants suggest that the primary regulators of ABA responsiveness in seed development are different from those after imbibition of dry seeds (Yamagishi et al., 2009). In this hypothesis, we still cannot explain why p35S::CHO1 lines did not show the after-ripening-insensitive (-like) phenotype. Indeed, we cannot rule out the possibility that p35S::CHO1 lines do not represent the gain of function of CHO1.

For example, ABI5 protein levels are regulated at the level of protein turnover (Lopez-Molina et al., 2001). It is possible that CHO1 is posttranscriptionally regulated and that the p35S::CHO1 transgene functions under specific conditions. To clarify the relationship between CHO1 and after-ripening, it will be necessary to examine how after-ripening affects the CHO1 protein levels in imbibed seeds.

Loss of function of CHO1 reduced the ABA responsiveness, whereas its gain of function led to the opposite result (Fig. 1B). The ABA responsiveness of the wild type, cho1 mutants, and p35S::CHO1 lines decreased in a manner dependent on the period of dry storage. The reduction of ABA responsiveness continued to the 8-week storage period, even though all genotypes were fully germinated by 2 weeks of dry storage. This result indicates that after-ripening continued to reduce the ABA responsiveness even after the seed was completely released from primary dormancy, as is the case of after-ripening-dependent gene expression (Carrera et al., 2008). A prominent effect of after-ripening on all genotypes suggests that CHO1 is not directly involved in the mechanisms of after-ripening itself, but the function of CHO1 is possibly one of the targets of after-ripening.

Both cho1 Mutations and After-Ripening Alter the Levels of Multiple Plant Hormones in Imbibed Seeds

It has been reported that the reduction in ABA levels in imbibed seeds is exaggerated by after-ripening prior to imbibition (Ali-Rachedi et al., 2004; Millar et al., 2006). Our hormone profiling also confirmed that after-ripening treatments enhanced the reduction in ABA levels after imbibition in a manner dependent on the storage period (Fig. 2). In our storage conditions, after-ripening did not affect ABA levels in dry seeds (Fig. 2). The effect of after-ripening on reducing ABA levels was more prominent in cho1 mutants. One week of dry storage reduced the ABA levels more rapidly in cho1 mutants than in the wild type after seed imbibition (Fig. 2). After 4 weeks of dry storage, the ABA levels in both the wild type and cho1 mutants were similarly low in imbibed seeds. In this regard, cho1 seeds are more sensitive to after-ripening than wild-type seeds (Fig. 1A). The cyp707a2-1 cho1-3 double mutant (as well as 35S::CHO1 lines) indicates that the primary action of CHO1 is to regulate ABA responsiveness. However, it is also noticeable that the ability of cho1 mutations to reduce the ABA levels in imbibed seeds (Fig. 2) was restored in the cyp707a2-1 cho1-3 mutant (Fig. 5B). This suggests that the cho1 mutation can reduce the ABA levels in imbibed seeds through CYP707A2, although this may be a minor mode of CHO1 action.

In addition, our comprehensive plant hormone measurement revealed that after-ripening treatment decreased the levels of ABA and SA and increased GA, JA, and ip levels in imbibed wild-type seeds (Table I). It has been reported that SA and cytokinin are
involved in the regulation of seed germination (Riefler et al., 2006; Chandra et al., 2007). Thus, the changes in levels of these phytohormones may contribute to the regulation of germination potential. Interestingly, the levels of all of these hormones were changed in cho1 mutants similar to the after-ripened wild type regardless of the period of after-ripening (Table I). When compared with 1-week-stored wild-type seeds, both 1-week-stored cho1 seeds and 6-week-stored wild-type seeds decreased in ABA and SA levels and increased in GA, JA, and iP levels when seeds were imbibed (Table I). These hormone levels provide a useful fingerprint to indicate the similarity between shortly stored cho1 seeds and fully after-ripened wild-type seeds.

Altered ABA/GA Balance Is Associated with Changes in the Expression Patterns of Genes Involved in ABA and GA Metabolism

Our gene expression analysis showed that the transcript levels of genes involved in ABA and GA metabolism were altered in cho1 mutants (Fig. 3). When 1-week-stored cho1 seeds were imbibed, transcript levels of ABA biosynthesis genes (ZEP, NCED9) were down-regulated, whereas those of ABA inactivation genes (CYP707A2, CYP707A3) and GA biosynthesis genes (GA3ox1, GA3ox2) were up-regulated in cho1 mutants relative to the wild type (Fig. 3, A and B). Among these genes, the changes of GA3ox1 and GA3ox2 mRNA levels were remarkable and increased more than 10-fold in cho1 mutants when compared with the wild type. These expression patterns are correlated with changes in ABA and GA, levels (Table I; Fig. 2). In addition, we found that expression patterns of the regulatory genes involved in ABA and GA metabolism were also altered in cho1 mutants (Fig. 3C). These include PIL5 and SPT genes, which are involved in the negative regulation of seed germination (Oh et al., 2004, 2006; Penfield et al., 2005). Expression of FUS3 was also found to be down-regulated in cho1 mutants (Fig. 3C). FUS3 regulates ABA and GA metabolism and negatively regulates GA3ox expression during seed development (Nambara et al., 2000; Curaba et al., 2004; Gazzarrini et al., 2004). This is noteworthy, because the phenotypes of the cho1 mutants are correlated with after-ripening, thus raising the possibility that PIL5 and FUS3 are also involved in the process of after-ripening.

Recent transcriptome analysis using dormant Cvi-0 has revealed that after-ripening affects the transcription of ABA and GA metabolism genes after imbibition (Cadman et al., 2006; Finch-Savage et al., 2007). In barley and Arabidopsis, the CYP707A gene, encoding ABA 8’-hydroxylase, has been shown to be a target of after-ripening (Millar et al., 2006). Also, transcriptome analysis using Arabidopsis aba1 and ab1 mutants indicates that after-ripening alters a pathway distinct from the ABA signaling pathway (Carrera et al., 2008). In this study, we analyzed the effect of after-ripening on the transcript levels of ABA metabolism genes (ZEP, NCED9, CYP707A2, CYP707A3) and GA metabolism genes (GA3ox1, GA3ox2) after imbibition in the wild type and cho1 mutants (Fig. 4). In both the wild type and cho1 mutants, after-ripening affects the transcript levels of these genes even after seed dormancy was completely released by after-ripening (Figs. 1E and 4). This indicates that after-ripening progresses in dry-stored seeds beyond seed dormancy release, consistent with a previous report showing that after-ripening affects gene expression in nondormant aba1 mutants (Carrera et al., 2008). Importantly, the altered expression patterns of these hormone metabolism genes are correlated with endogenous levels of the hormones (Figs. 2 and 4). These results indicate that CHOI is involved in the regulation of endogenous ABA and GA balance by affecting transcription of these hormone metabolism genes in imbibed seeds.

The cho1 Mutations Attenuate ABA-Mediated Down-Regulation of GA3ox1 Expression

Hormone metabolism is mutually regulated and constitutes a network to coordinate hormone balance (Seo et al., 2009). GA has been shown to reduce the ABA level in lettuce (Lactuca sativa) seeds (Toyomasa et al., 1994; Sawada et al., 2008), while ABA reduces the GA level at least in part through down-regulation of GA3ox expression in Arabidopsis seeds (Seo et al., 2006; Toh et al., 2008). Analysis of the cyp707a2-1 cho1-3 double mutant showed that the cho1-3 mutation increased the germination potential of the seeds carrying a cyp707a2-1 mutation, even though the double mutant contains a high level of ABA. This result indicates that the increased germination potential of the cho1 mutants is primarily caused by the reduced ABA sensitivity. Moreover, the cyp707a2-1 cho1-3 double mutant partially alleviated the reduced levels of GA, consistent with the up-regulation of GA3ox1 and GA3ox2 after imbibition (Fig. 5, C and D). Analysis of the ga1-3 cho1-3 double mutant showed that GA is responsible for the altered germination potential and expression of ABA metabolism genes in cho1 mutants (Fig. 6A; Supplemental Fig. S3). These results showed that the cho1-3 mutation did not alter either the germination potential or the GA responsiveness in germination in a GA-deficient ga1-3 background (Fig. 6A). This result also suggests that CHOI is not involved in GA signaling. Importantly, the cho1-3 mutation up-regulated GA3ox1 expression, but not GA3ox2 expression, in the ga1-3 background. This indicates that CHOI primarily down-regulates GA3ox1 expression and that the altered expression of GA3ox2 is due to the GA deficiency.

GA biosynthesis genes are expressed in hypocotyl and radicle of imbibed Arabidopsis seeds (Yamaguchi et al., 2001). In this study, we showed that pCHO1::GUS was expressed in the radicle, except for the provascular tissues of the embryo in imbibed seeds (Fig. 7A). The site of the pCHO1::GUS expression

Yano et al.
overlapped with those of pGA3ox1::GUS expression, although GA3ox1 expression seems to be more intensely expressed in the hypocotyl than in the radicle (Mitchum et al., 2006). Considering the up-regulation of GA3ox1 expression in cho1 mutants (Table I; Figs. 4 and 5), it is most likely that CHO1 is involved in ABA-mediated negative regulation of GA3ox1 expression in the radicle during seed imbibition. In addition, expression of CHO1 was induced in imbibed seeds by the after-ripened treatment, while this up-regulation was not observed in freshly harvested seeds (Fig. 7B). Interestingly, the up-regulation of CHO1 was observed even in freshly harvested cho1-1 seeds, which is correlated with the enhanced ABA reduction in the fresh cho1-1 seeds (Fig. 2). We note that ABA and GA do not seem to regulate the expression of CHO1, because induction of CHO1 expression was unaffected in cyp707a2-1 and ga1-3 mutants (Fig. 7C).

Functions of CHO1 in Germinating Seeds

In this study, our gel mobility shift assay demonstrated that the AP2 domains of CHO1 bind to the DNA element recognized by ANT in a specific manner (Fig. 8; Nole-Wilson and Krizek, 2000). Failure of the cho1-1 mutant protein to bind to this element supports that this in vitro experiment is correlated with in vivo functions of CHO1. However, we could not find any similar binding sites of CHO1 within the genomic region of GA3ox1 (Figs. 3B, 5D, and 6B). In addition, p35S::CHO1 lines did not show the suppressed expression of GA3ox1 in vegetative tissues (data not shown). Thus, CHO1 regulates GA3ox1 expression possibly through collaboration with other seed-specific factors or indirectly. Further analyses will be necessary to uncover target genes of CHO1 and a detailed mechanism of CHO1-dependent regulation of GA3ox1 expression as well as the effects of JA, IP, and SA on GA3ox1 expression.

Amino acid sequences of AP2 domains are highly conserved among AP2-like family members. The AP2 domains of CHO1 are most similar to those of PLT1 and PLT2 (both 94% identity) and ANT (86%). It has been demonstrated that overexpression of the ANT gene results in increased size in leaves, seeds, and flowers (Krizek, 1999; Mizukami and Fischer, 2000). Recently, four PLETHORA-related genes have been shown to play a fundamental role in root growth and formation (Galinha et al., 2007). While the AP2 domain of CHO1 is structurally distinct from those of WRINKLED1 (69%), the wrinkled1 mutation has been shown to result in impaired seed germination and reduced seed oil accumulation (Cernac et al., 2006). To date, there is no evidence about the functional relationship between CHO1 and other members of the AP2-like family. However, our results suggest that other members might also bind to the ANT-recognition DNA element. In this sense, it is interesting to investigate phytohormone metabolism in mutants or transgenic plants of other AP2-like family members. These studies will uncover a more detailed function of AP2-like family members in plant growth regulation and development.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) Columbia accession was used as the wild type in this study. cho1-1, cho1-3, and cyp707a2-1 mutants were reported previously (Kushiro et al., 2004; Yamagishi et al., 2009). ga1-3 in the Columbia background (Tyler et al., 2004) was a gift from Dr. Tai-ping Sun (Duke University). To obtain dormant seeds, seeds were first sown and germinated on half-strength Murashige and Skoog medium buffered with 5 mM MES, pH 5.7. The seedlings were then transferred to pot moss vermiculite (1:2) and grown at 23°C under continuous white light as described previously (Kushiro et al., 2004). Freshly harvested seeds were immediately used for experiments or stored under constant conditions (23°C, approximately 30% humidity, in the dark) for after-ripening. To obtain different seed lots, plants were grown in pots placed in separate trays. For all experiments, surface-sterilized seeds were sown on 0.8% (w/v) water-agarose (LO-3 agarose; Takara), buffered with 5 mM MES, pH 5.7, and incubated at 23°C under continuous white light illumination (45 μmol m⁻² s⁻¹). Dry-stored seeds and imbibed seeds were frozen in liquid N₂ and stored at −80°C until use.

Seed Germination and Hormone-Responsiveness Test

To compare seed dormancy and germination, freshly harvested seeds and dry-stored seeds were sown and imbibed on water-agarose at 23°C under continuous white light as described above. Radicle emergence was scored daily as seed germination for up to 7 d. For ABA- and GA-responsiveness tests, freshly harvested seeds and dry-stored seeds were sown on wateragarose containing (+)-5'-ABA or GA₄ and imbibed at 23°C under continuous white light. Radicle emergence was scored after 5 or 7 d. In each germination test, approximately 50 seeds were used, and two experiments were carried out using two independent seed lots.

Measurement of Plant Hormones

Extraction and purification of ABA, GA₄, IAA, T, IP, SA, and JA were performed by solid-phase extraction as described (Dobrev and Kamínk, 2002). Stable isotope-labeled compounds used as internal standards in this study were as follows: D₆-ABA (Icon Isotopes); D₅-GA₄, D₅-IP, and D₅-IP (Olchemim); D₅-IAA and D₅-SA (Sigma-Aldrich); and D₅-JA (Tokyo Kasei). Extraction and purification of ABA were performed as described by Saika et al. (2007) except for using extraction buffer as 80% (v/v) methanol containing 1% (v/v) acetic acid. Ten milligrams of dry seeds or equivalent imbibed seeds was used for extraction of ABA.

For simultaneous measurement of ABA, GA₄, IAA, T, IP, SA, and JA, 50 mg of frozen seed samples was mixed with 500 μL of 80% (v/v) methanol containing 1% (v/v) acetic acid and internal standards (D₅-ABA, D₅-GA₄, D₅-IP, D₅-IAA, D₅-SA, and D₅-JA), mashed up with a TissueLyser (Qiagen), and then extracted at −30°C overnight. Samples were centrifuged at 14,000g for 10 min at 4°C, and the pellet was washed with 80% (v/v) methanol containing 1% (v/v) acetic acid. Combined supernatant extracts were evaporated to obtain extracts in water containing 1% acetic acid and applied to preequilibrated Oasis HLB column cartridges (Waters). After washing with 1 mL of water containing 1% (v/v) acetic acid, all hormones were eluted with 1 mL of 80% (v/v) methanol containing 1% (v/v) acetic acid. The eluting materials were evaporated to obtain extract in water containing 1% (v/v) acetic acid and applied to preequilibrated Oasis HLB column cartridges (Waters). After washing with the MCX cartridges with 1 mL of water containing 1% (v/v) acetic acid, the acidic and neutral fraction that contained ABA, GA₄, IAA, SA, and JA was eluted with 2 mL of methanol. Two hundred microliters of the acidic and neutral fraction was transferred, evaporated, and reconstituted with water for SA analysis. The MCX cartridges were further washed with 1 mL of water containing 5% (v/v) aqueous ammonia, and the basic fraction that contained T and IP was eluted with 2 mL of 60% (v/v) methanol containing 5% (v/v) aqueous ammonia. Acidic and neutral fractions were further applied to preequilibrated Oasis WAX column cartridges (Waters).
After washing the WAX cartridges with 2 mL of methanol and water containing 1% (v/v) acetic acid, the acidic fractions that contained ABA, GA₃, IAA, and JA were eluted with 2 mL of methanol containing 1% (v/v) acetic acid. Each eluting fraction was evaporated, reconstituted with water containing 1% (v/v) acetic acid, and injected into a LC-ESI-MS/MS apparatus (Agilent 6410; Agilent) equipped with a ZORBAX Eclipse XDB-C18 column (Agilent). The amount of each hormone was determined by spectrometer software (MassHunter version B.01.02). The LC conditions and MS parameters are listed in Supplemental Tables S1 and S2.

Gene Expression Analysis

Total RNA preparation from seeds, first-strand cDNA synthesis, and QRT-PCR were performed as described previously (Okamoto et al., 2006). Gene-specific primers and TaqMan probes were as described previously (Aizawa et al., 2004; Aizawa et al., 2006; Yano et al., 2008). The gene-specific primers for CHOI, AtGA3ox1, AtGA20ox3, DOG1, FUS1, PIL3, SPT, GA1, RGA, RGL2, RGL3, and RD29B were as follows: CHOI, 5'-TAACTCTCTTGGTGGTGCAG-3' and 5'-CACATGAGACTGGACAG-3'; AtGA3ox1, 5'-CTCTCCACACTCATCAATGTG-3' and 5'-GAC-TAACCAGCTCTGACAG-3'; AtGA20ox3, 5'-AGGTTGTAACCCAGTGACT-3' and 5'-TAGAGGAAAGTGGAGAGGATC-3'; DOGI, 5'-AGAT-CCGGATCCGGCTGACG-3' and 5'-CATCGTCAAGATTCTTCTCTGTCCTGACG-3'; FUS1, 5'-TTCTGGTCTGACAGGACCATCATA-3' and 5'-CTCAAGGAGCAGCCCAACCACAT-3'; PIL3, 5'-ATGCATATGTTTCCCTGGTCTC-3' and 5'-GGTTCTGAGTGAACGTGGACAG-3'; SPT, 5'-GGTTGGAATGGTGTGACT-3'.

Construction of Transgenic Lines

To construct p35S::CHOI, a 1.7 kb cDNA fragment of CHOI was amplified from a wild-type cDNA library by PCR using the following primers: 5'-CACCCTATTTCCACAAGTGAAGCACAAG-3' and 5'-TCTTCTTGAGCAGTACATCCTAGC-3'; RGL2, 5'-ATGAGAGAGCAGTACATCCTAGC-3' and 5'-CATCGTCAAGATTCTTCTCTGTCCTGACG-3'; RGL3, 5'-AGGCTCAAAAGAAGGCTTGAGAAG-3' and 5'-GTACTTTGAGGATCCATTGCGACAG-3'; RGA, 5'-ATGAGAGAGCAGTACATCCTAGC-3' and 5'-TCTTCTTGAGCAGTACATCCTAGC-3'. The oligonucleotides were annealed and labeled with [32P]dCTP and the Klenow fragment of DNA polymerase I (Takara). The binding reaction was performed by incubating 1 μg of purified CHOI-AP2 or CHOI-AP2m protein with 100 fmol of labeled probe in 20 μL of binding buffer (50 ng/mL poly[dI-dC], 20 μM Tris-HCl, pH 7.5, 3 μM MgCl₂, 50 μM KCl, 1 μM EDTA, 10% [v/v] glycerol, 0.3 mg/mL bovine serum albumin, and 1 μM dithiothreitol) at 4°C for 30 min. Excess amounts of nonlabeled DNA were used as competitors. The reaction mixtures were separated on a 4% polyacrylamide gel in 1× Tris-borate/EDTA buffer at 4°C and analyzed with BAS-2500 imaging analyzer (FujiFilm).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Expression analysis for ABI and RGA/GAI genes in 1-week-seeded stores.

Supplemental Figure S2. Expression analysis for CPS, KS, KO, and KAO genes before and after 24 h of imbibition in 4-week-seeded wild-type, cyp707a2-1, and cyp707a2-1 cho1-3 seeds.

Supplemental Figure S3. Effects of cho1 mutation on seed germination and gene expression in the GA-deficient ga3-3 mutant.

Supplemental Table S1. LC conditions for hormone profiling.

Supplemental Table S2. Parameters for LC-ESI-MS/MS analysis.

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Histochemical Analysis of GUS Reporter Expression

GUS staining was performed as described previously (Yamaguchi et al., 2001). Briefly, embryo and seed coat of pCHOI-GUS were separated using forceps after 16 h of incubation to facilitate infiltration of the substrate

5-bromo-4-chloro-3-indolyl-D-glucuronic acid (X-gluc). The separated tissues were vacuum infiltrated with 1 mL X-gluc solution and incubated at 37°C. To terminate the enzyme reaction, the tissues were incu•bated in 70% (v/v) ethanol at room temperature. To minimize diffusion of the hydrolyzed product, 1 mL potassium ferricyanide was used. To make 10-μm thin sections, X-gluc-stained embryos were fixed with 4% (w/v) paraformaldehyde in phosphate buffer (pH 7.0) overnight at 4°C and embedded in paraffin. Whole embryos were viewed with an SXZ12 dissecting microscope (Olympus) and photographed with a DP11 digital camera (Olympus). Thin sections were examined by bright-field microscopy using an Axioplan2 microscope (Zeiss), and the images were captured with a DP71 digital camera (Olympus).
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