High Temperature-Induced Abscisic Acid Biosynthesis and Its Role in the Inhibition of Gibberellin Action in Arabidopsis Seeds[1,2][C][W][OA]

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Suppression of seed germination at supraoptimal high temperature (thermoinhibiton) during summer is crucial for Arabidopsis (*Arabidopsis thaliana*) to establish vegetative and reproductive growth in appropriate seasons. Abscisic acid (ABA) and gibberellins (GAs) are well known to be involved in germination control, but it remains unknown how these hormone actions (metabolism and responsiveness) are altered at high temperature. Here, we show that ABA levels in imbibed seeds are elevated at high temperature and that this increase is correlated with up-regulation of the zeaxanthin epoxidase gene *ABA1/ZEP* and three 9-cis-epoxycarotenoid dioxygenase genes, *NCED2*, *NCED5*, and *NCED9*. Reverse-genetic studies show that *NCED9* plays a major and *NCED5* and *NCED2* play relatively minor roles in high temperature-induced ABA synthesis and germination inhibition. We also show that bioactive GAs stay at low levels at high temperature, presumably through suppression of GA 20-oxidase genes, *GA20ox1*, *GA20ox2*, and *GA20ox3*, and GA 3-oxidase genes, *GA3ox1* and *GA3ox2*. Thermoinhibition-tolerant germination of loss-of-function mutants of GA negative regulators, *SPINDLY* (*SPY*) and *RGL2*, suggests that repression of GA signaling is required for thermoinibition. Interestingly, ABA-deficient *aba2-2* mutant seeds show significant expression of GA synthesis genes and repression of *SPY* expression even at high temperature. In addition, the thermoinhibition-resistant germination phenotype of *aba2-1* seeds is suppressed by a GA biosynthesis inhibitor, paclobutrazol. We conclude that high temperature stimulates ABA synthesis and represses GA synthesis and signaling through the action of ABA in Arabidopsis seeds.

Seed germination is determined by a combination of the degree of dormancy and environmental factors such as light, oxygen, and temperature. Even after the loss of dormancy, seeds do not germinate in unfavorable conditions. Suppression of germination at supraoptimal high temperatures is called thermoinhibition (Reynolds and Thompson, 1971; Abeles, 1986; Gallardo et al., 1991). It has been shown that seed responsiveness to temperature is closely related to the level of dormancy in soil-buried seeds of winter and summer annuals (Baskin and Baskin, 1998). In the case of Arabidopsis (*Arabidopsis thaliana*), the maximal permissive temperature for germination rises gradually during an after-ripening period in summer, but germination is repressed by environmental temperature higher than the upper limit for germination. The seeds germinate in autumn when the temperature falls below the upper limit for germination (Baskin and Baskin, 1983). Therefore, the change in seed sensitivity to temperature plays an ecologically important role in the detection of the appropriate seasonal timing for germination in the field (Baskin and Baskin, 1998; Yoshioka et al., 1998, 2003).

Phytohormones abscisic acid (ABA) and GAs are well known to be involved in germination control. In lettuce (*Lactuca sativa*) seeds, reduction of ABA content is suppressed during imbibition at supraoptimal temperature, and de novo ABA biosynthesis is required for thermoinhibition (Yoshioka et al., 1998). Alleviation of thermoinhibition by exogenous GA has been reported on several plant species (Madakadze et al.,

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1993; Dutta et al., 1994; Carter and Stevens, 1998; Gonai et al., 2004). However, the effect of supraoptimal temperature on GA levels in the seeds and function of endogenous GA on thermoinhibition have not been clarified. We have isolated ABA-resistant germination mutants of Arabidopsis, *abi3-14* and *trg2*, as high temperature-resistant germination mutants, and showed that the seeds of the ABA-deficient mutant *aba1-1* and ABA-insensitive *abi1-1* were highly tolerant to thermoinhibition (Tamura et al., 2006). These reports suggest that ABA and GA are involved in the control of seed germination by temperature, but how these hormones mediate the high-temperature signal remains unknown.

ABA is a key regulator of seed development, dormancy, germination, and adaptive responses to abiotic stresses (Zeevaart and Creelman, 1988). Endogenous ABA content is a determinant of these physiological processes, and ABA-deficient mutants exhibit reduced seed dormancy and reduced drought tolerance (McCarty, 1995). Recent genetic and genomics analyses have revealed the molecular basis of the pathway and genes/enzymes involved in ABA biosynthesis and catabolism (Nambara and Marion-Poll, 2005). In plants, ABA is synthesized from carotenoids through the indirect pathway (Zeevaart and Creelman, 1988). Zeaxanthin is converted to all-trans-violaxanthin by two-step epoxidation catalyzed by zeaxanthin epoxidase (ZEP) in plastids (Marin et al., 1996; Thompson et al., 2000a; Agrawal et al., 2001; Audran et al., 2001). 9-cis-Epoxycarotenoid dioxygenase (NCED) catalyzes oxidative cleavage of the 9-cis isomer of violaxanthin or neoxanthin and produces a C_{15} product xanthoxin and a C_{25} metabolite (Tan et al., 1997; Burbidge et al., 1999; Qin and Zeevaart, 1999; Chernys and Zeevaart, 2000; Iuchi et al., 2000, 2001). The reactions following production of xanthoxin occur in the cytosol. Xanthoxin is converted to abscisic aldehyde by short-chain dehydrogenase/reductase (SDR; Cheng et al., 2002; González-Guzmán et al., 2002), and then abscisic aldehyde is oxidized to ABA by abscisic aldehyde oxidase (Seo et al., 2000a). On the other hand, ABA is inactivated through hydroxylation and conjugation (Zeevaart and Creelman, 1988; Nambara and Marion-Poll, 2005). Among these pathways, the ABA 8′-hydroxylation pathway is shown to be the regulatory step in a variety of physiological processes. In Arabidopsis, *CYP707A1* to *CYP707A4* encode ABA 8′-hydroxylase (Kushiro et al., 2004; Saito et al., 2004). Gene expression and reverse-genetic analyses indicated that *CYP707A2* has a major role in the rapid decrease in ABA content in the first 6 to 12 h of imbibition and suggest that *CYP707A1* to *CYP707A3* are involved in seed germination (Kushiro et al., 2004; Okamoto et al., 2006).

NCED was first identified by analysis of the maize (*Zea mays*) viviparous germination mutant *vp14* (Tan et al., 1997), and is considered to be a key regulatory enzyme in ABA biosynthesis (Qin and Zeevaart, 1999, 2002; Thompson et al., 2000b; Iuchi et al., 2001). *NCED* is a multigene family in all plant species examined (Tan et al., 1997; Burbidge et al., 1999; Qin and Zeevaart, 1999; Chernys and Zeevaart, 2000; Iuchi et al., 2000, 2001). In Arabidopsis, phylogenetic analysis has suggested that five genes (*NCED2*, *NCED3*, *NCED5*, *NCED6*, and *NCED9*) are possibly involved in ABA biosynthesis, and they have been shown to catalyze NCED reaction in vitro, with the exception of NCED5 (Iuchi et al., 2001). Each member of the *NCED* family plays a unique regulatory role in specific environmental responses and developmental processes. Expression of the *NCED3* gene is induced by drought stress, and it plays a major role in ABA synthesis in response to the stress (Iuchi et al., 2001; Ruggiero et al., 2004). *NCED6* expression is regulated in a photoreversible manner via phytochrome in seeds, and it plays a role in far-red light-induced suppression of seed germination (Seo et al., 2006). *NCED6* and *NCED9* play a major role in the control of seed development, including dormancy (Lefebvre et al., 2006). Real-time PCR and reporter gene expression analyses indicated that *NCED2* and *NCED5* are expressed in specific tissues and developmental stages (Tan et al., 2003), but physiological functions of these two *NCED* genes remain obscure.

GA is essential for stem elongation and flowering and is also a key regulator of seed germination. Previous studies have suggested that GA responses are regulated by the modulation of GA levels and by altering the ability of cells to respond to the hormone (Richards et al., 2001). Biologically active GAs, such as GA_1 and GA_4, are tetracyclic diterpenoids synthesized from geranylgeranyl diphosphate, and the GA biosynthesis pathway can be divided into three stages (Hedden and Kamiya, 1997; Olszewski et al., 2002). First, geranylgeranyl diphosphate is cyclized to *ent*-kaurene in plastids, then *ent*-kaurene is oxidized to GA_{12} by microsomal cytochrome P450 monooxygenases, and, finally, GA_{12} is converted to active GA in cytosol by 2-oxoglutarate-dependent dioxygenases, GA 20-oxidase and GA 3-oxidase. Bioactive GAs are well known to be deactivated by a 2-oxoglutarate-dependent dioxygenase, GA 2-oxidase, which also catabolizes immediate precursors of active GAs. Recently, additional deactivation mechanisms by a cytochrome P450 monooxygenase and methyltransferases were reported (Zhu et al., 2006; Varbanova et al., 2007). Environmental signals regulate GA level through modulation of the late steps of GA biosynthesis and catabolism (Olszewski et al., 2002). Light signals mediated by phytochromes are critical environmental determinants for photoblastic seed germination (Borthwick et al., 1952; Shinomura et al., 1996). GA levels are regulated by phytochrome in lettuce and Arabidopsis seeds, in which genes encoding GA 3-oxidase (*GA3ox*) and GA 2-oxidase (*GA2ox*) are regulated in a photoreversible manner (Toyomasu et al., 1998; Yamaguchi et al., 1998). In Arabidopsis, *GA3ox1* and *GA3ox2* are up-regulated and *GA2ox2* is down-regulated by red light, which promotes seed germination. In addition to

light, exposure of imbibed seeds to low temperature promotes seed germination of many plant species. In Arabidopsis, expression of GA 20-oxidase genes, *GA20ox1* and *GA20ox2*, and *GA3ox1* was reported to be up-regulated by low temperature in darkness, and germination of *GA3ox1* mutant (*ga4-2*) seeds are not stimulated by low temperature (Yamauchi et al., 2004).

Genetic studies have identified several GA-signaling components in Arabidopsis, some of which play a role in GA-induced seed germination, as shown by their loss-of-function mutant phenotypes (Olszewski et al., 2002). The DELLA subfamily of GRAS proteins and SPINDLY (SPY), a Ser/Thr *O*-linked *N*-acetylglucosamine transferase, acts as negative regulators of GA-dependent seed germination (Jacobsen and Olszewski, 1993; Jacobsen et al., 1996; Lee et al., 2002; Wen and Chang, 2002). SPY is thought to increase the activity of DELLA proteins by *N*-acetylglucosamine modification (Silverstone et al., 2007). SPY also acts as a positive regulator of cytokinin signaling (Greenboim-Wainberg et al., 2005). In rice (*Oryza sativa*), the *SPY* ortholog *OsSPY* works as a negative regulator of GA signaling as in Arabidopsis and may also function as a negative regulator of brassinosteroid biosynthesis (Shimada et al., 2006). One of the five DELLA protein genes in Arabidopsis, *RGL2*, plays a major role on GA-dependent germination of the seeds (Lee et al., 2002; Tyler et al., 2004). The germination phenotypes of multiple DELLA protein mutants in the *ga1-3* background indicated that *GAI*, *RGA*, and *RGL1* enhance the function of *RGL2* (Cao et al., 2005).

Seed germination is considered to be determined by the balance of the negative and positive effects of ABA and GA, respectively. Interaction between ABA and GA signaling has been well studied in cereal aleurone cells as a model system and recent reports shed light on the interaction between ABA and GA in their metabolism. Gonai et al. (2004) suggested that exogenously applied GA_3 alleviates thermoinhibition of lettuce seeds by enhancing the catabolism of ABA. Seo et al. (2006) indicated that endogenous ABA suppresses GA biosynthesis in developing seeds and in far-red light-treated mature seeds during imbibition through suppression of *GA20ox* and *GA3ox* genes in Arabidopsis. Zentella et al. (2007) showed that the transcript levels of *GA20ox1* in Arabidopsis plants are significantly reduced by exogenous ABA.

In this study, we show that both enhancement of ABA synthesis and suppression of GA synthesis and signaling during imbibition are required for thermoinhibition of Arabidopsis seeds. Our data suggest that high temperature enhances ABA synthesis by stimulating expression of *ZEP* and three of the five *NCED* genes, *NCED2*, *NCED5*, and *NCED9*, and suppresses GA synthesis through suppression of *GA20ox* and *GA3ox* genes. In addition, high temperature may suppress GA signaling by enhancing *SPY* gene expression and maintaining *RGL2* gene expression. Our data also suggest that GA synthesis and signaling are suppressed through the action of ABA in the seeds at high temperature.

RESULTS

ABA Synthesis in Imbibed Seeds Is Responsible for Thermoinhibition

To examine whether ABA synthesis is responsible for thermoinhibition of Arabidopsis seeds, we first applied an ABA biosynthesis inhibitor to Columbia (Col) accession seeds during imbibition at supraoptimal temperatures. We used fluridone, which inhibits phytoene desaturase in the carotenoid biosynthesis pathway, as a potent inhibitor of ABA biosynthesis. As shown in Figure 1A, 10 μM fluridone rescued germination of the seeds at supraoptimal high temperature (34°C). This suggests that thermoinhibition of Arabidopsis seeds requires ABA biosynthesis de novo after the onset of imbibition as observed in lettuce and several winter annual species (Yoshioka et al., 1998). To examine whether the ABA level is altered by high temperature, we quantitated ABA in the imbibed seeds by gas chromatography (GC)-mass spectrometry (MS; Fig. 1B). At optimal temperature for germination (22°C), ABA levels decreased remarkably by 6 h of imbibition and continued to decrease until 48 h as reported previously (Kushiro et al., 2004; Millar et al., 2006; Okamoto et al., 2006). At 36 h after the start of imbibition, when the radicle starts to emerge, the ABA level decreased to one-tenth of the initial content (Fig. 1B). The decrease within the first 6 h also occurred at 34°C, but the reduction speed slowed down after 6 h and the level turned to increase gently after 12 h. The application of 10 μM fluridone suppressed the increase of ABA completely at 34°C, and the levels were comparable to those at 22°C. These results suggest that high temperature stimulates synthesis of ABA in Arabidopsis seeds.

High Temperature Up-Regulated ABA Biosynthesis Genes, *ABA1/ZEP* and *NCED*, and Down-Regulated the ABA Catabolism Gene *CYP707A*

To investigate the regulation step of ABA synthesis by high temperature, we analyzed the expression of genes that code for ABA biosynthesis enzymes by the quantitative reverse transcription (RT)-PCR method. ABA biosynthesis enzymes, zeaxanthin epoxidase (*ABA1/ZEP*), short-chain dehydrogenase/reductase (*ABA2/SDR*), and abscisic aldehyde oxidase (*AAO3*) have been reported to be coded by single genes in Arabidopsis (Marin et al., 1996; Seo et al., 2000b; Cheng et al., 2002). Dry seeds contained a considerable level of *ABA1/ZEP* transcript, and the transcript decreased significantly in the first 12 h of imbibition at 22°C and continued to decrease until 36 h (Fig. 2). At 34°C, the transcript also decreased, but reduced the rate of decrease after 3 h and kept considerably higher level than at 22°C. *ABA1/ZEP* transcript levels were approximately 2 times higher at 34°C than at 22°C at 12 h when the reduction rate of the ABA level slowed down (Fig. 1B). *ABA2/SDR* transcript levels slightly increased during imbibition both at 22°C and 34°C,

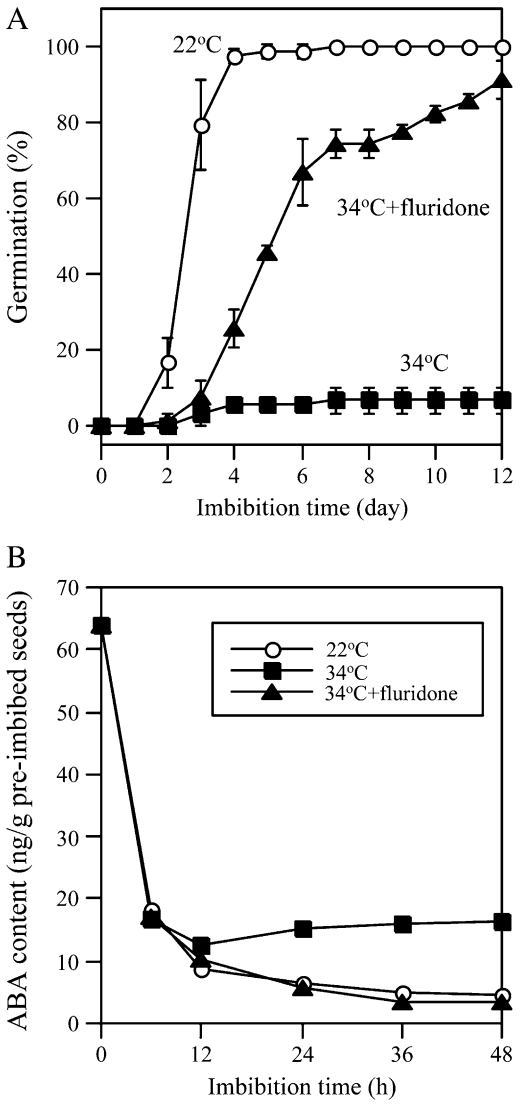

Figure 1. ABA biosynthesis in imbibed seeds is required for thermoinhibition. After-ripened Col seeds were imbibed at 22°C (white circles), 34°C (black squares), and at 34°C in the presence of 10 μM fluridone (black triangles) under continuous illumination. A, Effect of ABA biosynthesis inhibitor fluridone on thermoinhibition. Error bars show SD ($n = 3$). B, Effects of high temperature and ABA biosynthesis inhibitor fluridone on endogenous ABA levels in imbibed seeds. ABA levels were quantified by GC-MS using two independent seed batches and similar results were obtained.

and the levels were not remarkably different between the two temperature conditions. The levels of *AAO3* transcript were low throughout the imbibition period, and there was no significant difference between the two temperatures. These results suggest that *ABA2/SDR* and *AAO3* genes have no regulatory function on modulation of ABA level in response to high temperature in seeds.

In Arabidopsis, five *NCED* genes are reported to be involved in ABA biosynthesis, and NCED2, NCED3, NCED6, and NCED9 proteins have been reported to have the NCED activity to produce xanthoxin in vitro (Iuchi et al., 2001). To identify the enzyme activity of NCED5, the *NCED5* gene was expressed in *Escherichia coli* to obtain glutathione *S*-transferase (GST)-fused recombinant protein, and the proteins were incubated with 9′-cis-neoxanthin. HPLC analysis of the reaction products indicated that NCED5 catalyzes the cleavage of 9′-cis-neoxanthin to produce a C_{25} product (Supplemental Fig. S1A). The enzyme activity of the GST-NCED5 fusion protein was further confirmed by GC-MS analysis in which cis-xanthoxin was identified as another product from 9′-cis-neoxanthin (Supplemental Fig. S1B). In both experiments, GST-NCED3 recombinant protein was used as a control to give similar results (Supplemental Fig. S1, C and D). These results confirm that *NCED5* encodes an active NCED enzyme, which is probably engaged in ABA biosynthesis.

Relatively low levels of *NCED3* and *NCED6* transcripts were found in dry seeds, and their levels were not changed significantly by imbibition at 22°C and at 34°C (Fig. 2). The level of *NCED2* transcript was also low in dry seeds and did not increase during imbibition at 22°C. In contrast, a marked increase in the transcript level was detected at 12 h at 34°C, and the expression continued to increase until 36 h. In dry seeds, transcript levels of *NCED5* and *NCED9* were 2.5 to 6 times more abundant than those of other *NCED*s. They increased transiently and decreased to low levels during imbibition at 22°C. At 34°C, *NCED5* transcript level did not change significantly and the transcript was 4 to 8 times more abundant than at 22°C at 24 h and later time points. *NCED9* expression peaked at 3 h at 34°C, and its level was similar to that at 22°C at this point. However, the rate of decrease was significantly slower at 34°C than at 22°C and it ceased to decrease after 12 h. The *NCED9* transcript was the most abundant among five *NCED*s throughout the imbibition period at 34°C. *NCED2* and *NCED9* transcript levels were significantly higher at 34°C than at 22°C at 12 h after the start of imbibition when the decrease rate of ABA slowed down at 34°C (Fig. 1B). These observations suggest that *NCED2*, *NCED5*, and *NCED9* contribute to enhanced ABA biosynthesis ability in the seeds at supraoptimal temperature.

We analyzed transcript levels of ABA 8′-hydroxylase genes, *CYP707A1*, *CYP707A2*, and *CYP707A3*, and found that the expression of all three genes was significantly reduced at 34°C (Fig. 2). Expression of *CYP707A2* was most abundant among the three ABA 8′-hydroxylase genes and was transiently increased during imbibition even at 34°C. *CYP707A2* transcript in dry and imbibed seeds might be translated in the first 6 h and contribute to ABA catabolism in the thermoinhibited seeds as well as in germinating seeds because the reduction of ABA levels in the initial 6 h was commonly observed at 22°C and at 34°C, and the decrease rate of ABA at 34°C in the presence of fluridone was comparable to that at 22°C (Fig. 1B). ABA catabolism by the 8′-hydroxylation pathway may also work in thermoinhibited lettuce seeds because dihydrophaseic acid, which is an ABA catabolite in the

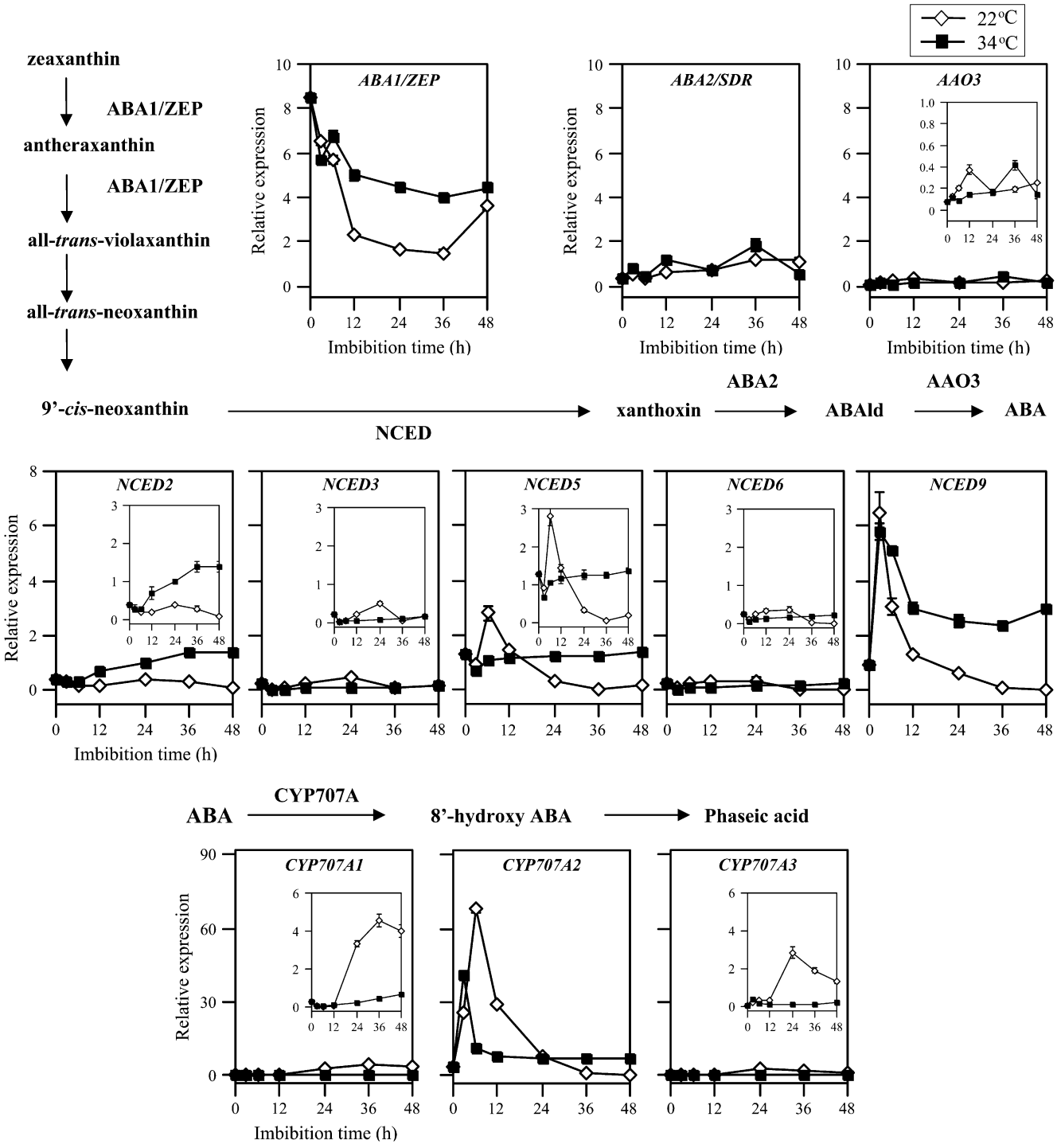

Figure 2. Effect of high temperature on expression of ABA biosynthesis and catabolism genes in imbibed seeds. Total RNA was prepared from after-ripened Col seeds imbibed at 22°C (white diamonds) or at 34°C (black squares) under continuous illumination. Transcript levels were quantified by quantitative RT-PCR as described in "Materials and Methods." Experiments were repeated twice with different seed batches and similar results were obtained. Data from one of the replicates are shown. Values are means with SDS from three measurements.

8′-hydroxylation pathway, is accumulated in the seeds imbibed at supraoptimal temperature (Chiwocha et al., 2003). ABA content in thermoinhibited seeds showed only a slight increase after 12 h of imbibition (Fig. 1B). This fine tuning of the ABA level may be achieved by the balance between synthesis and catabolism since *CYP707A2* transcript became more abundant at 34°C than at 22°C after 36 h of imbibition (Fig. 2).

NCED2, *NCED5*, and *NCED9* Have Unequally Redundant Functions on Thermoinhibition and ABA Level of the Seeds

To evaluate the physiological function of ABA biosynthesis genes on thermoinhibition, we analyzed germination phenotypes of their loss-of-function mutants. The seeds of *aba1-1*, *aba2-1*, and *aao3-4* showed apparent thermoinhibition resistance at 34°C (Supplemental Fig. S2B). *ABA1/ZEP*, *ABA2/SDR*, and *AAO3*, which are involved in the indirect pathway, should be responsible for ABA biosynthesis in Arabidopsis seeds also at supraoptimal temperature conditions. T-DNA insertion lines for *NCED2*, *NCED5*, and *NCED9* were obtained from the Arabidopsis Biological Resource Center (ABRC; Alonso et al., 2003), and we analyzed the corresponding T-DNA insertion homozygotes (Supplemental Fig. S2A). The seeds of *nced9* mutant alleles showed considerable tolerance to thermoinhibition, but those of *nced2* and *nced5* showed no apparent phenotypes (Fig. 3A; Supplemental Fig. S2B). This suggests that *NCED9* has a major role in thermoinhibition. To further evaluate the contribution of each *NCED* gene to thermoinhibition, we generated double and triple mutants of *NCED2*, *NCED5*, and *NCED9*, and investigated their germination phenotypes at supraoptimal temperature conditions. The seeds of the *nced5-1 nced9-1* double mutant showed higher thermoinhibition tolerance than *nced9-1*, but the *nced2-1 nced5-1* double mutant showed similar germination to its parents and the wild type (Fig. 3A). Seeds of the *nced2-1 nced5-1 nced9-1* triple mutant showed higher thermoinhibition tolerance than the double mutants. These results suggest that *NCED5* and *NCED2* genes have relatively minor, but unequally redundant (Briggs et al., 2006), function with *NCED9*, and their up-regulation by high temperature is critical for thermoinhibition of Arabidopsis seeds.

To evaluate the contribution of each *NCED* gene on de novo ABA biosynthesis during thermoinhibition, we analyzed the endogenous ABA level in *nced* mutants. The levels of ABA in dry seeds of single and multiple *nced* mutants were not lower, but rather higher in some cases, than those in wild-type seeds (Fig. 3B). It is possible that the mutations are compensated or overcompensated by other *NCED* genes during seed development. When seeds were imbibed at 34°C for 24 h, the *nced9-1* single mutant, double mutants except for *nced2-1 nced5-1*, and the triple mutant reduced ABA content considerably; ABA levels were comparable to those of wild-type seeds imbibed at 22°C for 24 h. The ABA content in the triple mutant was lower than that of the double mutants. These results indicate that *NCED2*, *NCED5*, and *NCED9* are responsible for ABA biosynthesis in the seeds imbibed at supraoptimal temperature conditions and support the idea that their up-regulation is a key regulatory point in ABA biosynthesis and germination inhibition by high temperature.

To investigate contributions of ABA biosynthesis in the embryo and the endosperm to thermoinhibition, we divided the 24-h imbibed Col seeds into the embryo and the remaining part, including endosperm and testa, and RNA was extracted from each tissue for quantitative RT-PCR. Quantification of an embryo-specific marker gene, *GA3ox2* (Yamaguchi et al., 2001), and an endosperm-specific marker gene, *EPR1* (Dubreucq et al., 2000; Penfield et al., 2004), indicated that there was no cross-contamination between the two fractions (Supplemental Fig. S3). In both the embryo and the endosperm, all three *NCED* transcripts were detected, and they were more abundant at 34°C than at 22°C (Fig. 4). Expression of *NCED9* was predominant over the other two *NCED* genes in the embryos from the seeds imbibed at 34°C. In the endosperm, on the other hand, *NCED5* transcript was most abundant among the three *NCED* genes. ABA synthesized in embryo may have a major contribution to germination inhibition at supraoptimal temperatures because seeds of the *nced9-1* single mutant showed thermoinhibition tolerance, but those of the *nced5-1* single mutant and the *nced2 nced5* double mutant were intolerant (Fig. 3A; Supplemental Fig. S2B).

Alleviation of Thermoinhibition by Exogenous GA and Suppression of Endogenous GA Level at High Temperature

GA is known to be essential for germination of Arabidopsis seeds (Koornneef and van der Veen, 1980). To investigate the effect of GA on thermoinhibition of Arabidopsis seeds, seeds were imbibed at 34°C in the presence of GA_3. Thermoinhibition of Col seeds was alleviated by 50 μM GA_3 (Fig. 5A). This result suggests that enhancement only of ABA biosynthesis in seeds is not sufficient, but suppression of an increase in bioactive GA level is also required for thermoinhibition of Arabidopsis seeds. To evaluate this possibility, we determined endogenous GA levels during imbibition at optimal and supraoptimal temperature conditions. In Arabidopsis, GA_4 is a main active GA and GA_1 is a minor component during seed germination. At 22°C, both active GAs increased in abundance at 12 h after the start of imbibition, and the level of GA_4 at 36 h was 5 to 6 times higher than the initial level (Fig. 5B). At 34°C, on the other hand, no increase in the levels of GA_4 and GA_1 was observed. Germination may be inhibited not only by ABA but also by the absence of active GAs at supraoptimal temperature conditions.

Suppression of GA Biosynthesis Gene Expression at High Temperature

To investigate the regulation mechanism of GA biosynthesis by high temperature in seeds, we analyzed the expression of *GA 20-oxidase* (*GA20ox1*, *GA20ox2*, and *GA20ox3*) and *GA 3-oxidase* (*GA3ox1* and *GA3ox2*) genes that are involved in the late steps of GA biosynthesis in Arabidopsis seeds (Ogawa et al., 2003). Expression of the three *GA20ox* genes was

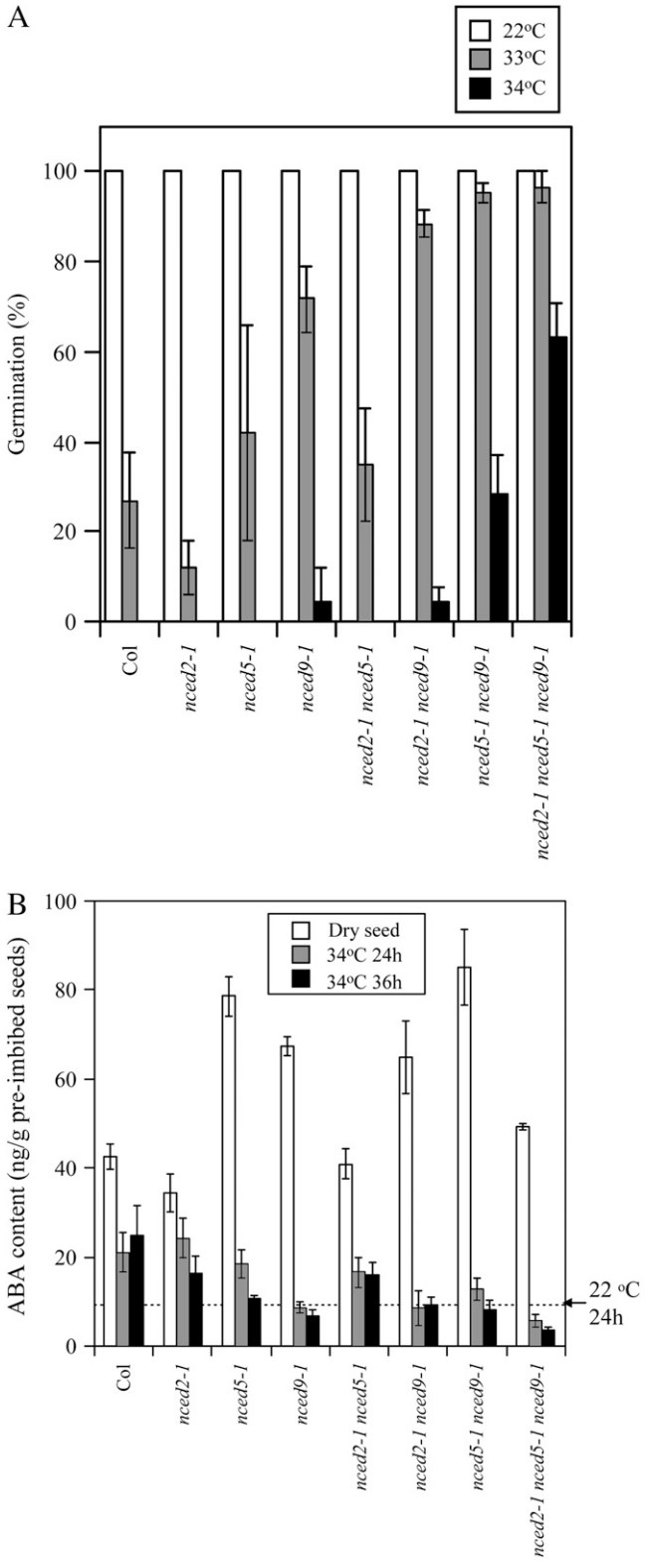

Figure 3. *NCED2*, *NCED5*, and *NCED9* are unequally redundant on thermoinhibition and ABA synthesis in the seeds. A, Thermoinhibition resistance of single and multiple mutants of *nced2*, *nced5*, and *nced9*. Seeds were imbibed at 22°C (white bars), 33°C (gray bars), and 34°C (black bars) under continuous illumination for 5 d. Error bars show SD ($n = 3$). Germination assays were confirmed in three independent batches. B, ABA content in seeds of *nced* single and multiple mutants. ABA was extracted from dry seeds (white bars) and the seeds imbibed at 34°C for 24 h (gray bars) and 36 h (black bars) under continuous illumination. ABA was quantified by liquid chromatography-tandem mass spectrometry. Dotted line indicates ABA content in wild-type seeds imbibed at 22°C for 24 h. Error bars show SD ($n = 3$).

induced significantly within 6 h of imbibition at 22°C (Fig. 6). The transcript level of *GA20ox1* was maintained from 6 to 48 h after the start of imbibition. *GA20ox2* and *GA20ox3* transcript levels peaked at 6 h and decreased thereafter. *GA20ox3* showed the highest expression among the three *GA20ox* genes at 6 h, but its expression decreased to very low level at 36 h. At 34°C, on the other hand, expression of all three *GA20ox* genes was greatly suppressed (Fig. 6). Expression of the two *GA3ox* genes was also up-regulated within the first 6 h of imbibition at 22°C. *GA3ox1* mRNA level was maintained from 6 h to 48 h of imbibition, but *GA3ox2* expression showed a peak at 24 h (Fig. 6). At 34°C, expression of the two *GA3ox* genes was greatly suppressed, as observed for *GA20ox* genes. These results suggest that high temperature repressed bioactive GA synthesis through suppression of *GA20ox* and *GA3ox* gene expression in Arabidopsis seeds. Suppression of *GA3ox1* and *GA3ox2* expression by high temperature may be critical for thermoinhibition of Arabidopsis seeds.

To evaluate the contribution of GA deactivation to thermoinhibition, we analyzed transcript levels of *GA2ox* genes in germinating and thermoinhibited seeds. Quantitative RT-PCR experiments indicated that expression of *GA2ox2*, whose expression is photoreversibly regulated and most highly expressed in seeds among the eight *GA2ox* genes (Oh et al., 2006; Seo et al., 2006), stayed at low level during imbibition even at 34°C (Fig. 6). Other *GA2ox* genes (*GA2ox1*, *GA2ox3*–*GA2ox8*) also showed low expression levels, and there was no difference in expression levels between germinating and thermoinhibited seeds (data not shown). These results suggest that active GA levels are dependent on their synthesis in thermoinhibited seeds in the light.

GA Negative Regulators, *SPY* and *RGL2*, Are Involved in Thermoinhibition of Arabidopsis Seeds

GA response is repressed by negative regulators, DELLA proteins and SPY, and GA signal is transduced through degradation of DELLA protein (Jacobsen and Olszewski, 1993; Jacobsen et al., 1996; Sun and Gubler, 2004). To evaluate the role of GA negative regulators in thermoinhibition of Arabidopsis seeds, we investigated thermoinhibition resistance of the loss-of-function mutants of them. Seeds of *spy-4* showed clear resistance to thermoinhibition (Fig. 7B). T-DNA insertion lines for all five DELLA protein genes were available at the ABRC, and we analyzed the homozygous T-DNA insertion mutants. Seeds of *gai*, *rga*, *rgl1*, and *rgl3* knockout lines showed no clear resistance to thermoinhibition (data

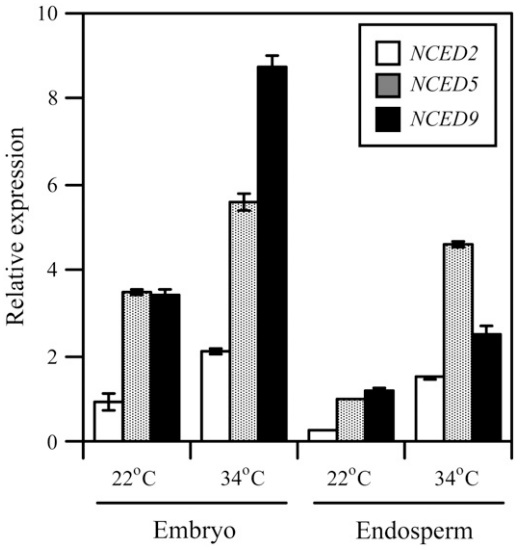

Figure 4. Expression of *NCED2*, *NCED5*, and *NCED9* in embryo and endosperm of thermoinhibited seeds. Total RNA was prepared from embryo and endosperm/testa separated from Col seeds imbibed at 22°C or at 34°C under continuous illumination for 24 h. Transcript levels of *NCED2* (white bars), *NCED5* (gray bars), and *NCED9* (black bars) were quantified by quantitative RT-PCR with a set of primers and a Taq-Man probe specific to each gene. To compare transcript levels across different *NCED* genes, standard curves were generated using a series of known concentration of target sequences (genomic DNA). Results for each tissue were normalized to the amplification of the 18S rRNA control and the amount of total RNA extracted from each tissue of one seed. Experiments were repeated three times with different seed batches and similar results were obtained. Data from one of the replicates are shown. Values are means with SDs from three measurements.

not shown). Seeds of *rgl2* alleles showed slightly, but significantly, higher germination percentages at supraoptimal temperature conditions (Fig. 7B). These germination studies suggest that *SPY* and *RGL2* are involved in the thermoinhibition mechanism of Arabidopsis seeds.

To investigate whether the expressions of GA negative regulators are regulated by high temperature or not, we quantified the transcripts in imbibed seeds by quantitative RT-PCR. *SPY* gene expression was induced rapidly and transiently during imbibition at 22°C and reduced to a low level at 48 h (Fig. 7C). Expression of *SPY* was also rapidly induced by imbibition at 34°C and maintained at high level by 48 h. *RGL2* showed significant induction by imbibition at 22°C and its expression peaked at 12 h. At 34°C, induction was delayed and a peak was observed at 36 h. These gene expression studies suggest that *SPY* and *RGL2* work as negative regulators of GA signaling in thermoinhibited seeds and that high temperature enhances suppressive function of *SPY* by maintenance of its transcript level.

High Temperature Suppresses GA Action through ABA

Recent studies have suggested that ABA suppresses GA levels in dark imbibed seeds treated with a far-red light pulse and in seedlings of Arabidopsis (Seo et al., 2006; Zentella et al., 2007). We therefore hypothesized that GA action might be suppressed by high temperature through the action of ABA in thermoinhibited seeds. To examine this possibility, we first analyzed germination of ABA-deficient mutant seeds imbibed at supraoptimal temperature in the presence of a GA biosynthesis inhibitor, paclobutrazol (PAC). The high temperature-resistant germination phenotype of *aba2-1* mutant seeds was almost completely suppressed by PAC and the suppression was recovered by GA_3 (Fig. 8A), demonstrating that GA biosynthesis is required for germination of *aba2-1* seeds at supraoptimal temperature. These observations suggest that GA action is

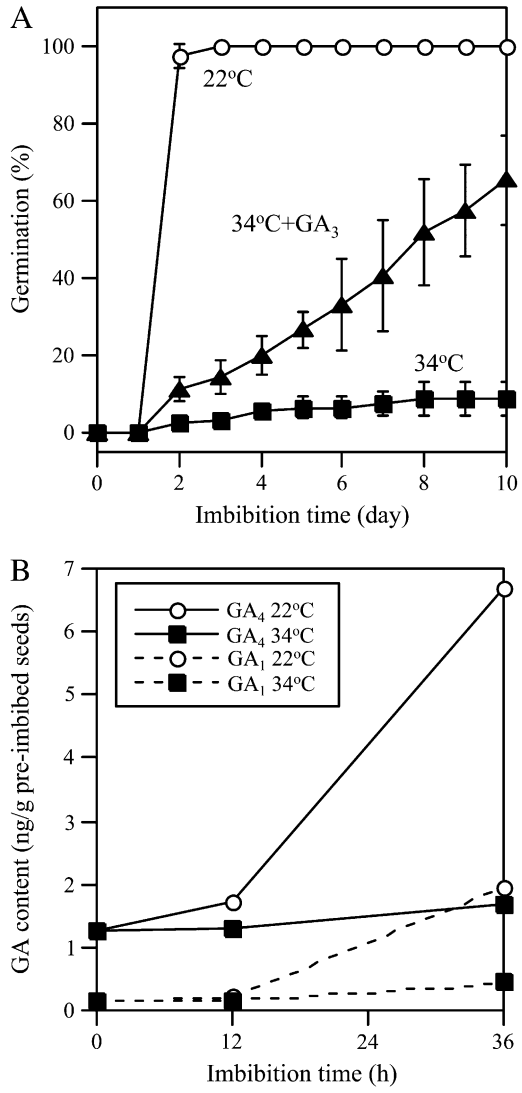

Figure 5. Alleviation of thermoinhibition by exogenous GA and suppression of bioactive GA levels by high temperature. A, Effect of exogenous GA on thermoinhibition. After-ripened Col seeds were imbibed at 22°C (white circles), 34°C (black squares), and 34°C with 50 μM GA_3 (black triangles) under continuous illumination. Error bars show SD (n = 3). B, Effect of temperature on bioactive GA levels in seeds. GA levels were quantified by GC-MS using two independent seed batches and similar results were obtained.

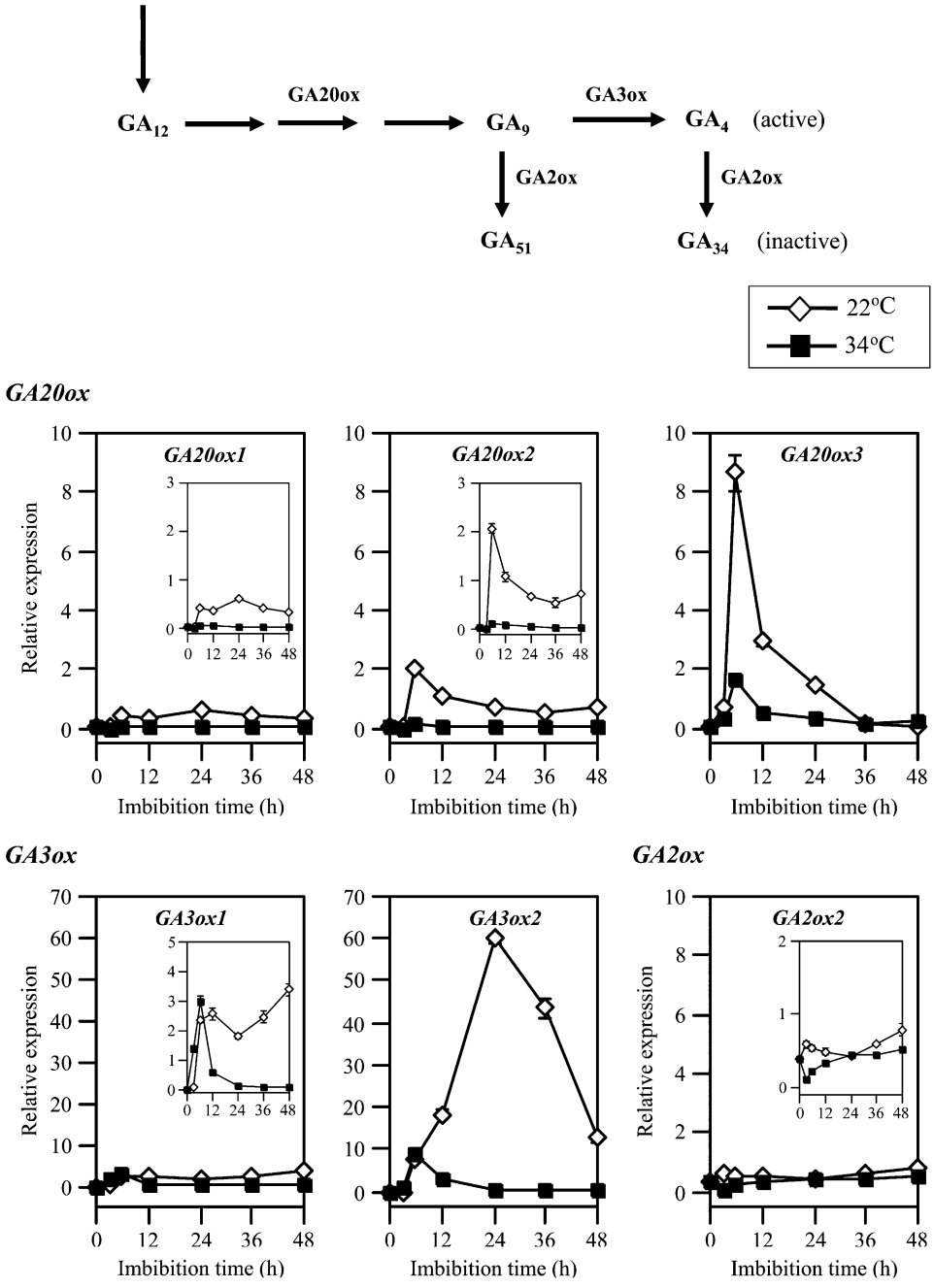

Figure 6. Expression of GA biosynthesis and deactivation genes was suppressed at high temperature. Total RNA was prepared from afterripened Col seeds imbibed at 22°C (white diamonds) or at 34°C (black squares) under continuous illumination. Transcript levels were quantified by quantitative RT-PCR as described in "Materials and Methods." Experiments were repeated twice with similar results. Data from one of the replicates are shown. Values are means with SDS from three measurements.

derepressed in the absence of ABA at supraoptimal temperature conditions. In other words, high temperature may regulate GA action through the action of ABA. To evaluate this possibility, expression of GA synthesis and signaling genes in *aba2-2* mutant seeds was analyzed at supraoptimal temperature conditions. In this experiment, germination of wild-type (Col) seeds was almost completely suppressed (5.2% germination after 7 d of imbibition), and the seeds of *aba2-2* showed thermoinhibition tolerance at 33°C (98.0% germination after 7 d of imbibition). Quantitative RT-PCR analysis indicated that the transcript levels of *GA3ox1* and *GA3ox2* in 24-h imbibed *aba2-2* seeds were similar between 22°C and 33°C (Fig. 8B). Transcript levels of *GA20ox2* and *GA20ox3* in *aba2-2* seeds imbibed at 33°C for 12 h were less abundant than those at 22°C, but more abundant than those of wild-type seeds imbibed at 33°C. These results suggest that expression of *GA3ox1* and *GA3ox2* genes is suppressed by ABA, and expression of *GA20ox2* and *GA20ox3* genes is suppressed by both ABA and other factors affected by temperature. These observations suggest that bioactive GA content in Arabidopsis seeds may be suppressed mainly through the action of ABA, which is enhanced by high temper-

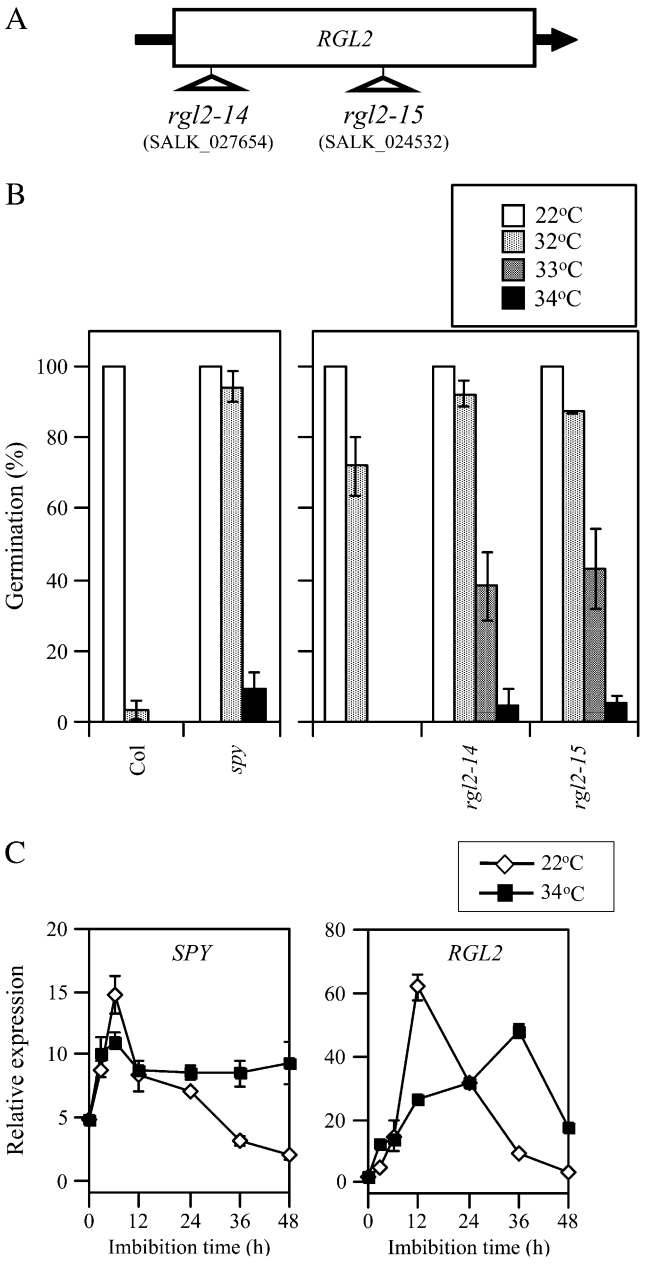

Figure 7. Thermoinhibition-resistant germination of *spy-4* and *rgl2* seeds and expression of the GA negative regulator genes at high temperature. A, T-DNA insertion sites in the *RGL2* gene. Arrow indicates 5′ to 3′ direction of the gene. B, Effect of imbibition temperature on germination of *spy* and *rgl2* mutant seeds. Seeds were imbibed at 22°C (white bars), 32°C (light gray bars), 33°C (dark gray bars), and 34°C (black bars) under continuous illumination for 5 d. Error bars show SD ($n = 3$). C, Effect of temperature on expression of *SPY* and *RGL2* genes. Total RNA was prepared from after-ripened Col seeds imbibed at 22°C (white diamonds) or at 34°C (black squares) under continuous illumination. Transcript levels were quantified by quantitative RT-PCR as described in "Materials and Methods." Experiments were repeated twice with similar results. Data from one of the replicates are shown. Values are means with SDS from three measurements.

ature in the light (Fig. 9). We also found that the transcript level of *SPY* in *aba2-2* seeds was not enhanced by high temperature (Fig. 8B). These results suggest that expression of *SPY* genes is up-regulated by ABA (Fig. 9).

DISCUSSION

Regulation of ABA Biosynthesis by High Temperature in Seeds

Pharmacological studies have suggested that ABA biosynthesis is required for thermoinhibition of the seeds of lettuce, chickweed, and several winter annual species when imbibed in darkness (Yoshioka et al., 1998, 2003). In this study, we confirmed that ABA synthesis de novo after the start of imbibition is essential for germination inhibition of Arabidopsis seeds at supraoptimal temperature in the light (Figs. 1 and 9). We have reported genetic evidence that shows that ABA has a critical function in thermoinhibition of Arabidopsis seeds and that not all, but a subset of ABA signaling components, including *ABI1*, *ABI3*, and possibly *ABI2*, are required for germination inhibition at high temperature (Tamura et al., 2006). These observations suggest that seeds have a specific mechanism to modulate ABA content in response to high temperature and use a specific ABA-signaling pathway for germination inhibition.

Our aim in this study was to elucidate how ABA and GA action (metabolism and responsiveness) is controlled by high temperature. We observed the up-regulation of *ABA1/ZEP* expression before the increase of ABA level in thermoinhibited seeds (Figs. 1 and 2), suggesting that enhanced expression of *ABA1/ZEP* contributes to the elevated ABA production at high temperature. In *Nicotiana plumbaginifolia*, overexpression of *NpZEP* by the cauliflower mosaic virus 35S promoter causes an increase in endogenous ABA content in the seed and delays germination (Frey et al., 1999). They recently demonstrated, however, that ABA content does not correlate with the level of *ABA1/ZEP* expression in transgenic *N. plumbaginifolia* seeds in the *NpZEP* loss-of-function mutant background (Frey et al., 2006). These results indicate that altered *ABA1/ZEP* expression alone is not sufficient to affect the level of ABA accumulation. However, it would still be possible for altered *ABA1/ZEP* expression to contribute to the change in ABA level if a downstream limiting step is coregulated. Analysis of endogenous epoxycarotenoids and tissue- and developmental stage-specific suppression/overexpression studies are required to evaluate the impact of *ABA1/ZEP* expression on the level of ABA during thermoinhibition of Arabidopsis seed germination.

NCED is considered to be a key regulatory enzyme in ABA biosynthesis in drought-stressed plants (Qin and Zeevaart, 1999, 2002; Iuchi et al., 2001). In this study, we identified catalytic activity of recombinant

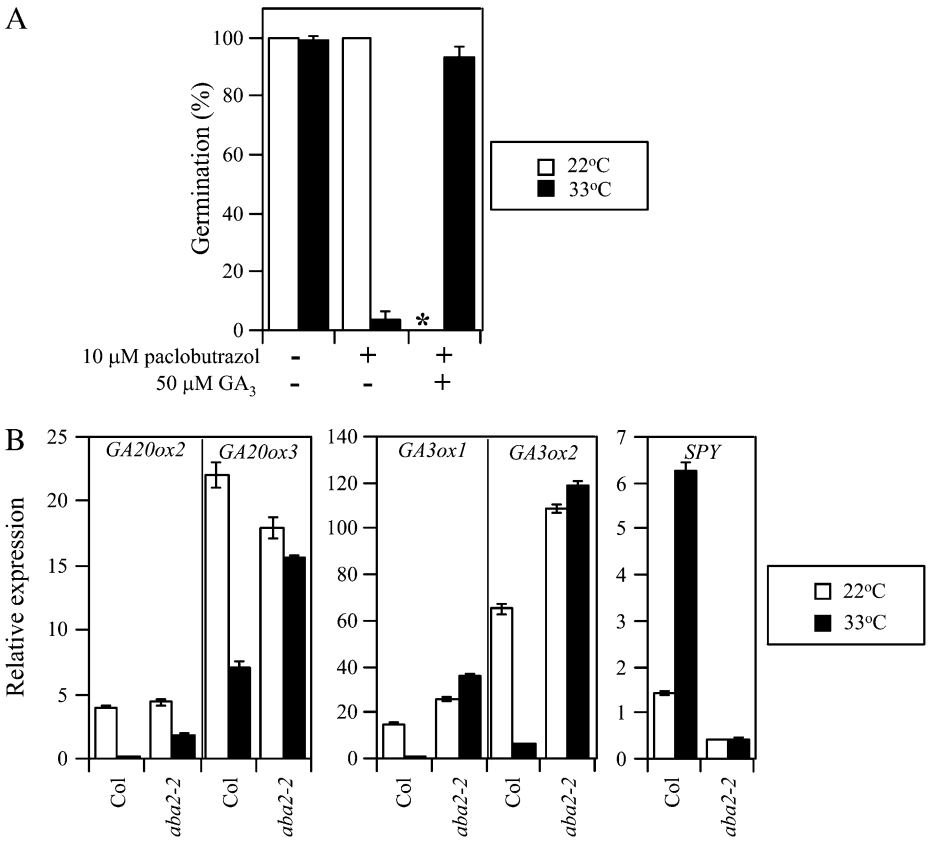

Figure 8. Modulation of GA synthesis and signaling by ABA in thermoinhibited seeds. A, Effect of GA biosynthesis inhibitor PAC on thermoinhibition-tolerant germination of ABA-deficient seeds. The seeds of *aba2-1* were imbibed at 22°C and at 33°C under continuous illumination for 6 d in the presence or absence of PAC and GA_3. All the imbibition solutions contained 10 μM fluridone to ensure ABA deficiency. Asterisk denotes that the germination test at 22°C in the presence of both PAC and GA_3 was not done. Error bars show SD ($n = 3$). B, Expression of GA synthesis and signaling genes in ABA-deficient mutant seeds. Seeds of wild type (Col) and *aba2-2* were imbibed at 22°C (white bars) and 33°C (black bars) under continuous illumination. Transcript levels were quantified by quantitative RT-PCR at 12 h (*GA20ox*) or at 24 h (*GA3ox* and *SPY*) after the start of imbibition. Experiments were repeated three times with different seed batches and similar results were obtained. Data from one of the replicates are shown. Values are means with SDs from three measurements.

NCED5 protein in vitro (Supplemental Fig. S1), and found that expression of *NCED2*, *NCED5*, and *NCED9* was up-regulated by high temperature in seeds (Fig. 2). We confirmed the physiological relevance of *NCED2*, *NCED5*, and *NCED9* genes to thermoinhibition by analyzing thermoinhibition-resistant germination phenotypes of the *nced9* single mutant and multiple *nced* mutants. Interestingly, the degree of reductions in ABA levels in single and multiple *nced* mutant seeds imbibed at 34°C were roughly correlated with the sum of the expression levels of each *NCED* gene during the imbibition period in wild-type seeds (Figs. 2 and 3B). In addition, the increase in ABA level was preceded by the increase in *NCED2* and *NCED9* transcript levels in thermoinhibited seeds. These results suggest that enhancement of ABA synthesis by high temperature is regulated at the transcript level of *NCED* as in drought-stressed plants. Transcript levels of *NCED2* and *NCED5* were about one-half those of *NCED9* in thermoinhibited wild-type seeds, but seeds of the *nced2 nced5* double mutant had a higher level of ABA than the *nced9* single mutant (Figs. 2 and 3B). The relatively high expression level of *NCED9* after 3 to 12 h of imbibition at 34°C may contribute to the above difference. It is also possible that the spatial distribution of carotenoid precursors and expression of ABA biosynthesis genes, including *NCED* itself, in a seed affect the contribution of each *NCED* to ABA biosynthesis.

Environmental, Developmental, and Spatial Regulation of *NCED* Gene Family Members Is Involved in Regulation of ABA Level and Germination

Among the five *NCED* genes in Arabidopsis, expression of *NCED3* is induced by drought stress in plants (Iuchi et al., 2001; Ruggiero et al., 2004). In this study, we found that expression of *NCED2*, *NCED5*, and *NCED9* was up-regulated by high temperature in after-ripened seeds under continuous illumination. *NCED6* and *NCED9* are responsible for ABA synthesis during seed development and dormancy (Lefebvre et al., 2006). In contrast to *NCED9*, *NCED6* showed weak expression during imbibition, and T-DNA insertion mutants of *NCED6* showed no resistance to thermoinhibition (Supplemental Fig. S2). Recently, by using a strongly dormant Arabidopsis accession, Cape Verde Islands (Cvi), Cadman et al. (2006) reported that expression of *NCED6* is up-regulated in primary dormant and secondary dormant seeds imbibed in the dark. Seo et al. (2006) reported that *NCED6* expression in seeds is induced during dark imbibition after far-red light treatment and suppressed by subsequent red-light treatment. Therefore, *NCED6* may work for ABA production and contribute to acquire dormancy during seed development and to maintain the resting/dormant state of mature imbibed seeds in the dark condition. These results indicate that a subset of *NCED* paralogs responds to specific environmental and de-

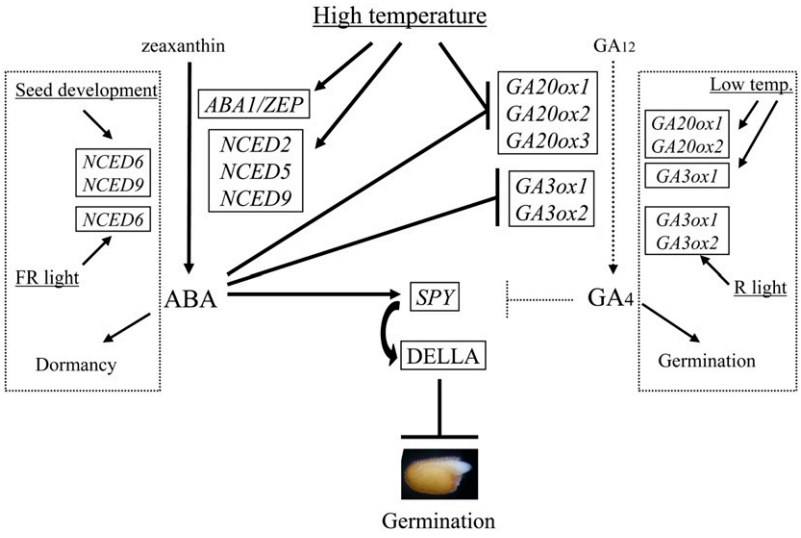

Figure 9. Regulation of ABA synthesis, GA synthesis, and GA signaling by high temperature in Arabidopsis seeds. High temperature enhances expression of specific ABA biosynthesis genes (*ABA1/ZEP*, *NCED2*, *NCED5*, and *NCED9*) under continuous illumination. High temperature also enhances expression of a GA negative regulator gene (*SPY*) and suppresses expression of GA biosynthesis genes (*GA20ox1*, *GA20ox2*, *GA20ox3*, *GA3ox1*, and *GA3ox2*) not directly, but through the action of ABA. Expression of *GA20ox2* and *GA20ox3* may be regulated by both ABA and high temperature. Transcripts of DELLA protein genes are not increased by high temperature, but their protein activity may be enhanced by *SPY*. A different set of *NCED* genes (*NCED6* and *NCED9*) works during seed development and contributes to dormancy development. Expression of *NCED6* is induced by far-red (FR) light, and those of *GA3ox1* and *GA3ox2* are induced by red (R) light. Expression of a set of GA biosynthesis genes (*GA20ox1*, *GA20ox2*, and *GA3ox1*) is induced by low temperature and suppressed by high temperature and may contribute to germination control in a wide range of temperatures. [See online article for color version of this figure.]

velopmental cues, and they encode enzymes that catalyze ABA production for proper response of the plant.

The endosperm is thought to be a barrier for radicle protrusion and ABA has been shown to inhibit the endosperm rupture of Arabidopsis and cress (*Lepidium sativum*) seeds (Müller et al., 2006). They also found that the endosperm weakens prior to its rupture and that ABA delays the onset and decreases the rate of this weakening process. In lettuce seeds, thermoinhibition is alleviated by removing or puncturing the endosperm (Dutta and Bradford, 1994). From spatial expression analysis of *NCED* genes during development of Arabidopsis seeds, Lefebvre et al. (2006) suggested that ABA synthesized in the endosperm contributes to the induction of seed dormancy. We found that expression of *NCED9* was predominant over other *NCED* genes in the embryo, whereas *NCED5* expression was predominant in the endosperm of thermoinhibited seeds (Fig. 4). Thermoinhibition tolerance of *nced9* and the thermoinhibition-sensitive phenotype of the *nced5* single mutant and the *nced2 nced5* double mutant may suggest that ABA synthesis in the embryo is critical for suppression of germination at supraoptimal temperature. Expression of the three *NCED* genes in the embryo and the endosperm and enhancement of their expression at high temperature in both tissues may suggest that ABA synthesized in the endosperm is also involved in thermoinhibition of the seeds. It is also possible that compensation by *NCED9* in the absence of *NCED2* and *NCED5* expression in the endosperm enhances the barrier function of the endosperm. Whatever the case, both tissues may cooperatively regulate seed germination by sensing and responding to temperature.

Suppression of GA Synthesis Is Critical for Thermoinhibition

Alleviation of thermoinhibition by exogenous GA_3 in lettuce and Arabidopsis seeds suggests that suppression of active GA synthesis is required for thermoinhibition (Gonai et al., 2004; Fig. 5A). Lettuce seeds do not increase bioactive GA (GA_1) content by 12 h of imbibition at supraoptimal temperature (33°C) in the dark (Gonai et al., 2004). Because bioactive GA synthesis is regulated by light during seed germination (Yamaguchi and Kamiya, 2002), it is not clear whether low GA content is due to the absence of light or to high temperature in thermoinhibited lettuce seeds. In this study, we showed that thermoinhibited Arabidopsis seeds did not increase bioactive GA content even in the light condition. This indicates that the bioactive GA level is controlled by high temperature regardless of the light condition. Gonai et al. (2004) reported that thermoinhibition of lettuce seeds at 33°C is not rescued by fluridone or GA_3 alone, but by combined application of fluridone and GA_3. They also showed that exogenous GA_3 lowers ABA content by enhancing catabolism of ABA presumably through the 8′-hydroxylation

pathway. In Arabidopsis seeds, application of fluridone or GA_3 alleviated thermoinhibition, but the seeds did not show 100% germination at 34°C (Figs. 1 and 5A). Combined application of fluridone and GA_3 allowed almost 100% germination at 34°C (data not shown). We also examined germination at 36°C and found that fluridone or GA_3 alone rescued germination only slightly, but the combined application allowed 60% to 70% of the seeds to germinate (data not shown), as observed in lettuce seeds (Gonai et al., 2004). Thermoinhibition might be achieved by maintaining ABA content above the threshold level of germination not only through its biosynthesis, but also through repression of its catabolism mediated by GA also in Arabidopsis seeds. We found that expression of three ABA 8′-hydroxylase genes, *CYP707A1*, *CYP707A2*, and *CYP707A3*, was suppressed at 34°C, but the ABA content showed a similar decreased rate in the presence of fluridone at 34°C and in the absence of fluridone at 22°C (Figs. 1 and 2). This suggests that ABA catabolism has little contribution to ABA content at 34°C, but it is possible that suppression of ABA catabolism contributes to up-regulation of ABA levels at higher temperature conditions. It is interesting to know whether expression of *CYP707A* genes is modulated by the action of GA or not.

During germination of Arabidopsis seeds, *GA20ox* genes, *GA20ox1*, *GA20ox2*, and *GA20ox3*, and *GA3ox* genes, *GA3ox1* and *GA3ox2*, are involved in GA synthesis (Ogawa et al., 2003). Suppression of all these *GA20ox* and *GA3ox* genes at supraoptimal temperature (Fig. 6) may be responsible for suppression of GA synthesis. Mitchum et al. (2006) reported that seeds of the *ga3ox1 ga3ox2* double mutant do not germinate in the light at 22°C and their germination is rescued by application of bioactive GA. Therefore, suppression of *GA3ox1* and *GA3ox2* genes at high temperature should be critical for thermoinhibition. Interestingly, expression of *GA20ox1*, *GA20ox2*, and *GA3ox1* has been reported to be up-regulated by low temperature (Yamauchi et al., 2004). Thus, GA synthesis is likely to be regulated by a wide range of temperature through modulation of *GA20ox* and *GA3ox* gene expression (Fig. 9). Expression of *GA3ox* genes in lettuce and Arabidopsis is well known to be photoreversibly regulated by light through phytochrome (Toyomasu et al., 1998; Yamaguchi et al., 1998). Understanding the cross talk between light and temperature signals on the regulation of *GA3ox* gene expression may give us insight into how plants transduce multiple signals and how plants respond to complex environmental stimuli.

The *GA2ox2* gene showed little expression during imbibition even at supraoptimal temperature conditions (Fig. 6). Expression of *GA2ox2* is regulated via phytochrome in a photoreversible manner as observed for *NCED6* (Seo et al., 2006). Therefore, this gene might be suppressed because of continuous illumination conditions adopted in this study. In fact, expression of *GA2ox2* is up-regulated in primary dormant and secondary dormant Cvi seeds imbibed in the dark (Cadman et al., 2006). Thus, it will be informative to examine the effect of high temperature on *GA2ox2* expression in imbibed seeds in darkness. In the dark condition, for example, when the seeds are buried in the soil in summer, GA deactivation may contribute to thermoinhibition of the seeds.

Function of GA Negative Regulators on Thermoinhibition

Loss-of-function mutations in GA negative regulators, SPY and DELLA proteins, allow GA response in the absence of active GA (Jacobsen and Olszewski, 1993; Peng and Harberd, 1997; Silverstone et al., 1997, 1998; Dill and Sun, 2001; King et al., 2001; Lee et al., 2002). Overexpression of *SPY* in the wild-type and *spy-3* backgrounds reduces GA response and enhances sensitivity to ABA during seed germination (Swain et al., 2001). Overexpression lines also show slow germination under normal conditions. Our results indicated that the seeds of loss-of-function mutant *spy-4* were highly resistant to thermoinhibition (Fig. 7B). *SPY* transcripts decreased to low level at 22°C, but maintained relatively high level at 34°C (Fig. 7C). In addition to its role as a GA negative regulator, SPY also acts as a positive regulator of cytokinin signaling (Greenboim-Wainberg et al., 2005). If SPY exerts its function on thermoinhibition through cytokinin signaling, cytokinin should inhibit germination of seeds. However, application of cytokinin alleviates thermoinhibition of lettuce seeds (Odegbaro and Smith, 1969). These observations support the idea that up-regulation of *SPY* expression by high temperature is involved in the thermoinhibition mechanism of Arabidopsis seeds through regulation of the GA response (Fig. 9). In rice, the *SPY* ortholog (*OsSPY*) is suggested to work as a negative regulator of brassinosteroid biosynthesis (Shimada et al., 2006). Application of brassinosteroids to the seeds of GA-deficient and GA-insensitive mutants of Arabidopsis partially rescues germination phenotypes (Steber and McCourt, 2001). It is therefore also possible that *SPY* suppresses germination by repressing brassinosteroid biosynthesis.

High temperature-resistant germination of *rgl2* seeds suggests that *RGL2* works as a repressor of Arabidopsis seed germination at supraoptimal temperature conditions. The thermoinhibition-resistant germination phenotype of *rgl2*, however, was mild when compared with the germination of *spy-4* and ABA-deficient mutant seeds (Figs. 3 and 7B; Supplemental Fig. S2). It is possible that SPY acts as a positive regulator of ABA action in addition to its role as a GA negative regulator, as reported in barley (*Hordeum vulgare*) aleurone (Robertson et al., 1998). A germination study of multiple DELLA protein mutants suggested that *RGL2* is the predominant repressor of Arabidopsis seed germination in the light and *GAI*, *RGA*, and *RGL1* enhance the function of *RGL2* (Cao et al., 2005). Recently, RGA and GAI have been shown to play a role in inhibiting

germination of dark-imbibed seeds in the absence of active phytochromes (Oh et al., 2007). DELLA proteins other than *RGL2* may enhance *RGL2* function also in supraoptimal temperature conditions. It is also possible that high temperature stabilizes DELLA protein through an increase in ABA levels because GFP-fused RGA protein was reported to be stabilized by exogenous ABA in root cells (Achard et al., 2006). However, a recent report showed that ABA has no effect on the stability of endogenous RGA protein in seedlings of the GA-deficient mutant, *ga1-3*, in the absence or presence of exogenous GA (Zentella et al., 2007). Further analysis on the levels of DELLA proteins in seeds will increase our knowledge on the effect of ABA on GA signaling and germination inhibition.

Regulation of GA Action by ABA

Suppression of the thermoinhibition-resistant germination phenotype of ABA-deficient mutant seeds by a GA biosynthesis inhibitor, PAC, indicates that GA is necessary for germination in the absence of ABA at supraoptimal temperature conditions. This suggests the possibility that GA biosynthesis is suppressed at high temperature through the action of ABA (Figs. 8 and 9). The significant expression of *GA3ox1*, *GA3ox2*, *GA20ox2*, and *GA20ox3* genes in ABA-deficient mutant seeds at high temperature strongly suggests that these GA biosynthesis genes are regulated by ABA. The partial derepression of *GA20ox2* and *GA20ox3* expression at the ABA-deficient conditions may indicate that these two genes are regulated by both ABA and other factors induced by high temperature. Expression of *GA20ox1* is reported to be significantly reduced by exogenous ABA in seedlings (Zentella et al., 2007). Seo et al. (2006) suggested that ABA down-regulated GA levels through suppression of *GA3ox1* and *GA3ox2* genes in far-red light-irradiated and dark-imbibed seeds, but ABA plays a relatively minor role in regulating GA levels compared with the role played by light. In our illuminated conditions, expression of *GA3ox1* and *GA3ox2* was severely repressed at high temperature in wild-type seeds, but their expression was completely derepressed in ABA-deficient mutant seeds at high temperature (Fig. 8). Our present data suggest that ABA plays a major role in the suppression of GA levels at high temperature in the light. The suppression of *SPY* gene expression in ABA-deficient mutant seeds imbibed at high temperature suggests that expression of *SPY* is up-regulated through the action of ABA. ABA may inhibit GA action by suppressing GA biosynthesis and also by suppressing GA signaling (Fig. 9).

A Common Germination Control Mechanism in Dormant and After-Ripened Seeds Changes Sensitivity to Temperature during After-Ripening?

Fluridone and GA treatments induce germination of dormant seeds of *N. plumbaginifolia* and Arabidopsis (Grappin et al., 2000; Jullien et al., 2000). De novo ABA synthesis and suppression of GA synthesis may be required for maintaining dormancy as well as for thermoinhibition of the after-ripened seeds. Expression of *ABA1/ZEP*, *NCED2*, and *NCED9* is up-regulated and *GA3ox2* is down-regulated in primary and secondary dormant Cvi accession seeds as in thermoinhibited Col seeds (Fig. 2; Cadman et al., 2006). Up-regulation of *NCED6* and *GA2ox2* in the dormant seeds imbibed in darkness, which was not observed in the thermoinhibited seeds imbibed in the light, may be the result of the difference in the light condition because the expression of these two genes is regulated by phytochrome (Seo et al., 2006). The significant decrease in ABA level, which was observed in the thermoinhibited seeds in the first several hours of imbibition, was also reported for dormant seeds of Arabidopsis C24 and Cvi accessions imbibed at room temperature (Ali-Rachedi et al., 2004; Millar et al., 2006). *CYP707A2* expression in dormant C24 seeds also increases transiently during the first 3 h of imbibition, but the peak level is about one-half of after-ripened seeds, which is similar to what we observed in thermoinhibited seeds (Millar et al., 2006; Fig. 2). These results suggest that a common genetic mechanism is involved in ABA and GA regulation in dormant seeds imbibed at room temperature and in after-ripened seeds imbibed at high temperature. Seed responsiveness to temperature is closely related to the level of dormancy, and the maximal temperature for germination rises gradually during an after-ripening period in field-grown soil-buried seeds of winter annual species and in laboratory-grown Arabidopsis seeds (Baskin and Baskin, 1998; Tamura et al., 2006). Tamura et al. (2006) isolated high temperature-resistant germination mutants of Arabidopsis from after-ripened seed batches and showed that they have reduced seed dormancy. They also indicated that the seeds of Arabidopsis reduced-dormancy mutants *rdo1*, *rdo2*, *rdo3*, and *rdo4* are thermoinhibition tolerant. Among five ABA-insensitive mutants of Arabidopsis, the seeds of *abi1-1*, *abi2-1*, and *abi3* alleles show reduced dormancy and thermoinhibition tolerance and the seeds of *abi4* and *abi5* alleles show normal levels of dormancy and normal thermoinhibition sensitivity. Dormant and after-ripened seeds may utilize a common genetic mechanism to modulate germination and the change in sensitivity to temperature may occur during after-ripening on the modulation mechanism of ABA and GA levels in the seeds.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

We used the Col-0 accession of Arabidopsis (*Arabidopsis thaliana*) for germination, hormone, and gene expression analyses. Seeds of *spy-4* were kindly provided by Stephen M. Swain. Seeds of *aba1-1* (CS21); T-DNA insertion lines of SALK (Alonso et al., 2003), *nced2-1* (SALK_026541), *nced2-2* (SALK_052555), *nced2-3* (SALK_090937), *nced2-4* (SALK_004231), *nced9-1* (SALK_033388), *nced9-2* (SALK_123975), *rgl2-14* (SALK_027654), and *rgl2-15* (SALK_024532); and Wisconsin, *nced5-1* (CS853457, WiscDsLox377–380M20)

and *nced6-3* (CS852600, WiscDsLox356H02), were obtained from the ABRC (The Ohio State University). Plants were grown as described previously (Tamura et al., 2006).

Isolation of T-DNA Insertion Homozygotes and *nced* Multiple Mutants

Plants homozygous for T-DNA insertion in each gene were identified by the method described in SIGnAL (http://signal.salk.edu) of the Salk Institute Genomic Analysis Laboratory with T-DNA left-border primers LB_6313R for SALK lines and p745 (http://www.hort.wisc.edu/krysan/2010/default.htm) for WiscDsLox lines and the gene-specific primers listed in Supplemental Table S1. T-DNA insertion points were identified by sequencing the amplified left-border flanking regions.

To generate multiple *nced* mutants, we first crossed *nced2-1* and *nced9-1*, and genotypes of F_2 plants were identified by PCR with the left-border primers and the gene-specific primers. Then we crossed the *nced2 nced9* double mutant with *nced5-1*, and the triple and *nced2 nced5* and *nced5 nced9* double mutants were isolated from the segregants by PCR as described above.

Germination Tests

Seeds were harvested at physiological maturity when about one-half of the fruits on a plant turned to yellow and were stored in a desiccator for after-ripening for about 2 months at room temperature. Thirty seeds were imbibed with 300 μL of water in a well of a 24-well plate. Seeds were imbibed at constant temperature under continuous fluorescent light (18 μmol m^{-2} s^{-1}). Fluridone (recrystallized; Daw Elanco) and GA_3 (Sigma) were first dissolved in dimethylsulfoxide (DMSO) and then diluted to the final concentrations with water. The final concentration of DMSO was usually 0.01%, and the maximal concentration was restricted to 0.1% because DMSO affects germination of Arabidopsis seeds at higher than 0.1%. Germination was scored as radicle protrusion. All germination tests were done at least in two independent seed batches with three replicates in each. A typical result was presented because there were some differences in germination percentages from batch to batch, but the results were almost parallel.

GA and ABA Analysis

One gram and 0.4 g of dry seeds were used for GA and ABA measurement, respectively. Quantitative analysis of GA and ABA by GC-MS was performed using ^{2}H-labeled GAs and ^{13}C-labeled ABA as internal standards, as described previously (Gawronska et al., 1995; Cheng et al., 2002). GA and ABA levels were determined twice using independent seed batches. In the case of *nced* single and multiple mutants, ABA was extracted from 5 (nonimbibed) or 10 mg (imbibed) seeds with deuterium-labeled d_6-ABA purchased from ICON SERVICES as an internal standard, and quantified by liquid chromatography-tandem mass spectrometry as described previously (Saika et al., 2007).

Quantitative Real-Time PCR

Total RNA was isolated using an RNAqueous kit with plant RNA isolation aid (Ambion). Total RNA (2 μg) was treated with RNase-free DNase (Promega) to eliminate genomic DNA contamination. First-strand cDNA was synthesized with random hexamers using a SuperScript first-strand synthesis system according to the manufacturer's instructions (Invitrogen). Quantitative RT-PCR with Taq-Man technology (Holland et al., 1991) was performed using first-strand cDNA as a template on a sequence detector system (model 7700; Applied Biosystems). For quantitative RT-PCR, 12.5 μL of QuantiTect Probe PCR kit (Qiagen), 5 μL of cDNA, 0.2 μM of Taq-Man probe, and 0.8 μM of each primer were used in triplicate 25-μL reactions and subjected to the following cycling conditions: 50°C for 2 min, followed by 95°C for 15 min, then 45 cycles of 95°C for 15 s, and 60°C for 1 min. The sequence of primers and Taq-Man probes for *ZEP*, *NCED*s, *ABA2*, *AAO3*, *CYP707A*s, *GA20ox*s, *GA3ox*s, and *GA2ox2* were as described previously (Ogawa et al., 2003; Kushiro et al., 2004; Seo et al., 2004; Yamauchi et al., 2004). Primers and probes for *SPY* and *RGL2* are listed in Supplemental Table S2. Data were analyzed using ABI Prism 7700 SDS software (Applied Biosystems). To compare mRNA levels across different genes, the absolute amount of each target transcript was determined by generating standard curves using a series of known concentrations of target sequences. Genomic DNA (*ABA1/ZEP*, *ABA2*, *AAO3*, *NCED2*, *NCED3*, *NCED5*, *NCED6*, *NCED9*; Figs. 2 and 4) and cDNA (*GA20ox1*, *GA20ox2*, *GA20ox3*, *GA3ox1*, *GA3ox2*, *GA2ox2*, *SPY*, *RGL2*; Figs. 6, 7C, and 8B) were used as the target sequences. Then the data were normalized to the amplification of 18S rRNA internal control. For each sample, the mean value from triplicate real-time PCRs was adapted to calculate the transcript abundance, and the mean values were plotted with the SDS. To confirm biological reproducibility, experiments were performed at least twice using different seed batches and we obtained similar results. We also obtained similar results by two-color microarray analysis (Arabidopsis II 22k array; Agilent) with RNA samples from another two different seed batches (A. Imamura, S. Toh, and N. Kawakami, unpublished data).

Assay of NCED Activity

The procedure used for the assay of NCED enzyme activity was as described previously (Iuchi et al., 2000). Recombinant NCED5 or NCED3 protein was incubated with 9′-cis-neoxanthin under 37 mM BisTris, pH 6.7, 0.05% (v/v) Triton X-100, 1 mM ascorbate, and 500 μM $FeSO_4$ at room temperature for 1 h (total volume 100 μL). After the addition of 1 mL of water, the reaction mixture was extracted twice with 1 mL of ethyl acetate. The ethyl acetate fractions were combined, concentrated, and analyzed by HPLC on a column of Senshu Pak ODS H 3151 (150 mm length, 8 mm i.d.; Senshu Scientific). The column was eluted with a linear gradient between solvent A (85:15 [v/v], methanol:water) and solvent B (1:1 [v/v], chloroform:methanol) at a flow rate of 1.5 mL min^{-1}. The concentration of solvent B was increased from 10% to 50% in 25 min and kept at 50% for 5 min. The elution from the column was monitored with a UV/visible detector at 440-nm wavelength to detect substrate carotenoids and C_{25} products.

To identify cis-xanthoxin, the ethyl acetate extract of the reaction mixture was submitted to HPLC purification using the column described above. The column was eluted with 50% (v/v) aqueous methanol at a flow rate of 1.5 mL min^{-1}, and the elution from the column was monitored with a UV detector at 260-nm wavelength. The predicted cis-xanthoxin fraction was collected and submitted to GC-MS analysis.

Separation of Embryo and Endosperm with Testa

Col seeds were imbibed at 22°C and 34°C for 24 h and endosperm/testa and embryo were separated under a microscope. Approximately 850 embryos and endosperms/testas were pooled for each RNA isolation. RNA extraction, cDNA synthesis, and quantitative RT-PCR were done as described above, but cDNA was made from 1 μg of total RNA and quantitative RT-PCR was done with 10-μL reactions by the following cycling conditions: 95°C for 20 s, 45 cycles of 95°C for 3 s, and 60°C for 30 s. Results from embryo and endosperm/testa were normalized to the amplification of the 18S rRNA control and the amount of total RNA extracted from each tissue of one seed.

Supplemental Data

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

Supplemental Figure S1. NCED5 has 9-cis-epoxicarotenoid dioxygenase activity in vitro.

Supplemental Figure S2. Thermoinhibition-resistant germination of seeds from ABA-deficient mutants and lines with T-DNA insertion in *NCED* genes.

Supplemental Figure S3. Evaluation of embryo and endosperm separation by quantitative RT-PCR of *EPR1* and *GA3ox2* transcripts.

Supplemental Table S1. Gene-specific PCR primer sequences for T-DNA insertion analysis.

Supplemental Table S2. Sequences of gene-specific primers and Taq-Man probes for quantitative RT-PCR.

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