Isolation and Characterization of High Temperature-Resistant Germination Mutants of Arabidopsis thaliana

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Abstract

Temperature is a primary environmental cue for seed germination of many weeds and vegetables. To investigate the mechanism of germination regulation by temperature, we selected five high temperature (thermoinhibition)-resistant germination mutants (TRW lines) from 20,000 T-DNA insertion lines of Arabidopsis. Segregation analyses indicated that each of the five lines had single locus recessive mutations. The seeds of TRW134-15 and TRW187 showed reduced sensitivity to ABA and also to the gibberrellin biosynthesis inhibitor, paclobutrazol. Genetic and nucleotide sequencing analyses indicated that TRW187 is a new allele of abi3 (abi3-14). TRW71-1 exhibited a maternal effect for both thermoinhibition-resistant and transparent testa phenotypes, and genetic analysis revealed that the mutation was allelic to tt7 (tt7-4 sib). Interestingly, the seeds of reduced dormancy mutants rdo1, rdo2, rdo3 and rdo4 were also thermoinhibition tolerant, and all the TRW seeds showed reduced dormancy. Like rdo3, TRW13-1 had shorter siliques and slightly shorter stems than the wild type. The mutation of TRW13-1 was mapped to the bottom arm of chromosome 1 where rdo3 has also been mapped, but the two mutants are not allelic. We designated TRW13-1 as thermoinhibition-resistant germination 1 (trg1). We also mapped the ABA-insensitive mutation of TRW134-15 to the bottom arm of chromosome 5 and named it trg2. These results show that both embryo/endosperm and maternal factors contribute to germination inhibition at supraoptimal temperatures in Arabidopsis. In addition, we confirm the role of ABA in thermoinhibition of seed germination and a link between seed physiological dormancy and response to high temperature.

Keywords: Abscisic acid — Germination — Seed dormancy — Thermoinhibition — transparent testa — trg.

Introduction

Seed dormancy is defined as the failure of an intact viable seed to complete germination under favorable conditions. Types of dormancy vary between species, and are categorized as physiological dormancy, morphological dormancy, physical dormancy, chemical dormancy, mechanical dormancy and their combinations (Baskin and Baskin 1998). Physiological dormancy is caused by a physiological inhibiting mechanism of the embryo that prevents radicle emergence, and is common in seeds of most weeds and vegetables (Baskin and Baskin 1998). We call physiological dormancy simply dormancy herein. Dormancy enables the seeds to germinate at a season and place favorable not simply for germination but also for the vegetative and reproductive growth of the plants. Studies with ABA-deficient and insensitive mutants of maize, Arabidopsis, tomato and tobacco have indicated the crucial role of ABA in determining dormancy (Hilhorst 1995, McCarty 1995, Marin et al. 1996). Pharmacological studies have shown that ABA synthesis in developing and imbibing seeds is involved in the acquisition and the maintenance of dormancy, respectively (Le Page-Degivry et al. 1990, Grappin et al. 2000, Julien et al. 2000). Significant induction of the ABA 8'-hydroxylase gene, CYP707A2, of Arabidopsis during imbibition of after-ripened seeds, and...
hyperdormancy of the seeds of the knockout mutant, cyp707a2, suggest that catabolism of ABA also has an important role in dormancy and germination (Kushiro et al. 2004). In wheat grains, the sensitivity of the embryo to ABA but not the ABA content of embryos has been shown to be correlated with variation in dormancy among cultivars (Walker-Simmons 1987). The embryos of a reduced dormancy mutant of wheat showed a delayed increase and accelerated decrease in the ABA level during seed development and reduced sensitivity to ABA (Kawakami et al. 1997). In Arabidopsis, a subset of ABA signaling factors may be involved in dormancy; the seeds of ABA-insensitive mutants abi1-1, abi2-1 and abi3-1 showed reduced dormancy (Koornneef et al. 1984), but those of abi4 and abi5 were reported to have the same degree of dormancy as their wild types (Finkelstein 1994). Reduced dormancy mutants of Arabidopsis, rdo1, rdo2, rdo3 and rdo4, were neither ABA deficient nor ABA insensitive (Léon-Kloosterziel et al. 1996, Peeters et al. 2002). The seeds of rdo1, rdo2 and rdo3 showed reduced gibberellin requirement for germination (Peeters et al. 2002), but the molecular basis for this remains unclear.

Physiological and genetic studies have indicated that germination is determined by a combination of the growth potential of the embryo and the restrictive potential of the tissues surrounding the embryo. Arabidopsis mutants with abnormal seed coats, aberrant testa shape (ats) and transparent testa (tt), showed reduced dormancy phenotypes (Léon-Kloosterziel et al. 1994, Debeaujon et al. 2000). In wheat, the lines with a red seed coat color usually show stronger dormancy than those with a white coat color (Gale 1989). This indicates the importance of the testa as a factor in determining dormancy. The endosperm surrounding an embryo is thought to be another mechanical barrier to germination (Bewley 1997a, Bewley 1997b, Leubner-Metzger 2003). Endosperm weakening has been proposed as a prerequisite for radicle protrusion in several species. Cell wall-hydrolyzing enzymes, such as endo-β-mannanase in tomato (Nonogaki and Murohashi 1996) and β-1,3-glucanase in tobacco (Leubner-Metzger 2003), and a cell wall-loosening protein, expansin, in tomato (Chen and Bradford 2000), have been reported to be involved in endosperm weakening. Although these cell wall-weakening activities were induced by gibberellin, inhibition of this activity by ABA was protein and species dependent (Dulson et al. 1988, Toorop et al. 1996, Chen and Bradford 2000, Leubner-Metzger 2003). A causal link between the regulation of cell wall-weakening activities and germination has not been conclusively shown.

Seed germination is determined by a combination of the degree of dormancy and environmental factors such as light, oxygen, water potential and temperature. Seed dormancy is lowered by after-ripening (dry condition), environmental conditions such as cold or warm stratification (wet condition), and mechanical scarification or removal of the tissues surrounding the embryo. Even after the loss of dormancy, seeds do not germinate in unfavorable conditions. The suppression of germination at supraoptimal temperatures is called thermoinhibition (Reynolds and Thompson 1971, Abeles 1986, Gallardo et al. 1991). Winter annual species such as Arabidopsis shed seeds in spring, but their germination is inhibited by high temperatures during summer, and they germinate in autumn. 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, the maximum temperature for germination rises gradually during an after-ripening period in the summer, but germination is repressed by environmental temperatures higher than the upper limit for germination. The seeds become germinable in high temperature conditions in autumn, and germinate in the field when the temperature falls below the upper limit for germination (Baskin and Baskin 1983). Therefore, the change in seed sensitivity to thermoinhibition plays an ecologically important role in the detection of the appropriate seasonal timing for germination in soil-buried seeds (Baskin and Baskin 1998, Yoshioka et al. 2003). High temperatures often cause delayed or poor germination of cultivated plant seeds which have relatively low optimal temperatures for germination, and consequently reduce the efficiency of the crop production.

Lettuce (Lactuca sativa L. cv. Grand Rapids) seeds germinate well at 10–22°C but the germination is suppressed above 25–30°C (Reynolds and Thompson 1971, Thomson et al. 1979). In lettuce seeds, the ABA content was maintained during imbibition at high temperatures, whereas it decreased rapidly at optimal temperatures (Yoshioka et al. 1998). It was also shown that application of the ABA biosynthesis inhibitor, fluridone, during imbibition alleviated the thermoinhibition of lettuce and 17 out of 19 species of winter annuals (Yoshioka et al. 1998). Gibberellins, cytokinins and ethylene have been shown to alleviate the inhibitory effect of high temperatures on lettuce seed germination (Odegbaro and Smith 1969, Abeles 1986, Gonai et al. 2004). Ethylene production in chick-pea seeds was inhibited at supraoptimal temperatures, and their thermoinhibition was alleviated by treating the seeds with ethylene (Gallardo et al. 1991). Gonai et al. (2004) suggested that exogenously applied GA3 alleviates thermoinhibition of lettuce seeds by enhancing the catabolism of ABA.

To identify new genetic factors involved in the germination response to supraoptimal temperature, we used after-ripened seed pools of T-DNA insertion lines, and selected five mutants whose germination was tolerant...
to thermoinhibition. We characterized these mutants by their ABA sensitivity, seed coat color and dormancy. We discuss the mechanism of thermoinhibition and the link between seed response to high temperature and dormancy.

**Results**

**Isolation of high temperature-resistant germination mutants**

To investigate the effect of imbibition temperature on germination of *Arabidopsis* seeds and to determine the conditions for high temperature-resistant germination (thermoinhibition-resistant) mutants, freshly matured Wassilewskija (Ws) ecotype seeds were stored at room temperature for different time periods, then imbibed at four different temperatures in the light. Freshly harvested Ws seeds showed relatively weak but detectable dormancy. At harvest time, 59% of the seeds germinated at 22°C, and the germination was repressed at higher temperatures (Fig. 1A). The seeds became more resistant to high temperatures during after-ripening, and about 55% of the seeds germinated at 30°C 3 weeks after the harvest, but their germination was repressed severely at 32°C.

About 90% of fully after-ripened seeds germinated after 3–5 d of imbibition at 22 and 28°C (Fig. 1B). Germination was inhibited moderately and severely at 30 and 32°C, respectively, and there was no germination at 34°C. The low germination percentages at the high temperatures were not due to mortality of the seeds by heat stress, because the seeds germinated after cold stratification on agar plates supplemented with GA3 and Murashige–Skoog salts (data not shown).

To eliminate fluctuation of the germination response to supraoptimal temperature dependent on the variation of dormancy level between seeds, we used fully after-ripened seed batches for the selection. From 20,000 T-DNA insertion lines, we selected 701 T3 seedlings that germinated after 3–5 d of imbibition at 32°C. Progeny testing with after-ripened seeds showed that five mutants were recovered, and the phenotypes were stably inherited. The mutant lines were tentatively named TRWs (temperature-resistant Ws mutants; TRW13-1, TRW71-1, TRW124-4, TRW134-15 and TRW187) and crossed to the wild type reciprocally. F1 seeds from these crosses did not germinate at 32°C except for those from TRW71-1 which showed a maternal effect as described in the next section. Segregation analyses indicated that all five lines had single gene recessive mutations. Allelism tests among these mutants failed to show complementation (data not shown), showing that the mutations affect independent loci.

The after-ripened seeds of the mutants showed different degrees of thermoinhibition resistance (Fig. 1C). TRW187 seeds showed the shortest time to germination and also the highest final percentage of germination at 32°C.
The seeds germinated well even at 36°C (95% after 7 d). TRW13-1, TRW71-1 and TRW134-15 showed germination at 34°C (59, 58 and 65% after 7 d, respectively), but the germination of TRW124-4 seeds was almost completely repressed at 34°C (4%). TRW13-1 seeds showed relatively slow germination at 32°C when compared with other mutants (Fig. 1C).

We analyzed T-DNA insertion in the mutants by kanamycin resistance (conferred by the nptII gene in the T-DNA region of pGKB5) and PCR with the specific primer set (left border region of the T-DNA) as described in Materials and Methods. Kanamycin-sensitive phenotypes of TRW187 and TRW71-1 mutant seedlings (T4) and no amplification of T-DNA fragment from their genomic DNAs indicate that these two mutations are not tagged with a full-length T-DNA. F2 segregation analyses for kanamycin resistance and T-DNA amplification indicated that TRW13-1 and TRW134-15 contained a single locus of T-DNA insertions, but the insertions were not linked to thermoinhibition-resistant phenotypes. TRW124-4 also contained a single T-DNA insertion locus, but fluctuation of the germination response between seed batches combined with the mild phenotype (about 50% germination at 32°C) often made segregation analysis ambiguous, and further analysis is necessary to confirm a linkage between T-DNA insertion and the thermoinhibition-resistant phenotype.

**Seed color**

TRW71-1 had a pale brown seed coat, and reduced accumulation of anthocyanin pigment around the base of the petioles and the upper part of the hypocotyl of the seedling (Fig. 2B, D). A maternal effect was seen on both the coat color and thermoinhibition resistance of F1 seeds from the reciprocal crosses with the wild-type (Ws × TRW71-1, 0%; TRW71-1 × Ws, 74% at 32°C). Almost no germination of F2 seeds from the reciprocal cross at supraoptimal temperatures indicated that the thermotolerant germination of TRW71-1 was not provided by a maternal inheritance. Only F3 seeds which had a pale brown coat color showed thermoinhibition resistance.

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**Fig. 2** Seed color phenotypes of TRW71-1 and TRW187. (A and B) Transparent testa phenotype of TRW71-1. Arrows indicate seed coats with endosperm but no embryo, and arrowheads show non-germinated seeds imbibed for 5 d at 22°C. (A) Wild type (Ws). (B) TRW71-1 (tt7-4 sib). (C-E) Seed color of TRW71-1 and TRW187. (C) Wild type (Ws). (D) TRW71-1 (tt7-4 sib). (E) TRW187 (abi3-14).
and germination of F3 seeds with a normal brown color was repressed severely at 32°C. These results suggest that seed coat but neither the embryo nor the endosperm is responsible for the thermoinhibition resistance of this mutant.

TRW71-1 closely resembled tt7 in color phenotypes, and moreover, a tt7 allele had been isolated from the same pool of T-DNA insertion lines by visible screening (N. Nesi, I. Debeaujon and L. Lepiniec, unpublished) and named tt7-4 (Routaboul et al. 2006). The seeds of tt7-1 have been reported to have reduced dormancy when compared with the background Ler (Debeaujon et al. 2000), but thermoinhibition resistance has not been studied. Ler seeds were slightly more resistant to high temperatures than Ws (Table 1), but their germination was almost completely inhibited at 34°C (Fig. 3C). The seeds of tt7-1 showed a higher germination percentage than Landsberg erecta (Ler) at 32°C (Table 1) and at 34°C (data not shown). F1 seeds from the reciprocal cross between tt7-1 and TRW71-1 showed thermoinhibition resistance (tt7-1 × TRW71-1, 81%; TRW71-1 × tt7-1, 90% at 32°C). This does not mean the loss of complementation but means maternal effects of the mutations. If TRW71-1 has a mutation in a different locus from tt7, F1 plants produce F2 seeds with brown testa, and the seeds show normal germination phenotype. We confirmed that TRW71-1 was allelic to tt7 since F2 seeds from every F1 showed a pale brown color and thermoinhibition resistance (Table 1).

**ABA and thermoinhibition**

ABA sensitivity of TRW seeds was tested since ABA has been shown to be involved in the thermoinhibition of lettuce seeds (Yoshioka et al. 1998). Germination of TRW71-1 (tt7-4 sib) was slightly more sensitive to ABA than the wild type (Fig. 3A). This is probably because of enhanced permeability of the testa to chemical compounds observed in tt mutants (Debeaujon and Koornneef 2000). The seeds of TRW134-15 and TRW187 showed apparent resistance to 3–10 μM ABA (Fig. 3A). In the presence of 3 μM ABA, elongation of the hypocotyls and the roots, and greening of the cotyledons were observed in TRW187 seedlings but not in TRW134-15 seedlings (Fig. 3B). TRW187 mutation might affect post-germinative growth in addition to the germination process. The seeds of a known ABA-deficient mutant, aba1-1 showed strong resistance to thermoinhibition (Fig. 3C). ABA may have a decisive role in thermoinhibition of Arabidopsis seeds in the same way that it does for lettuce seeds. The seeds of ABA-insensitive abi1-1 showed relatively strong resistance to thermoinhibition, and abi3-1 and abi3-8 showed a moderate level of thermoinhibition resistance at 34°C. On the other hand, the seeds of ABA-insensitive abi2-1, abi4-3 (E8-4) and abi5-7 (E74-1) did not show resistance at 34°C (Fig. 3C). The seeds of abi2-1 showed a higher germination percentage than the wild type (Ler) at 32°C (Ler, 27 ± 25%; abi2-1, 84 ± 8%; mean ± SD of three replicates), but germination of abi4-3 and abi5-7 seeds was inhibited severely the same as their wild type [Columbia (Col)] at 32°C. In Arabidopsis seeds, not all but just a subset of ABA signaling factors may be involved in the thermoinhibition mechanism.

Mature dry seeds of TRW187 showed a grayish green appearance (Fig. 2E), and this was due to the green colored embryo but not to testa color (data not shown). We crossed TRW187 with abi3-8 to test allelism, since embryos of strong abi3 alleles, abi3-3, abi3-4, abi3-5 and abi3-6, remain green even at maturity (Nambara et al. 1992, Ooms et al. 1993, Nambara et al. 1994). Preliminary complementation experiments suggested that TRW187 is a new abi3 allele; the F1 seeds germinated at supraoptimal temperature and in the presence of ABA in the same way as their parents. To confirm the mutation at the nucleotide level, we first amplified and analyzed ABI3 gene sequence of the wild type, Ws. The sequence (DDBJ accession No. AB253328) was 99.8% identical to that of Col and Ler, including the introns. The deduced sequence of Ws ABI3 protein had two amino acid substitutions when compared with strong abi3 alleles, one amino acid insertion in the introns (N481) and one substitution (P555 in Col to L556) when compared with Ler. The ABI3 gene of TRW187 had a 54 bp deletion at the fourth exon/intron boundary region, a 22 bp duplicated insertion from the fourth intron of the wild type, and a 3 bp insertion, but no T-DNA sequence (Fig. 4). This rearrangement resulted in the loss of exon/intron boundary consensus sequence (GT)

<p>| Table 1 Genetic complementation analysis between TRW71-1 and tt7-1 |
|-------------------------|---------------------|</p>
<table>
<thead>
<tr>
<th>Parental seeds</th>
<th>Germination (%)</th>
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<tbody>
<tr>
<td>Ws</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>TRW71-1</td>
<td>45 ± 12</td>
</tr>
<tr>
<td>Ler</td>
<td>38 ± 2</td>
</tr>
<tr>
<td>tt7-1</td>
<td>68 ± 5</td>
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<td>F2 seeds</td>
<td>tt7-1×TRW71-1</td>
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tt7-1 was crossed with TRW71-1, and F2 seeds from 13 independent F1 plants were used for the germination test at the supraoptimal temperature. Typical data of F2s are presented here with those of the parental lines and their wild types. The seeds were imbibed for 7 d at 32°C. Values are the mean ± SD of three replicates with 30 seeds.
from the fourth intron, and may cause substitution of five amino acids and a premature stop codon in the B3 region of ABI3 (Fig. 4B). TRW187 is the first \textit{abi3} allele with a Ws background and is designated as \textit{abi3-14}.

\textbf{Gibberellin requirement for TRW seed germination}

Gibberellin has been shown to be required for \textit{Arabidopsis} seed germination, but not for the seeds of ABA-deficient and ABA-insensitive mutants (Karssen and Lačka 1986). The seeds of \textit{tt} mutants \textit{tt4} and \textit{ttg1}, and reduced dormancy mutants \textit{rdo1}, \textit{rdo2} and \textit{rdo3} also reduced the requirement for gibberellin for germination (Debeaujon and Koornneef 2000, Peeters et al. 2002). To investigate the gibberellin requirement for germination of TRW mutants, the seeds were imbibed in the presence of a gibberellin biosynthesis inhibitor, paclobutrazol (PAC).
Germination of ABA-insensitive TRW134-15 and TRW187 (abi3-14) seeds was highly resistant to PAC, and TRW13-1 seeds showed partial resistance (Fig. 3D). Germination of TRW71-1 (tt7-4 sib) and TRW124-4 seeds was almost completely repressed by 10 μM PAC, as it was for the wild type. Hypersensitivity of tt mutants (including tt7-1) to PAC has been reported and explained by increased uptake of chemical compounds (Debeaujon and Koornneef 2000). We have not confirmed the hypersensitivity by low concentrations (e.g., 1 μM) of PAC, but the hypersensitivity of TRW71-1 seeds to exogenous ABA (Fig. 3A) supports this explanation. None of the TRW mutant plants showed the pale green color or slender phenotype which is typical for a constitutive gibberellin response and gibberellin overaccumulation mutants.

Seed dormancy

Buried seeds of Arabidopsis exposed to natural seasonal change have been reported to germinate at progressively higher temperatures from spring to autumn until they are non-dormant by October (Baskin and Baskin 1983). The seeds from laboratory-grown Ws plants showed a similar modification during dry after-ripening (Fig. 1A). These physiological correlations between dormancy and temperature response of the seeds, and the recovery of alleles of known reduced dormancy mutants (tt7 and abi3) in our high temperature germination screening prompted us to investigate the dormancy of TRW seeds.

Dormancy of the mutant seeds was evaluated by germination of freshly harvested seeds imbibed at 22°C, both in the light and in the dark. All the mutant seeds showed apparently reduced dormancy in the light (Fig. 5A). The seeds of TRW71-1 (tt7-4 sib), TRW134-15 and TRW187 (abi3-14) also showed high germination percentages in the dark. TRW13-1 seeds often showed low but detectable levels of germination in the dark. TRW124-4 seeds showed a moderately reduced level of dormancy in the light and no germination in the dark.

Germination of rdo seeds at high temperature

Since the seeds of reduced dormancy mutants rdo1 and rdo2 have been shown to germinate in the dark at moderately high temperatures (Léon-Kloosterziel et al. 1996), thermoinhibition resistance of rdo mutant seeds was evaluated under our experimental conditions (Fig. 5B). The seeds of all four rdo mutants showed
higher germination percentages than wild-type (Ler) seeds at 32°C in the light. The seeds of rdo3 showed high germination percentages even at 34°C. The linkage between thermoinhibition resistance and reduced dormancy in TRWs and rdo3 suggests a close relationship between the genetic mechanisms of thermoinhibition and dormancy.

Genetic complementation analysis of TRW13-1 and rdo3, and molecular mapping of TRW mutations

TRW13-1 plants were slightly shorter than wild-type (Ws, 38.1 ± 3.7 cm; TRW13-1, 33.8 ± 2.8 cm; n = 10), and produced short siliques (Ws, 14.49 ± 1.18 mm; TRW13-1, 11.48 ± 0.77 mm; n = 30, measurement of siliques at the middle part of the main stem when the plant had almost...
stopped flowering). Genetic analysis indicated that the plant height and siliqua length phenotypes were tightly linked with the germination phenotypes (data not shown). These characters were very similar to those of the rdo3 mutant (Peeters et al. 2002). The mutation of TRW13-1 was mapped between single-strand length polymorphism (SSLP) markers on chromosome 1, NGA280 and NGA111, where rdo3 had also been mapped (Fig. 6, Peeters et al. 2002). The TRW13-1 mutation was mapped further between cleaved amplified polymorphic sequence (CAPS) markers 14G4 and KNAT2. To test allelism between TRW13-1 and rdo3, we crossed them reciprocally and germination of the offspring was observed under supraoptimal conditions. If the two mutations are allelic, F1 and F2 seeds should show high temperature resistance as the same level as their parents. However, all the F1 seeds showed no germination even at 32°C. The complementation was confirmed by low germination percentages of F2 seeds at supraoptimal temperatures, demonstrating that TRW13-1 and rdo3 are not allelic (Fig. 5C). We designated TRW13-1 as thermoinhibition-resistant germination 1 (trg1).

We failed to map the mutation of TRW124-4 since the seeds of Col and Ler were slightly more tolerant to high temperature than Ws and showed almost the same germination at supraoptimal temperature conditions. We could not identify the mutant homozygote in the mapping population. The ABA-insensitive mutation of TRW134-15 was mapped between SSLP markers on chromosome 5, JV57/58 and MBK-5, and designated as trg2 (Fig. 6).

Discussion

Genetic evidence for ABA function in thermoinhibition

Selection for mutants whose seeds germinate at supraoptimal temperatures yielded two ABA-insensitive mutants, TRW134-15 and TRW187. A complementation test and sequencing analysis identified TRW187 as a new allele of abi3 (abi3-14). The seeds of another known ABA-insensitive mutant, abi1-1, and of an ABA-deficient mutant, aba1-1, were also highly resistant to thermoinhibition (Fig. 3C). These results indicate that ABA has a major role in the thermoinhibition mechanism of Arabidopsis seeds. The seeds of ABA-insensitive abi4-3 (E8-4) and abi5-7 (E74-1), however, did not show resistance at 34°C (Fig. 3C). These two mutations might be severe since 100% of the seeds germinated in the presence of 3 μM (+)-ABA (Nambara et al. 2002), and they have point mutations which induce premature stop codons in their N-terminal regions (abi4-3, W62 to stop; abi5-7, W75 to stop, E. Nambara, personal communication). In Arabidopsis seeds, not all but only a subset of ABA signaling factors may be involved in the thermoinhibition mechanism.

Based on experiments with the ABA biosynthesis inhibitor, fluridone, Yoshioka et al. (1998) suggested that de novo ABA synthesis during imbibition is required for therminobilization of lettuce and several other winter annual seeds. In several plant species including Arabidopsis, ABA levels have been found to increase as a result of imbibition in dormant seeds but not in non-dormant seeds, and the germination of dormant seeds was induced by application of an ABA biosynthesis inhibitor (Ried and Walker-Simmons 1990, Le Page-Degivry and Garello 1992, Wang et al. 1995, Grappin et al. 2000). These observations suggest that the regulation of ABA synthesis during imbibition is important for dormancy maintenance and for therminobilization.

It has been suggested that ABI3 works not only in seeds, but also during post-germination seedling growth (Finkelstein and Somerville 1990) and on lateral root formation (Brady et al. 2003). Nambara et al. (2000) suggested that ABI3 has a role in the phase transition from late embryo development to germination. Germination of TRW134-15 (trg2) seeds was resistant to 10 μM ABA (Fig. 3A), but in contrast to abi3 alleles, post-germination processes such as greening of the cotyledons and elongation of roots and hypocotyls were severely inhibited by 3 μM ABA (Fig. 3B). TRW134-15 may have a defect of ABA signaling specific to the germination process but not for the phase transition from seed development to post-germination growth. It is also possible, however, that the mutation of TRW134-15 (trg2) is not null and that the mutant showed a leaky phenotype.

The two ABA-insensitive mutants, TRW134-15 (trg2) and TRW187 (abi3-14), showed reduced gibberellin requirement for germination (Fig. 3D). This is consistent with previous results obtained with ABA biosynthesis and ABA-insensitive mutants (Karssen and La¨cka, 1986, Nambara et al. 1991, Léon-Kloosterziel et al. 1996). TRW13-1, which had slightly reduced ABA sensitivity, showed a little resistance to PAC, but other TRW mutant seeds were not resistant to it. In addition, none of the five TRW mutants showed a wilty phenotype. These results suggest that the five TRW mutants are not ABA deficient.

The seeds of rdo2 and rdo3 showed high temperature resistance (Fig. 5B), and were reported to have a reduced gibberellin requirement for germination although not being affected in ABA sensitivity and ABA content (Peeters et al. 2002). They also reported that rdo1 showed a reduced gibberellin requirement for germination in a gibberellin-deficient gal-3 background but not in the presence of PAC since the growth retardants may not be fully specific to gibberellin biosynthesis. It is possible that TRW13-1 (trg1) and TRW124-4 show a reduced gibberellin requirement in a gibberellin-deficient mutant background. Exogenously applied gibberellin has been reported to rescue germination.
of lettuce seeds under supraoptimal temperature conditions (Gonai et al. 2004). These observations suggest that an increase in the bioactive gibberellin level or gibberellin sensitivity is suppressed in Arabidopsis seeds under supraoptimal temperature conditions.

Germination response to high temperature and dormancy of Arabidopsis seeds

Dormancy does not begin or end abruptly (Baskin and Baskin 1998). During after-ripening of Arabidopsis and other winter annual seeds in natural conditions, the maximum temperature for germination rises as the dormancy decreases (Baskin and Baskin 1983, Baskin and Baskin 1998). After-ripening of Ws seeds in dry and room temperature conditions also allowed the seeds to germinate at progressively higher temperatures (Fig. 1A). To eliminate variation of dormancy between seeds, we used after-ripened seed batches for the selection, and isolated the mutants that germinate at supraoptimal temperatures. The seeds of all the five mutants showed reduced seed dormancy at harvest ripeness (Fig. 5A). The seeds of reduced dormancy mutants, rdo1 and rdo2, have a higher capacity for germination in darkness at 30°C than the wild type (Léon-Kloosterziel et al. 1996). In addition to rdo1 and rdo2, rdo3 and rdo4 also showed thermoinhibition resistance (Fig. 5B). The seeds of the abi1-1, abi2-1 and abi3 alleles have reduced dormancy (Koornneef et al. 1984) and showed thermoinhibition resistance, whereas those of abi4 and abi5 did not show reduced dormancy (Finkelstein 1994) and thermoinhibition resistance (Fig. 3C). The genetic linkage between dormancy and the responsiveness to temperature, and the physiological correlation between after-ripening and the responsiveness to temperature suggest that a temperature sensing and responding mechanism in the seeds is essential for dormancy of winter annual species. Understanding of the molecular mechanism of the temperature response may help in elucidating some of the mechanisms regulating seed dormancy.

Seed coat color and thermoinhibition

An allele of tt7, TRW71-1 (tt7-4 sib), was recovered as a thermoinhibition-resistant mutant in our selection.
This indicates that both seed coat and embryo (including endosperm) factors are involved in the germination response to high temperature. Most of the tt mutants have a reduced dormancy (Debeaujon et al. 2000). The seed coat is thought to be act as a germination barrier through mechanical resistance to radicle protrusion, impermeability to water and/or oxygen, the existence of some germination-inhibiting substance(s) or impermeability to an endogenous germination inhibitor (Bewley and Black 1994). The seeds of tt mutants had reduced amounts of proanthocyanidins (except for tt10-1 and tt14-1), increased permeability to chemical compounds such as tetrazolium salts (except for tt10-1) and, in the case of tt4-1, a reduced gibberellin requirement for germination in a gibberellin-deficient mutant background (Debeaujon and Koornneef 2000, Debeaujon et al. 2000). To determine what factor is responsible for the thermoinhibition resistance of tt7 seeds, further physiological and biochemical analyses of the different tts are required. Our results demonstrated, however, that some seed coat factor(s) have some role in germination response to high temperature. It is also possible that some factor transported from the maternal tissue into the developing seeds is responsible for thermoinhibition. Recently, Salaita et al. (2005) reported that a tt7 allele (tt7-3) is one of the cold temperature germinating mutants which complete germination at 10°C faster than wild type. TT7 may work on the process of the germination response to both low and high temperatures.

Materials and Methods

Mutant isolation and growth conditions

The mutant screen used T-DNA insertion lines from INRA produced by infiltration of Arabidopsis thaliana (L.) Heynh. ecotype Ws with pGKB5-bearing Agrobacterium (Bechtold et al. 1993, Bouchez et al. 1993). After-ripened T1 seeds from 20,000 insertion lines were pooled into 200 groups (100 lines/pool), and about 500 seeds from each pool (about five seeds from each line) were sown on water-saturated filter paper (Whatman No. 3 for the first 100 pools and Schleicher and Schuell #595 for the second 100 pools) in Petri dishes (9 cm diameter). The dishes were incubated at 32°C under continuous illumination. Seeds that had germinated within 5 d of imbibition were selected and transferred to growing medium (oasis™, H-1.5, Smithers-Oasis, Kent, OH, USA) and grown in a chamber (16/8 h light/dark cycle at 22°C) with nutrient solution (Fujiwara et al. 1992). The mutant phenotype was confirmed by retesting the thermoinhibition resistance of the after-ripened T4 and T5 seeds at 32 and 34°C.

Col™ and Ler seeds were provided by Dr. Naoto Yabe. The seeds of aba1-1, abi1-1, abi2-1, abi3-1, tt7-1, rdo1, rdo2, rdo3 and rdo4 were supplied by ABRC. The seeds of E4-6 (abi3-8), E8-4 (abi4-3) and E74-1 (abi5-7) were provided by Dr. Eiji Nambara. Seeds were surface sterilized with 0.5% sodium hypochlorite solution containing 0.2% Triton X-100 for 5 min and washed four times with sterilized water, then sown on agar plates (Murashige-Skoog medium supplemented with 1.5% sucrose) (Murashige and Skoog 1962), stratified at 4°C for 4 d and germinated in the chamber described above. After 10 d, the seedlings were transferred onto the growing medium and grown as described above.

Germination tests

The seeds were harvested at physiological maturity when about half of the siliques on a plant turned to yellow and were stored in a desicator for after-ripening for 1–3 months at room temperature. Thirty seeds were imbibed with 300 μl of H2O in a well of a 24-well plate. The seeds were imbibed at constant temperature in continuous light. ABA (Sigma) and PAC (Wako Pure Chemical, Osaka, Japan) were first dissolved in dimethylsulfoxide (DMSO), and then diluted to the final concentrations with H2O. The final concentration of DMSO was usually 0.01%, and the maximum concentration was restricted to 0.1% since DMSO affects germination of Arabidopsis seeds at higher concentrations than this. ABA and PAC sensitivities were tested at 22°C in the light after stratification at 4°C for 4 d. When the dormancy was tested, freshly matured seeds were stored in a desiccator for 2 d, and imbibed at 22°C in the light and in the dark. The plates were wrapped with two sheets of aluminum foil and covered with a black plastic bag immediately after sowing to produce complete darkness. Germination was scored as radicle protrusion. All the 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 rates from batch to batch, but the results were almost parallel.

DNA extraction

Five to six rosette leaves were homogenized in 500 μl of CTAB buffer (3% cetyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 0.2% mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0), and incubated for 30 min at 60°C. The homogenate was extracted once with chloroform, and the nucleic acids were precipitated using isopropanol. After centrifugation, the pellet was dissolved in 300 μl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and treated with RNase A followed by chloroform extraction. DNA was precipitated with 1/3 vol. of 7.5 M ammonium acetate and an equal volume of ethanol. After centrifugation, the pellet was rinsed with 70% ethanol, dried and dissolved in 50 μl of TE.

Segregation analyses of thermoinhibition resistance and T-DNA insertion

TRW mutants were crossed with Ws reciprocally, and after-ripened F1, F2 and F3 seeds were used for segregation analyses. Thermoinhibition resistance was tested at 32°C and at 34°C as described in ‘Germination tests’. T-DNA insertions were detected by antibiotic resistance and by PCR amplification. Antibiotic resistance conferred by the nptII gene in the T-DNA was evaluated by root elongation and the appearance of green rosette leaves on a Murashige-Skoog agar plate containing 0.1 mg ml⁻¹ kanamycin. About 30 F3 seeds from each F2 were used for evaluating segregation of kanamycin resistance. To detect T-DNA directly, genomic DNA was extracted from the F2 leaves and used for PCR analysis. Primers for the left border region of pGKB5 were 5'-CCGTAGCCCTGTCCTCCTTG-3' and 5'-CTTTTCTTGC CGTTTTCGTC-3'. PCR was performed as described in ‘Molecular mapping’, with an annealing temperature of 55°C. The amplified fragment (1,030 bp) was separated on a 0.8% agarose gel (Agarose L03, TAKARA BIO INC., Ohtsu, Japan) and detected by ethidium bromide staining. About 100 F2 plants were used for the segregation analyses.
PCR cloning and sequencing of ABI3 gene

Two overlapping ABI3 gene fragments of Ws and TRW187 were amplified by PCR using two primer sets (P1 5'-GAAAA GCTTGCATGGGCG-3' + P3 5'-ACAAACAAACATCAG CAG-3', and P4 5'-CATTAGCCGATACATCC-3' + P6 5'-AA CAGTTTGAAGAGTGGTG-3') designed from the ABI3 mRNA sequence of Col (X681141). PCR s (total 50 µl) contained 1× PCR buffer (TAKARA SHUZO CO. LTD, Ohtsu, Japan, containing 2 mM MgCl2, 0.2 mM of each dNTP, 1 µM primers, 2.5 U of Taq DNA polymerase (ExTaq™, TAKARA BIO INC.) and about 20 ng of genomic DNA. PCR was performed as described in the next section, with an annealing temperature of 55 °C. Amplified DNA fragments were separated on a 1% agarose gel, detected by SYBR Green, extracted and purified using a QIA quick Gel Extraction kit (Qiagen), and cloned into pT7Blue T-Vector (Novagen) by TA cloning. Cloned DNA fragments were sequenced on both strands by a DNA sequencer ABI Prism 3100 Genetic Analyzer (Applied Biosystems/HITACHI).

Molecular mapping

TRW13-1 and TRW134-15 (Ws background) were crossed with the Col+ ecotype to create mapping populations. After ripened F3 seeds from each F2 were used for the germination test at 32 °C (TRW13-1) or at 22 °C in the presence of 10 µM ABA (TRW134-15), and the lines which showed the thermoinhibition-resistant (TRW13-1) or ABA-insensitive (TRW134-15) phenotypes were used for linkage analysis with molecular markers. The TRW13-1 mutation was initially roughly mapped to a chromosome using the SSLP and CAPS markers, NGA63 and NGA280 (chromosome 1), M246 and NGA168 (chromosome 2), NGA162 and NGA6 (chromosome 3), NGA8 and NGA1107 (chromosome 4), and NGA151, NGA76 and NGA129 (chromosome 5). Since NGA280 showed the lowest recombination value with TRW13-1, the mutation was mapped in detail on the bottom arm of chromosome 1 using SSLP markers (NGA111 and F51449495) and CAPS markers (1G4 and KNAT2). The mutation of TRW134-15 showed the lowest recombination value with NGA129 and mapped further with SSLP markers on the bottom arm of chromosome 5 (Jv61, Jv5f, Jv5t, and MBK-5). Primer sequences and the restriction enzymes were used as listed at The Arabidopsis Information Resource (TARI; http://www.arabidopsis.org). SSLP and CAPS fragments were digested with restriction enzymes of KNAT2 and the restriction enzyme of KNAT2 were used as described by Laswell et al. (2000).

The reaction mixture for PCR (total 20 µl) was composed of 0.5 U of DNA polymerase (TaKaRa Taq™, TAKARA BIO INC.), 2 µl of 10× PCR buffer (TAKARA SHUZO Co. LTD), 0.2 mM dNTPs (TAKARA SHUZO Co. LTD), >2 ng of genomic DNA and 1 µM forward and reverse primers. PCRs were performed in a programmed incubator (Thermal Cycler MP, TAKARA SHUZO CO. LTD) which was programmed for 4 min at 94 °C and then 30 cycles of 30 s at 94 °C, 30 s at 55–65 °C and 1 min at 72 °C. PCR products with CAPS primers were digested overnight with >0.5 U of restriction enzymes [MaeI (Roche Diagnostics GmbH, Mannheim, Germany) for M246, ScfI (Roche Diagnostics GmbH) for 1G4 and Atbl (TAKARA BIO INC.) for KNAT2]. SSLP and CAPS fragments were separated on 2–3% agarose gels (agarose L03, TAKARA BIO INC.) or 4–5% agarose gels consisting of a half-and-half mixture of agarose L03 and NuSieve GTG agarose (Cambrex Bio Science Rockland, Inc., ME, USA), and stained with ethidium bromide.

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References


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