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Gibberellin Requirement for Arabidopsis Seed Germination Is Determined Both by Testa Characteristics and Embryonic Abscisic Acid[1]

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The mechanisms imposing a gibberellin (GA) requirement to promote the germination of dormant and non-dormant Arabidopsis seeds were analyzed using the GA-deficient mutant *ga1*, several seed coat pigmentation and structure mutants, and the abscisic acid (ABA)-deficient mutant *aba1*. Testa mutants, which exhibit reduced seed dormancy, were not resistant to GA biosynthesis inhibitors such as tetcyclacis and paclobutrazol, contrarily to what was found before for other non-dormant mutants in Arabidopsis. However, testa mutants were more sensitive to exogenous GAs than the wild-types in the presence of the inhibitors or when transferred to a GA-deficient background. The germination capacity of the *ga1-1* mutant could be integrally restored, without the help of exogenous GAs, by removing the envelopes or by transferring the mutation to a *tt* background (*tt4* and *ttg1*). The double mutants still required light and chilling for dormancy breaking, which may indicate that both agents can have an effect independently of GA biosynthesis. The ABA biosynthesis inhibitor norflurazon was partially efficient in releasing the dormancy of wild-type and mutant seeds. These results suggest that GAs are required to overcome the germination constraints imposed both by the seed coat and ABA-related embryo dormancy.

The important role of the plant hormones gibberellins (GAs) in promoting seed germination is indicated by several observations. In plant species such as Arabidopsis and tomato, the strong alleles of GA-deficient mutants are unable to germinate without exogenous GAs (Koornneef and van der Veen, 1980; Groot and Karssen, 1987). A de novo biosynthesis of GAs is required during imbibition, as concluded from the observation that inhibitors of GA biosynthesis such as paclobutrazol and tetcyclacis prevent germination (Karssen et al., 1989; Nambara et al., 1991). A germination-promoting role for GAs has also been deduced from their ability to overcome germination constraints that exist in seeds requiring after-ripening (Metzger, 1983; Grappin et al., 2000), light (Hilhorst and Karssen, 1988; Derkx and Karssen, 1993a; Yang et al., 1995; Toyomasu et al., 1998), and cold. This led to the suggestion that such environmental factors may induce GA biosynthesis during the early phases of germination. Indeed, this light effect has been shown convincingly by Toyomasu et al. (1998) for lettuce and by Yamaguchi et al. (1998) for Arabidopsis. In the latter species, two 3-β-hydoxylases enzymes encoded by the *GA4* and *GA4H* genes are induced by phytochrome. Cold treatments do not stimulate GA biosynthesis in Arabidopsis seeds but, rather, increase their sensitivity to GAs (Derkx and Karssen, 1993a).

Two different mechanisms of action have been proposed to explain the role of endogenous GAs in the control of germination. The first one is the induction of the expression of genes encoding enzymes hydrolyzing the endosperm. This tissue confers part of the mechanical resistance to radicle protrusion, as demonstrated in tomato (Groot and Karssen, 1987; Groot et al., 1988), tobacco (Leubner-Metzger et al., 1996), and barley (Schuurink et al., 1992). The second mechanism consists of a direct stimulating effect on the growth potential of the embryo, as suggested for Arabidopsis (Karssen and Laçka, 1986). This growth potential is assumed to be restricted by the plant hormone abscisic acid (ABA), which is produced in the embryo (Karssen et al., 1983). ABA has been suggested to induce a dormant state during the later phases of seed maturation; after this point its function is limited because the concentration falls below an inhibiting level. GA is required to overcome this ABA-induced dormant state. However, the finding that ABA levels increase upon imbibition in dormant seeds and not in non-dormant seeds (Le Page-Degivry and Garello, 1992; Wang et al., 1995; Grappin et al., 2000) may indicate that the actual level of ABA during imbibition is important. Therefore, as in the induction of genes involved in reserve mobilization in the cereal aleurone system (Skadsen, 1998), GA and ABA can act antagonistically. These two different mechanisms, one targeted to the envelopes and one to the embryo, do not have to be mutually exclusive, because dormancy and germination are probably the net result of a balance between many promoting and inhibiting factors.

GAs may not be the only factor through which environmental factors modify dormancy in seeds. Only after-ripening, not GA application, was found to regulate seed dormancy release in wild oat (*Avena fatua* L.; Fennimore

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and Foley, 1998). Similarly, in *Sisymbrium officinale*, Derkx and Karssen (1993b) showed that seasonal dormancy patterns were regulated by sensitivity to light and nitrate rather than by GAs.

The aim of the present study was to investigate the role of GAs in dormancy and germination of Arabidopsis seeds. Special attention was paid to the seed envelopes, and particularly to the seed coat, as a factor interfering with germination and dormancy. For this purpose, we studied the germination behavior of several testa mutants affected in flavonoid pigmentation or in structural characteristics, in combination with GA deficiency conferred by the *ga1* mutation. In addition, we compared the effect of various compounds inhibiting GA and ABA biosynthesis with biosynthesis mutants. The use of such inhibitors allows a more specific analysis of the time when de novo synthesis is playing a role, but the interpretation of the results can be biased by differences in uptake of the compounds. Using testa mutants that were shown to take up tetrazolium dyes much more easily than the wild types (Debeaujon et al., 2000), we show here the importance of these permeability factors.

MATERIALS AND METHODS

Genotypes

The origin and genetic background of the seed coat mutant alleles *tt2-1*, *tt4-1*, *tt7-1*, *ttg1-1*, *tt12-1*, *ats-1*, *gl2-1*, and *ap2-1* of Arabidopsis used in this experiment are described in Debeaujon et al. (2000). The *tt* mutants are characterized by a yellow (*tt2-1*, *tt4-1*, and *ttg1-1*) or pale brown (*tt7-1* and *tt12-1*) seed color. The *ttg1*, *gl2*, and *ap2* mutants are characterized by an aberrant testa surface that excretes very little mucilage (Koornneef, 1981) and the *ats* mutant has a heart-like seed shape due to the absence of two integument layers (Léon-Kloosterziel et al., 1994). The *ap2* and *ats* mutants are assumed to have structural testa defects that allow them to take up tetrazolium salts, as is the case with *tt* mutants but not with the wild types or the *gl2-1* mutant (Debeaujon et al., 2000).

The isolation of the non-germinating GA-deficient mutants *ga1-1* (W58) and *ga1-3* (W113) in the Landsberg *erecta* (L*er)* background was described by Koornneef and van der Veen (1980) and the molecular defects of these alleles by Sun et al. (1992). The T-DNA-tagged *ga1-11* allele in the Wassilevskija (Ws) background was isolated from the Versailles T-DNA transformant collection after a screen for non-germinating mutants (Dubreucq et al., 1996).

The ABA-deficient allele *aba1-1* (A26) was obtained by screening for germination after an ethyl methanesulfonate mutagenesis of *ga1-1* mutant seeds (Koornneef et al., 1982). The *aba1-6* allele in the Ws background was recovered from the Feldmann T-DNA transformant collection after screening for seeds germinating in presence of a 10 μM concentration of the GA biosynthesis inhibitor tetcyclacis (BASF, Ludwigshafen, Germany). A cross with *aba1-1* gave non-dormant seeds and F_1 seedlings with the typical phenotype of *aba1* mutants, which indicated that this mutant was an allele of *aba1*. The resistance to tetcyclacis, which is a characteristic of all ABA-deficient mutants isolated thus far (Léon-Kloosterziel et al., 1996), was confirmed in this mutant (data not shown).

Growth Conditions

The seed lots were harvested on plants grown as previously described (Debeaujon et al., 2000). Seed lots to be compared were grown in the same environmental conditions, harvested the same day from mature siliques, and stored at room temperature in cellophane bags. The *ga1* mutant seeds were sown on filter paper soaked with 10 μM GA_{4+7} (ICI) to enable germination. Once in the greenhouse, the *ga1* plants were sprayed once a week with 100 μM GA_{4+7} to stimulate elongation growth, anther development, and seed production.

Construction of Double Mutants

Double mutants of *tt* mutants and *ga1* were obtained by crossing *ga1-1* with *tt4-1* and *ttg1-1* and by crossing *tt12-1* with *ga1-11*. F_2 seeds originating from these crosses were first germinated on water after 5 d of cold treatment to break dormancy. Germinating seeds were discarded and the remaining ones were put on 10 μM GA_{4+7} to induce germination before planting in the greenhouse. Dwarf plants were selected and sprayed with GA_{4+7} for seed set. The F_3 seeds with a *tt* phenotype harvested on GA-deficient F_2 plants were retained as double mutants. The double mutants with *tt4* and *ttg1* could be selected as F_2 plants on the basis of a lack of anthocyanins in their leaves.

Double mutants between *ga1* and *aba1* were obtained by crossing *ga1-3* with *aba1-1* and by crossing *ga1-11* with *aba1-6*. F_2 seeds germinating on 10 μM tetcyclacis were grown in the greenhouse. Dwarf plants were selected and sprayed with GA_{4+7} for double mutant seed set.

Germination Assays

All germination experiments were performed in 6-cm Petri dishes on filter paper (no. 595, Schleicher & Schuell, Dassel, Germany). Each genotype was sown in triplicate (80–100 seeds from one individual plant per Petri dish). The average germination percentage was determined after 7 d of incubation in a climate room (25°C, 16 h light/d; TL57 bulbs, Philips, Eindhoven, The Netherlands). Average germination percentages were calculated with SES of the triplicates. For dark germination experiments, Petri dishes were wrapped in two layers of aluminum foil and stored in a closed box. In some experiments, the seeds sown on water-soaked filter paper were submitted to 5 d of cold treatment at 6°C (chilling) to break dormancy.

Filter papers were soaked either with water or with solutions of the growth regulators GA_{4+7} and ABA (mixed isomers, Sigma-Aldrich, St. Louis), of the GA biosynthesis inhibitors tetcyclacis and paclobutrazol (ICI) or of the ABA biosynthesis inhibitor norflurazon. The pH of the aqueous solutions of GA_{4+7} and ABA was adjusted to 7.0 with KOH. Norflurazon was dissolved in pure dimethyl sulfoxide (DMSO). A preliminary experiment showed that the

maximum DMSO dose used in our experiment (400× dilution of the 99% [w/v] solution) did not have any effect on seed germination (data not shown).

Microscopy

The cellular aspect of the aleurone layer was observed in mature seeds before and after germination. Whole seeds and remaining seed coats were dissected under a stereomicroscope (Zeiss, Jena, Germany). Pieces of aleurone layer were mounted in an aqueous solution of 0.03% (w/v) ruthenium red and observed under a light microscope (Optiphot, Nikon, Tokyo).

RESULTS

Germination Behavior of *ga1* Single and Double Mutants

In our previous report (Debeaujon et al., 2000), we showed that most testa mutants exhibited reduced seed dormancy. To investigate to what extent the GA requirement for germination may be imposed by the testa, the embryos of the GA-deficient mutants *ga1-1*, *ga1-3*, and *ga1-11* were excised from the envelopes. The germination behavior of these embryos without the surrounding testa and aleurone layer was found to be 100% for all *ga1* alleles. In contrast, none of the intact seeds germinated, demonstrating the restrictive effect of the envelopes on germination. Results of this experiment did not reveal which component(s) of the envelopes, i.e. the testa versus the aleurone layer or both tissues, restrict(s) germination of the *ga1* mutants. Therefore, we examined the effect of mutations altering testa pigmentation (*tt4*, *ttg1*, and *tt12*) on the germination of *ga1* GA biosynthetic mutants in the absence of exogenous GA.

The germination response of the various single and double mutants to light and chilling was investigated. The *ga1-3 aba1-1* double mutant was included in this analysis because it had previously been shown (Koornneef et al., 1982) that ABA deficiency alleviates the GA requirement for germination conferred by the *ga1-1* and *ga1-2* alleles. The data presented in Figure 1 show that freshly harvested seeds of the wild types Ws and L*er* require light for germination but are still partially dormant in light. The cold treatment results in 100% germination in light and abolishes the light requirement in L*er* but not fully in Ws. The *tt* and *aba* mutants all show a higher germination percent-

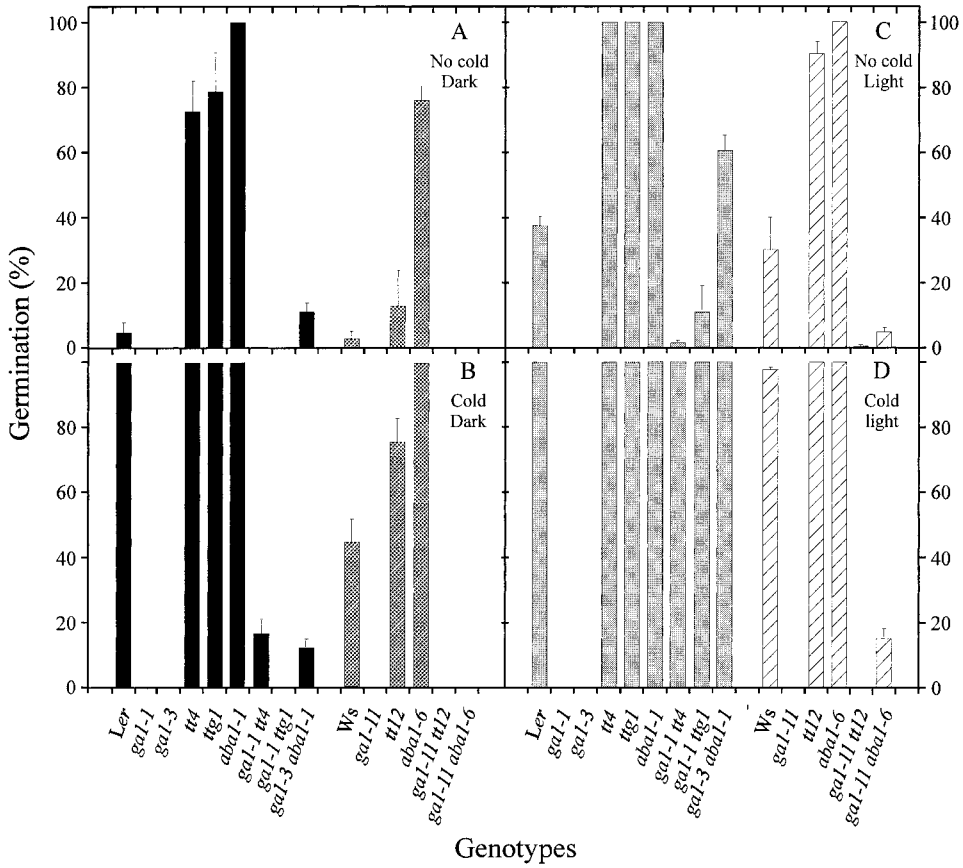

Figure 1. Effect of light and chilling on dormancy breaking and germination of wild types, single mutants, and double mutants. Black bars represent germination of genotypes in L*er* background in darkness; the dotted bars (in A and B) represent germination of genotypes in Ws background in darkness. The gray bars (in C and D) represent germination of L*er* genotypes in light, and the striped bars (in C and D) represent germination of Ws genotypes in light. Seeds were sown on water 11 d after harvest.

age compared with their wild types, as shown previously (Debeaujon et al., 2000; Koornneef et al., 1982). None of the four environmental conditions induced germination of the three *ga1* alleles.

The *ga1-1* mutant germinated at 100% only in a *tt4-1* and *ttg-1* background when submitted to a cold treatment followed by germination in the light. In *ga1-3* background, the *aba1-1* mutation abolished the GA requirement under these germination conditions. Chilling alone led to 17% germination in the *ga1-1 tt4-1* double mutant and to 12% in the *ga1-3 aba1-1* double mutant, but did not induce germination in any other *ga1* genotype. Light without a cold treatment induced some germination in the double mutants of *ga1-1* with *tt4* (2%) and with *ttg1* (11%) and even 60% germination in *ga1-3 aba1-1*. The *ga1-11 aba1-6* double mutant in the Ws background (and therefore using other alleles) germinated only 15% when both light and chilling were applied. The *ga1-11 tt12* double mutant did not germinate at all without exogenous GAs. However, the *tt12* mutation strongly increased the GA responsiveness of the *ga1-11* mutant, as shown in Figure 2. Germination is induced at a 100-fold lower GA_{4+7} concentration compared with the monogenic *ga1-11* mutant in Ws background (Fig. 2B). The overall sensitivity of the *ga1-11* allele to GAs was reduced compared with the L*er ga1-1 and ga1-3* alleles, which only slightly differed from each other (Fig. 2A).

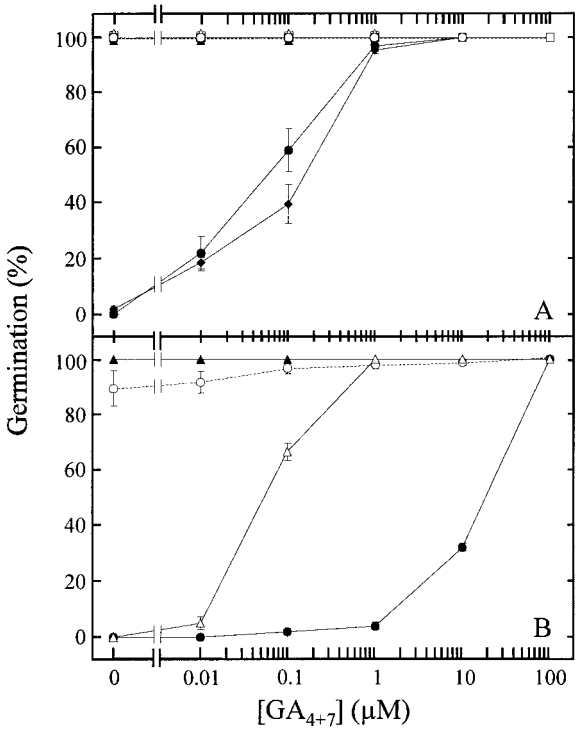

Figure 2. Sensitivity to GA_{4+7} of wild types, single, and double mutants between *tt* and *ga1* mutants. A cold treatment was applied to seeds before germination. A, ○, L*er*; ▲, *tt4*; ■, *ttg1*; ●, *ga1-1*; ♦, *ga1-3*; △, *ga1-1 tt4*; □, *ga1-1 ttg1*. B, ○, Ws; ▲, *tt12*; ●, *ga11-11*; △, *ga1-11 tt12*. Dashed lines represent wild types (Ws and L*er*).

Sensitivity to GA Biosynthesis Inhibitors

From the experiments described above it appears that a pigment defect in the testa reduces the GA requirement for germination. While in the testa mutants germination does not require GAs, it can be expected that this will lead to insensitivity or an increased resistance to GA biosynthesis inhibitors, as shown before for ABA-deficient mutants (Léon-Kloosterziel et al., 1996). To test this hypothesis, a larger number of *tt* mutants and other mutants with structural testa defects, e.g. *ats*, *ap2*, and *gl2* (Debeaujon et al., 2000), were investigated for their resistance to tetcyclacis (Fig. 3, A–C) and paclobutrazol (Fig. 3, D–F). Surprisingly, the testa mutants, with the exception of *tt7* and the structural mutants *ats*, *ap2*, and *gl2*, which are more sensitive, are only slightly more resistant to tetcyclacis. However, all are even more sensitive to paclobutrazol than the corresponding wild types. It appears also that Ws is far more sensitive to tetcyclacis than L*er*, but that both wild types are equally sensitive to paclobutrazol (Fig. 3, A and C).

Apparently, the tetcyclacis sensitivity of the genotypes in the L*er* background depends on the time of dry storage (after-ripening). Indeed, resistance is higher when the same seed lots were tested 3 months later (Fig. 4), with the testa mutants having a higher germination percentage than wild type on 100 μM inhibitor (except for *gl2*, which behaves similarly to the wild type).

When endogenous GA biosynthesis is inhibited, the application of GAs should reflect exclusively the responsiveness to GAs. Genotype differences, then, might be interpreted as differences in the GA requirement for germination. The data presented in Figure 4 show that all testa mutants require less GA than the wild type to germinate at 100% after a combined application of 100 μM tetcyclacis or paclobutrazol together with GA. Only the *gl2* mutant was not that different from the wild type. The dependency of germination on GA was stronger when paclobutrazol instead of tetcyclacis was applied. Only Ws needed more GAs on tetcyclacis than on paclobutrazol. Moreover, the difference between this wild type and the *tt12* mutant was larger on tetcyclacis than on paclobutrazol.

The large difference between Ws and *tt12* in GA sensitivity was found to be maternally inherited, as indicated by the difference between the reciprocal crosses (Fig. 5). This indicates that the primary defect of the *tt12* mutant, which is an altered testa pigmentation, is the cause of the apparent increased sensitivity to exogenous GAs.

Sensitivity of Dormant Seed Lots to the ABA Biosynthesis Inhibitor Norflurazon

To determine whether de novo biosynthesis of ABA taking place during seed imbibition may impose dormancy and therefore a higher GA requirement for germination, dormant seed lots of wild types, *tt*s, *ga1*s, and double mutants between *tt*s and *ga1*s were germinated on increasing concentrations of the bleaching herbicide norflurazon (Fig. 6). This product is an inhibitor of carotenoid biosynthesis, and therefore of ABA biosynthesis (Chamovitz et al., 1991). Norflurazon had a significant stimulating effect on

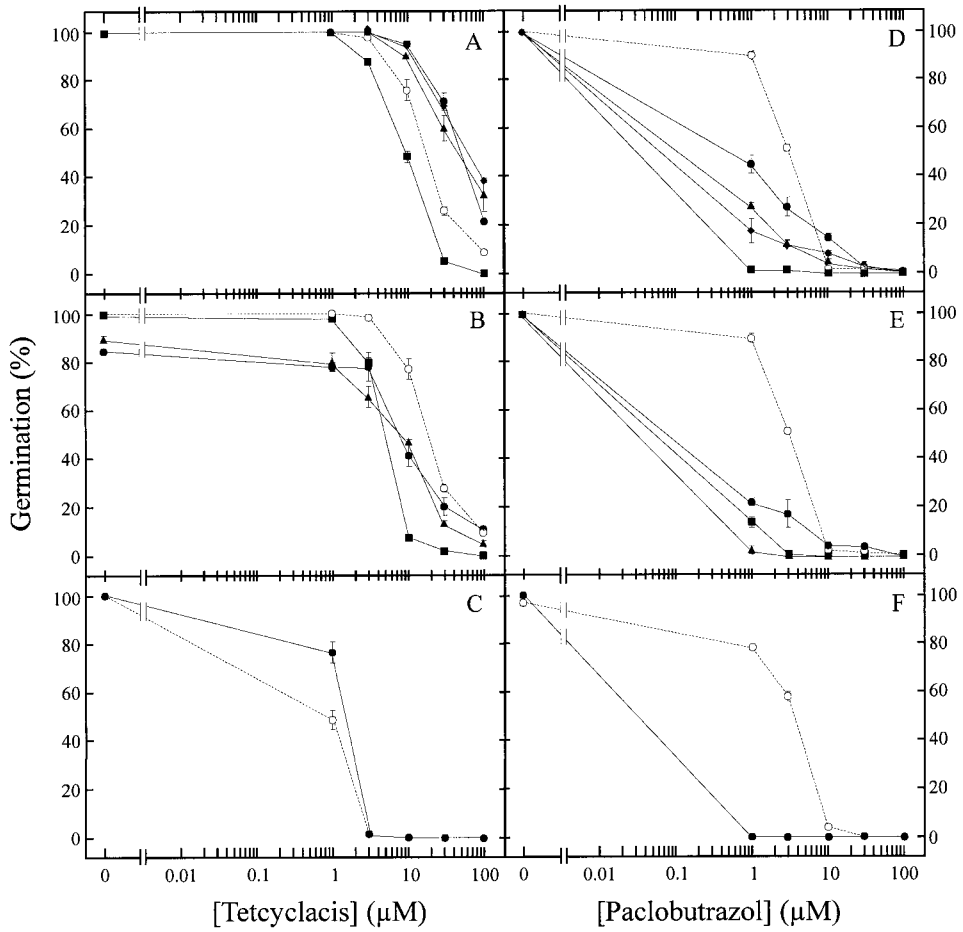

Figure 3. Sensitivity of wild-type and seed coat mutant seeds to the GA-biosynthesis inhibitors tetcyclacis and paclobutrazol. A cold treatment was applied to seeds before germination. Sowing was done 3 months after seed harvest. A and D, ○, L*er*; ●, *tt2*; ▲, *tt4*; ■, *tt7*; ◆, *ttg1*. B and E, ○, L*er*; ●, *ats*; ▲, *ap2*; ■, *gl2*. C and F, ○, Ws; ●, *tt12*. Dashed lines represent the wild types L*er* and Ws.

germination of all the genotypes that were still dormant after 10 d of after-ripening. Germination was increased from 38% to 85% for the *tt12* mutant and from 0% up to 42% for Ws. However, even in the *tt12* mutant, 100% germination could not be obtained. The norflurazon treatment could not restore the germination capacity of the *ga1* single mutants. Only when the mutation was in a *tt4* or *ttg1* background did the germination percentage increase to nearly 50%. In contrast, the *ga1-11 tt12* double mutant did not respond to this treatment.

Morphological Changes in the Aleurone Layer due to Germination

To determine whether GAs may influence the metabolism in the outer layer of the peripheral endosperm (aleurone layer) in Arabidopsis mature seeds, we analyzed microscopically aleurone cells before (30-min imbibition) and after (seedlings protruding from the testa) germination in the genotypes L*er*, *ga1-1*, *tt4* and *ga1-1 tt4*. The aleurone cells of the wild type L*er* before germination (Fig. 7a) appeared to be loaded with refringent inclusions, which are essentially storage reserves (Mansfield and Briarty, 1994). After germination (Fig. 7b), these structures completely disappeared and the cells became distended. The same was observed for the two other germinating genotypes *tt4* and *tt4 ga1*, but not for the non-germinating *ga1* mutant, which indicated that this morphological change at the level of the aleurone layer occurred independently from GA but depended on germination. Slightly damaging the testa of the *ga1-1* mutants resulted in germination and in the disappearance of the inclusions in the remaining endosperm as well. On the other hand, when the embryo was removed completely from the envelopes, no modification of the aleurone cells could be detected.

DISCUSSION

The *GA1* gene encodes the enzyme copalyl diphosphate synthase, which catalyzes the conversion of geranylgeranyl pyrophosphate into copalyl pyrophosphate (Sun and Kamiya, 1994). Therefore, the *ga1* mutants are affected in GA biosynthesis and, as a consequence, depend on exogenous GAs for germination (Koornneef and van der Veen, 1980).

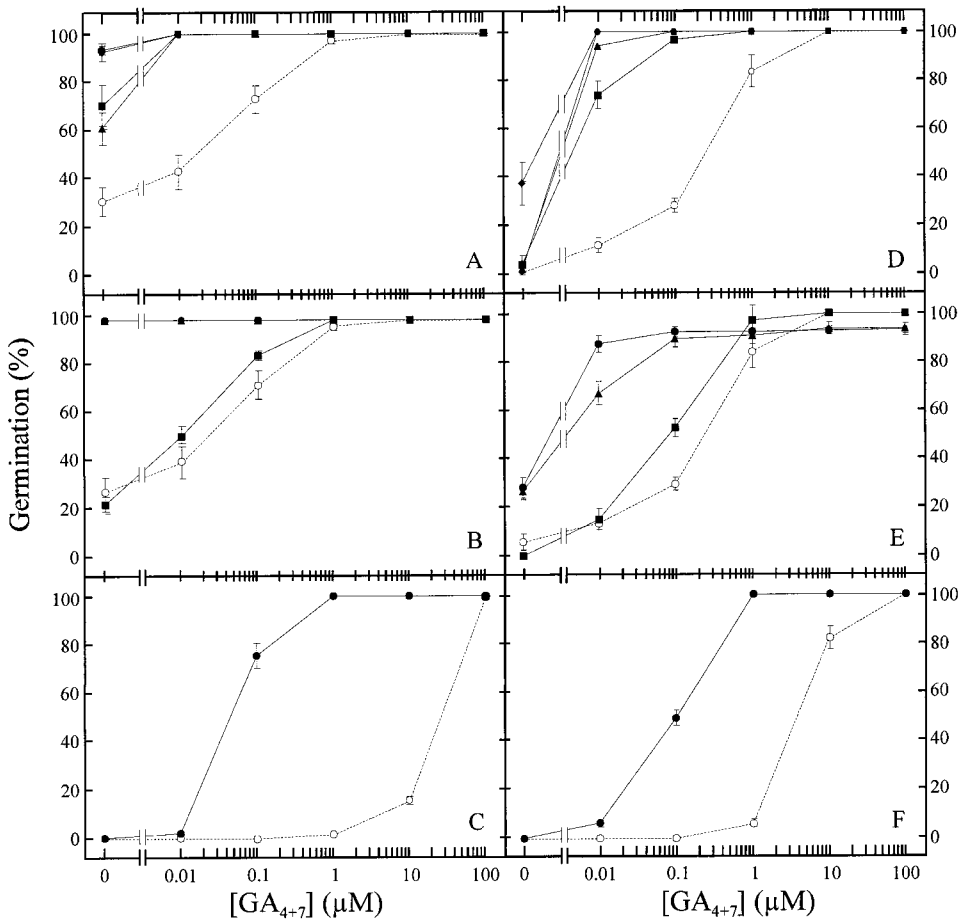

Figure 4. Sensitivity of wild-type and seed coat mutant seeds to GA_{4+7} in presence of either 100 μM tetcyclacis (A–C) or 100 μM paclobutrazol (D–F). A cold treatment was applied to seeds before germination. Sowing was done 6 months after seed harvest. A and D, ○, L*er*; ●, *tt2*; ▲, *tt4*; ■, *tt7*; ♦, *ttg1*. B and E, ○, L*er*; ●, *ats*; ▲, *ap2*; ■, *gl2*. C and F, ○, Ws; ●, *tt12*. The dashed lines represent the wild types L*er* and Ws.

As observed for *ga1-3* (Telfer et al., 1997) and confirmed for the two additional alleles *ga1-1* and *ga1-11*, mechanical removal of the seed envelopes can substitute for the GA requirement for germination. The low percentage of germination observed in the *ga1-3* mutant by Silverstone et al. (1997) was postulated to be due to a testa damage and/or a carryover of exogenous GA_3 used to stimulate growth of the GA-deficient mother plants.

In the present paper we show that the double mutants of some *tt* and *ga1* mutants are able to fully germinate in the absence of exogenous GAs when imbibed in optimal germination conditions with respect to light and temperature regime. These data imply that GAs are necessary during Arabidopsis seed germination to overcome the germination-restricting properties of the testa itself. In other words, the specific weakening of the testa is sufficient to enable germination in absence of GAs. In this, the effect of the testa mutations resembles the effect that *aba* mutations have on the GA requirement for germination (Koornneef et al., 1982). However, for ABA the effect is under embryonic genetic control (Karssen et al., 1983).

The abolishment of the GA requirement for germination by *aba* or *tt* mutations is not seen for the *ga1-11* allele. This difference might be due to the *ga1* allele that was used; for the testa mutant, the difference in locus may be relevant. However, we favor the hypothesis that the capacity to alleviate the GA requirement depends on the genetic background. The *ga1-3* mutation, which is a 5-kb deletion from 1 kb upstream ATG to exon 11, is assumed to be the null allele in L*er*, in contrast to *ga1-1*, which results from a single base pair substitution at the level of a splice junction (Sun and Kamiya, 1994). Despite this, both alleles are non-germinating under all conditions tested and both have the same GA sensitivity (Fig. 2A). The *ga1-11* allele, which most likely is also a null mutation (Dubreucq et al., 1996), is much less responsive to exogenous GA (Fig. 2B). Although this may be due to differences in GA uptake, it seems likely that in the more dormant Ws ecotype, GA sensitivity is strongly reduced. In what way this reflects differences in GA metabolism is not known.

It cannot be excluded that in Arabidopsis, testa weakening is the consequence of GA action. This action may not be required anymore when the testa is genetically weakened. Therefore, the low water potential of the wild type and GA-deficient embryos (for tomato, see Liu et al., 1996) may result in a passive water uptake not prevented anymore by

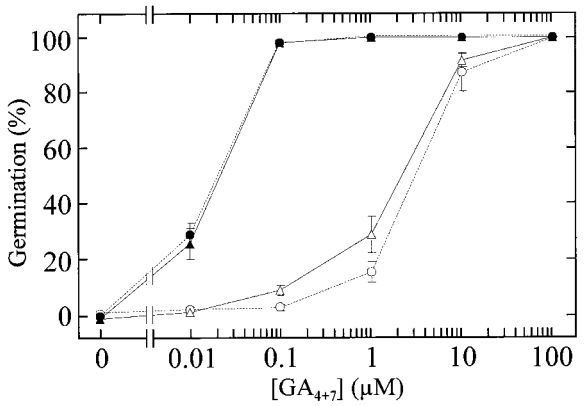

Figure 5. Genetic determinism of the increased sensitivity to GA_{4+7} exhibited by the *tt12* mutant in the presence of 100 μM tetcyclacis. The parent mentioned first was used as the female parent and the second as the pollen parent. A cold treatment was applied to seeds before germination. ○, Ws; ●, *tt12*; △, Ws × *tt12*; ▲, *tt12* × Ws.

the restraint of the envelope structures and may subsequently lead to radicle protrusion. The further growth of the embryo might not require GA as such, although cell elongation is affected. The fact that germination and not GA induces metabolic changes is also indicated by the disappearance of the storage reserves in the aleurone layer during the process of germination (Fig. 7). This metabolic activity was independent from the presence of GAs, as it also took place in *ga1-1 tt4* double mutants but not in non-germinating *ga1-1* mutants, and therefore correlated with the germination event itself.

The role of ABA as an antagonist of GA in germination of Arabidopsis (Karssen et al., 1983; Steber et al., 1998; this report) might be through its inhibiting effect on the growth potential of the embryo, which is required to overcome the restraint imposed by the envelope structures. This agrees with the observation that ABA effects are controlled by the genotype of the embryo (Karssen et al., 1983), in contrast to the testa, which is of maternal origin.

It has been suggested that environmental factors such as light and cold treatment can promote germination by inducing GA biosynthesis (Yamaguchi et al., 1998) or by increasing GA sensitivity (Karssen and Laçka, 1986). With both hypotheses it is expected that when GA biosynthesis is not possible, as should be the case in GA-deficient mutants, these treatments should have no germination-promoting effect. However, this is not what was found for the *ga1* double mutants, for which the GA requirement for germination was abolished under certain conditions. An explanation for this observation might be that light and cold can have a stimulating effect on dormancy breaking and/or seed germination-promoting factors other than GAs, such as the increase of ABA degradation, as postulated by Kraepiel et al. (1994).

Karssen et al. (1989) observed that ethylene could induce full germination of *ga1* seeds in the absence of GAs but in the presence of light, suggesting that ethylene may act in parallel to GAs. However, it may not be excluded that other factors, apart from plant hormones, might be affected by light and chilling. This interpretation is valid if the *ga1* mutants are completely deprived of GAs or if GAs are not present in a concentration sufficient to trigger a germination response, when the sensitivity of the system is maximal due to, for example, a cold treatment (Karssen and Laçka, 1986). However, Zeevaart and Talon (1992) could detect the presence of a very low amount of GA in the *ga1-3* mutant, despite the fact that this allele is a null one (Sun et al., 1992). Silverstone et al. (1997) proposed that the remaining GA may have been due to a carryover of the GAs sprayed on the mother plant to sustain a normal growth and fruit set.

The *ga1-3 aba1-1* double mutant responded much less to cold treatment than the *ga1-1 tt* double mutants, which might suggest that a chilling treatment is only effective when ABA is present in mature seeds or was present during seed development. Effects of a cold treatment on the *aba* mutants, which are known to be somewhat leaky (Rock and Zeevaart, 1991), have been reported for dark germination (Koornneef et al., 1982), but are minimally detected because of the high germination percentage without the promotive factors light and cold. It cannot be excluded that cold treatment may act both through GA and ABA, which would explain why only a double mutant would not be responsive.

The data obtained with the *ga1 tt* double mutants seem to be in conflict with the increased sensitivity to inhibitors of GA biosynthesis in the monogenic *tt* mutants. This is in

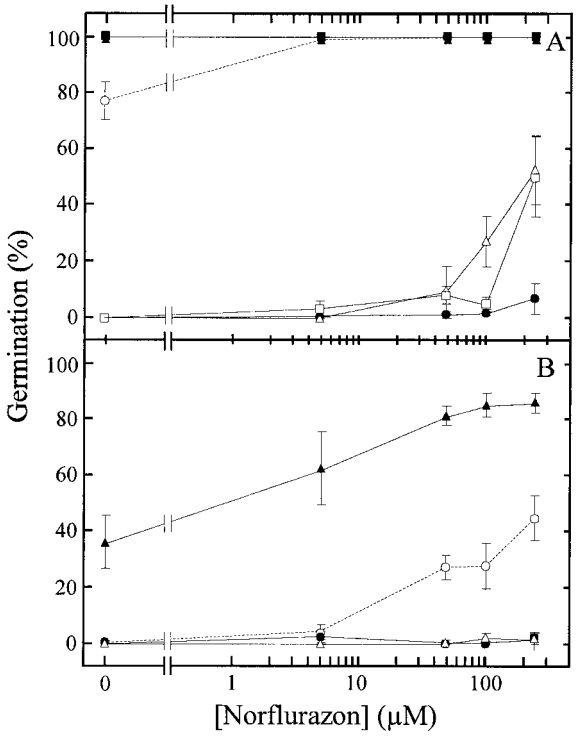

Figure 6. Sensitivity to the ABA biosynthesis inhibitor norflurazon of wild types, single mutants, and double mutants between *tt* and *ga1* mutants. Seeds were sowed 10 d after harvest. A, ○, L*er*; ▲, *tt4*; ■, *ttg1*; ●, *ga1-1*; △, *ga1-1 tt4*; □, *ga1-1 ttg1*. B, ○, Ws; ▲, *tt12*; ●, *ga1-11*; △, *ga1-11 tt12*.

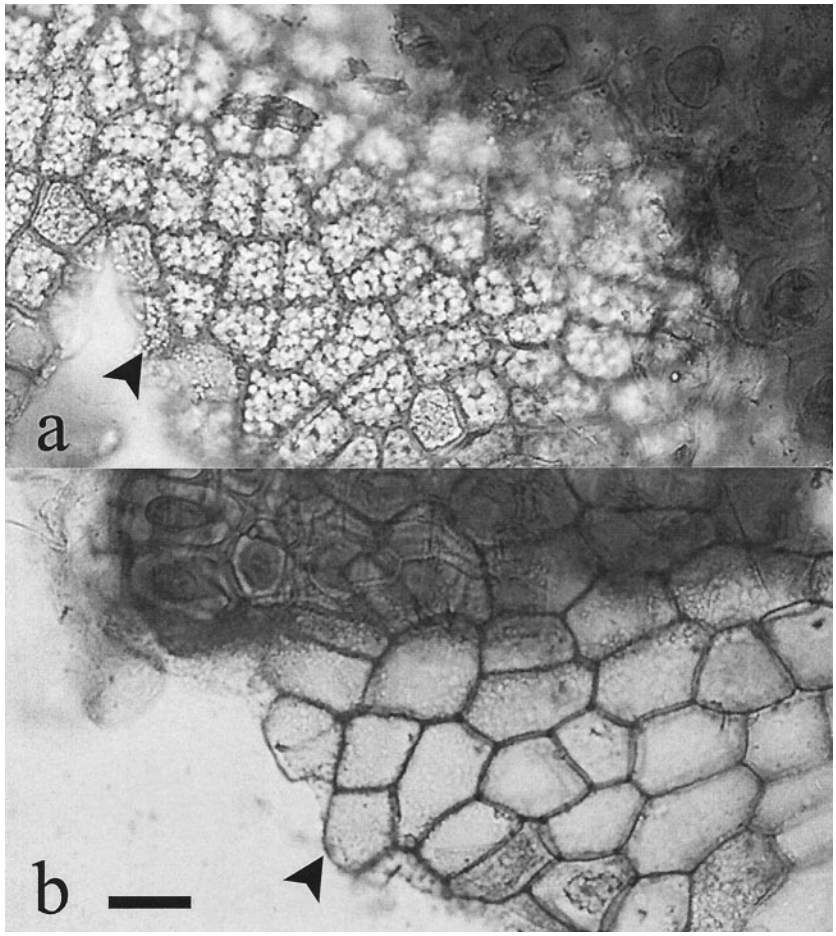

Figure 7. Aleurone layer of the wild-type L*er* before (a) and after (b) seed germination. The aleurone layer (arrowheads) is still associated with pieces of the seed coat. Cell walls are stained with ruthenium red. Bar = 20 μm.

contrast to *aba* mutants, which show a clear resistance to these inhibitors (Léon-Kloosterziel et al., 1996) in agreement with the abolishment of the GA requirement observed in the *ga1 aba1* double mutants. One difference between GA deficiency in the mutants and the application of inhibitors upon imbibition is that in the mutants, GA deficiency was experienced not only during imbibition but also during seed development, and that this affected the properties of the testa.

Another effect that might be important is the increased uptake of inhibitors by testa mutants. In another study (Debeaujon et al., 2000), we found that tetrazolium salts are taken up easily by the seeds of mutants, whereas these compounds could not be taken up by wild-type seeds. Differences in uptake may also explain the different effect of tetcyclacis and paclobutrazol on seed germination. Both tetcyclacis and paclobutrazol belong to the group of norbornanodiazetins and act as inhibitors of the oxidative steps from *ent*-kaurene to *ent*-kaurenoic acid, their target enzymes being cytochrome P450 monooxygenases (Rademacher, 1991). However, they differ considerably in their chemical formulas, and therefore may have a differential inhibitory action on the P450 monooxygenase involved in the oxidative catabolism of ABA into phaseic acid (Zeevaart and Creelman, 1988).

Differences in uptake of compounds by testa mutants might also explain or partially explain the increased sensi-

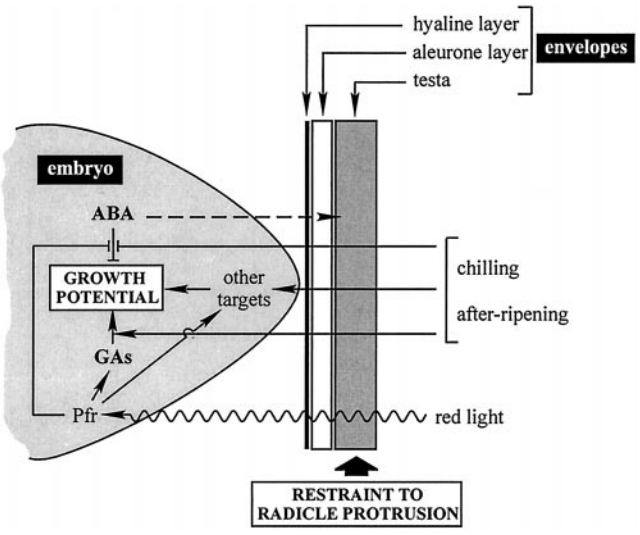

Figure 8. Schematic presentation of the interactions between the envelopes and the embryo affecting dormancy breaking and germination of Arabidopsis seeds. Germination occurs when the growth potential of the embryo is sufficient to overcome the restraint to radicle protrusion imposed by the seed envelopes. When the restraint of the testa is weakened by mutations, the growth potential threshold required for germination is lowered. The sharp arrows and the blunt arrows stand for a promotive and an inhibitory action, respectively. Dashed arrow indicates leaching of ABA through the testa.

tivity to GAs applied together with the inhibitor. It is also possible that the tannins present in the testa may act as specific antagonists of GAs, as was suggested previously (Corcoran et al., 1972; Green and Corcoran, 1975), which may explain the particularly dramatic effect of the testa mutation on GA uptake compared with inhibitor uptake.

It was reported for sunflower (Le Page-Degivry and Garello, 1992), barley (Wang et al., 1995), and *Nicotiana plumbaginifolia* (Grappin et al., 2000) that de novo ABA biosynthesis during imbibition took place in dormant seeds and was absent in non-dormant seeds, thus pointing to a role of ABA in dormancy imposition. The inhibiting effect of the ABA biosynthesis inhibitor norflurazon also indicates that in Arabidopsis there is a de novo ABA biosynthesis during imbibition and that its suppression by the inhibitor restores germination in dormant seeds. However, this restoration is only partial. In particular, it is unsuccessful in restoring the germination capacity of the *ga1* mutants. These results agree with the observation that *ga1 aba1* double mutants do not reach 100% germination when placed in limiting environmental conditions compared with the wild types. This might be an indication that not only does the ABA produced upon imbibition have to be overcome by GAs, but also that ABA produced in the developing seeds and/or the state of dormancy set by ABA during development plays a role (Karssen et al., 1983; Karssen and Laçka, 1986). To distinguish between the various temporal hormonal effects, the seed-specific immunomodulation of ABA activity postulated by Phillips et al. (1997) looks very promising in this respect. The various aspects discussed above are summarized in Figure 8.

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