

Isolation and characterization of abscisic acid-deficient *Arabidopsis* mutants at two new loci

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Summary

Novel *Arabidopsis* mutants with lowered levels of endogenous abscisic acid (ABA) were isolated. These were selected in a screen for germination in the presence of the gibberellin biosynthesis inhibitor paclobutrazol. Another mutant was isolated in a screen for NaCl tolerance. The ABA-deficiency was caused by two monogenic, recessive mutations, *aba2* and *aba3*, that were both located on chromosome 1. The mutants showed a phenotype that is known to be characteristic for ABA-deficiency: a reduced seed dormancy and excessive water loss, leading to a wilted phenotype. Double mutant analysis, combining different *aba* mutations, indicated the leaky nature of the mutations.

Introduction

The plant hormone abscisic acid (ABA) is a sesquiterpenoid molecule that is derived from xanthophyll carotenoids (C₄₀) via the C₁₅ intermediate xanthoxin (Taylor, 1991; Zeevaart and Creelman, 1988). Biochemical analysis of mutants that are deficient in ABA has facilitated the elucidation of the biosynthetic pathway of ABA in higher plants. Most of the mutants have been isolated on the basis of their wilted phenotype or on the basis of seed dormancy characteristics (Koornneef, 1986). In maize, viviparous seeds are easy to identify on cobs segregating for this trait (Robertson, 1955). In *Arabidopsis*, *aba1* mutants were identified among the revertants of non-germinating gibberellin-deficient

mutants (Koornneef *et al.*, 1982). In addition, mutants at the *abi1* and *abi2* loci are wilted mutants with a reduced seed dormancy but with normal or enhanced ABA levels (Koornneef *et al.*, 1984). These mutants were shown to be relatively insensitive to the inhibiting effect of ABA and apparently have a defect in ABA response. For most of the ABA-deficient loci the biosynthetic defect is known. Maize *viviparous* (*vp*) mutants have an early block in the biosynthesis of carotenoids (Neill *et al.*, 1986). The *Arabidopsis aba1* mutant (Duckham *et al.*, 1991; Rock and Zeevaart, 1991) and the *Nicotiana plumbaginifolia aba2* mutant (Marin *et al.*, 1996) are affected in the conversion of zeaxanthin to antheraxanthin and in the subsequent conversion to all-*trans*-violaxanthin, the direct C₄₀ precursors of ABA. The tomato *not* mutant and the pea *wil* mutant may be affected in the cleavage of 9'-*cis*-neoxanthin to xanthoxin, but to date this has not been convincingly proven (Taylor, 1991). A large number of mutants are known that affect the last step in ABA biosynthesis, that is, the conversion from ABA-aldehyde to ABA. These include *flc* and *sit* in tomato, *dr* in potato, *aba1* in *N. plumbaginifolia*, and *nar2a* in barley (Leydecker *et al.*, 1995; Taylor, 1991).

Shortly after its discovery, ABA was thought to be mainly a growth inhibitor, but it has become clear that it is involved in several important physiological processes. The phenotype of ABA-biosynthetic mutants indicates its function, for example, failure of stomatal closure leads to the wilted phenotype. The possible mechanisms by which ABA works on the ion fluxes associated with stomatal closure have been reviewed by MacRobbie (1991). Another aspect is the involvement of ABA in stress adaptation. Many genes have been isolated that show expression in correlation with the occurrence of ABA and/or stress such as osmotic stress or cold. Studies on the regulation and expression of these genes make clear that ABA is involved in at least part of the complex regulation of stress tolerance (Chandler and Robertson, 1994). The involvement of ABA in stress tolerance is also indicated by mutant analyses. ABA-biosynthetic mutants of *Arabidopsis* do not develop freezing tolerance (Gilmour and Tomashow, 1991; Heino *et al.*, 1990) and drought-resistant root structures (Vartanian *et al.*, 1994).

The involvement of ABA in the induction of dormancy has been demonstrated by studies on ABA-deficient mutants in *Arabidopsis* (Karssen *et al.*, 1983) and tomato (Groot and Karssen, 1992). In plants that are homozygous for both the *aba1* and the *abi3-1* mutation, or for severe alleles of *abi3*, dormancy as well as other aspects of seed maturation

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are abolished. These include the accumulation of storage proteins and the development of desiccation tolerance (Koornneef *et al.*, 1989; Nambara *et al.*, 1992; Ooms *et al.*, 1993).

To date, no mutants of *Arabidopsis* that affect other loci than the *ABA1* locus have been reported, whereas mutants at several other steps of the ABA biosynthesis are known in other species. The present paper describes the isolation and characterization of mutants at additional loci in *Arabidopsis* that result in ABA-deficiency, by means of selection on a GA biosynthesis inhibitor.

Results

Genetic analysis

EMS-mutagenized *Arabidopsis* M₂ seeds were screened for germination in the presence of the GA biosynthesis inhibitor paclobutrazol (Jacobsen and Olszewski, 1993). This screen yielded 22 mutants with a 'wilty' phenotype: the plants showed a tendency to wilt, especially in low relative humidity, were slightly darker green and less vigorous than wild-type plants. Allelism tests were performed among the new mutants mutually and with the wilted *aba1* mutant. Of these wilted mutants, 10 were allelic to the *aba1* mutant, including the line J11. The other lines represented two new complementation groups; one group of three lines including J12 and J14 and the other group consisting of nine lines, including lines J25, J210 and J212.

Table 1. Segregation of mutant phenotypes in F₂s derived from crosses of mutant lines to the wild-type (WT)

Cross	Segregating locus	No. of plants		χ^2_{a}
		Total	Wilty	
J11 × WT	<i>ABA1/aba1-5</i>	141	38	0.29
J14 × WT	<i>ABA2/aba2-1</i>	146	30	1.54
J12 × WT	<i>ABA2/aba2</i>	143	39	0.39
J25 × WT	<i>ABA3/aba3-1</i>	146	34	0.23
J212 × WT	<i>ABA3/aba3</i>	134	32	0.09

^a χ^2 calculation for expected ratio of 3:1.

Table 2. Estimates of recombination percentages with standard errors of the *aba* loci and morphological marker loci on chromosome 1

Marker	Population ^a	Rec.%	SE	Marker	Population ^a	Rec.%	SE
<i>aba2-ch1</i>	F ₃ R	7.9	1.5	<i>aba3-alb1</i>	F ₃ R	6.1	3.0
<i>aba2-ap1</i>	F ₂ R	20.4	4.8	<i>aba3-dis1</i>	F ₃ R	3.7	2.4
<i>aba2-gl2</i>	F ₂ R	32.3	4.4	<i>aba3-dis1</i>	F ₂ R	12.7	4.4
<i>aba2-th1</i>	F ₂ R	34.9	6.0	<i>aba3-an</i>	F ₂ R	17.4	4.4
				<i>aba3-an</i>	F ₂ C	15.7	2.6
				<i>aba3-cer1</i>	F ₂ R	18.3	5.8
				<i>aba3-th1</i>	F ₂ R	23.0	6.5

^aData from F₂ and F₃ populations in repulsion (R) or coupling (C) phase.

The segregation of the wilted phenotype in the F₂s derived from crosses between the wild-type and the mutants fitted to a 3:1 ratio, indicating that the wilted trait was controlled by single recessive mutations (Table 1). The expectation that paclobutrazol-resistant, wilted mutants are ABA-deficient was confirmed by the finding of lowered ABA levels in the plant (see below). Therefore the two new loci were named *ABA2* and *ABA3*. No allele numbers could be assigned to all members of the allelic groups because they came from the same bulked M₂ seed stock. The J11, J14, and J25 lines will be used as representative alleles and have been named *aba1-5*, *aba2-1*, and *aba3-1*, respectively. An additional mutant (line CB2a-9), was found in a screen aimed at the isolation of NaCl-tolerant mutants applied to γ -irradiation-mutagenized seeds. The selected seedlings reached the green cotyledon stage quickly, whereas control seeds did not germinate or seedlings remained yellow on culture medium with 200 mM NaCl. After transfer to the greenhouse the mutant appeared to be wilted. Allelism tests revealed that CB2a-9 was allelic to *aba3* and it was named *aba3-2*.

The *aba2* and *aba3* mutations were both located on chromosome 1. Close linkage was found between *aba2* and the morphological marker *ch1*, and *aba3* was found to be tightly linked to *alb1* and *dis1*. The recombination percentages between *aba2*, *aba3*, and the morphological markers used are given in Table 2 and map positions of *aba2* and *aba3* resulting from these recombination percentages are shown in Figure 1.

Physiological characterization

The mutants were isolated on the basis of their resistance to paclobutrazol. Figure 2 shows that all *aba* mutants were at least 10 times more insensitive to paclobutrazol than the wild-types. Apparently, a lower need for GA during germination as described for the *aba1* mutant (Karssen and Laćka, 1986) results in the ability of ABA-deficient mutants to germinate in the presence of GA biosynthesis inhibitors, such as paclobutrazol.

The mutant line CB2a-9 (*aba3-2*) has been isolated by means of a screen for salt (NaCl) tolerance. This mutant

and all other *aba* mutants were slightly insensitive to NaCl at germination (Figure 3) as determined in a germination assay on filter paper. To investigate whether this is a general effect due to lower water potential or a specific NaCl effect, germination on a range of PEG concentrations was determined (Figure 4). This indicated that the *aba* mutants were only slightly less sensitive to osmotic stress at germination than the wild-type seeds.

The induction of dormancy during development of the *aba2* and *aba3* mutant seeds was investigated (Figure 5). In contrast to the wild-type, but similar to *aba1* mutants (Karszen *et al.*, 1983), no dormancy was induced in *aba2* and *aba3*, resulting in a high germination percentage at maturity of the seeds (20 days after flowering).

The amount of ABA was measured in rosettes with or without water stress (Table 3). Wild type plants accumulated ABA approximately 10-fold upon water stress. The mutants *aba2* and *aba3* had lower levels of ABA in turgid condition and did not accumulate ABA upon water stress as strongly as the wild-type. Spraying the mutants with

the ABA analogue LAB 173.711 restored the wild-type phenotype. The lack of adequate amounts of ABA in the vegetative parts of the plants leads to a higher transpiration rate as was demonstrated by measuring the decrease in fresh weight of plants detached from their root system (Figure 6). The rate of water loss was higher in the mutants than in the wild-type. The relatively low rate of water loss of mutant line J25 correlated with the relatively high ABA content in this mutant (Table 3).

Double mutant analysis

Lines carrying two ABA-deficient mutations were constructed. The *aba* mutants were less vigorous, with smaller rosettes and thinner flower stems than the wild-type, resulting in a lower fresh weight. In Figure 7 the fresh weights of rosettes just after bolting and the degree of transpiration are given, showing that the *aba1-5,aba2-1* and the *aba2-1,aba3-1* plants were more reduced in growth and showed a higher transpiration rate than the single mutants. These results indicate a slightly additive effect of the mutations, which is in agreement with the lower ABA content measured in vegetative tissues of the double mutants (Table 3).

To investigate the effect of the combination of ABA insensitivity and ABA-deficiency of the new mutants on seeds, *aba2-1,abi3-1* and *aba3-1,abi3-1* double mutants were constructed. Seeds of the *aba3-1,abi3-1* double mutant were green, similar to the *aba1-1,abi3-1* seeds (Koorneef *et al.*, 1989), but were not as desiccation-intolerant as *aba1-1,abi3-1* seeds: after 2 months of dry storage of *aba3-1,abi3-1* seeds, 50% had retained the ability to germinate. Seeds of the *aba2-1,abi3-1* double mutant seeds were normally brown and desiccation-tolerant. With respect to dormancy both double mutants showed synergistic effects. Figure 8 shows the germination of freshly harvested seeds in light and dark conditions. Seeds of both wild-types were dormant, resulting in a very low germination percentage. The single mutants were non-dormant, reflected in the high germination percentage in

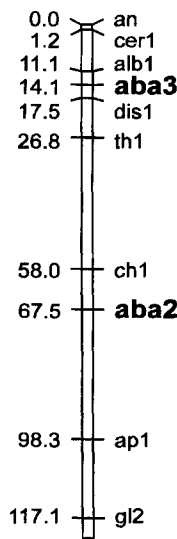


Figure 1. Genetic map of the *Arabidopsis* chromosome 1 with the map positions of *aba2* and *aba3* calculated from the data in Table 1 combined with published data of chromosome 1 markers.

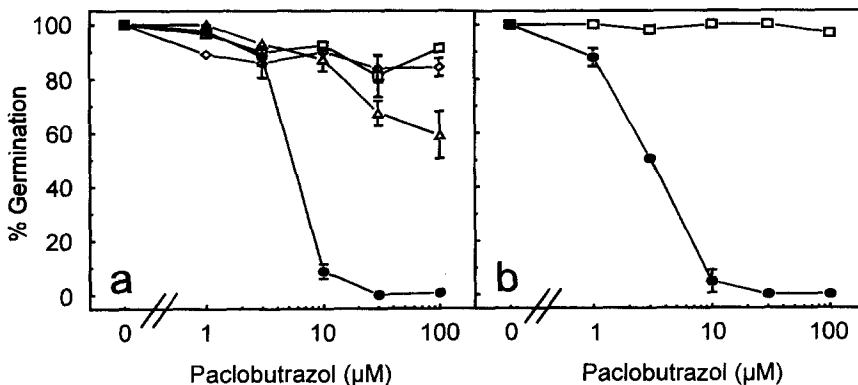


Figure 2. Paclobutrazol sensitivity of *aba* mutants. Germination of (a) wild-type Col (●), J11 (*aba1-5*) (◊), J12 (*aba2*) (Δ), and J25 (*aba3-1*) (◻) seeds and of (b) wild-type Ler (●) and CB2a-9 (*aba3-2*) (◻) seeds on different concentrations of paclobutrazol.

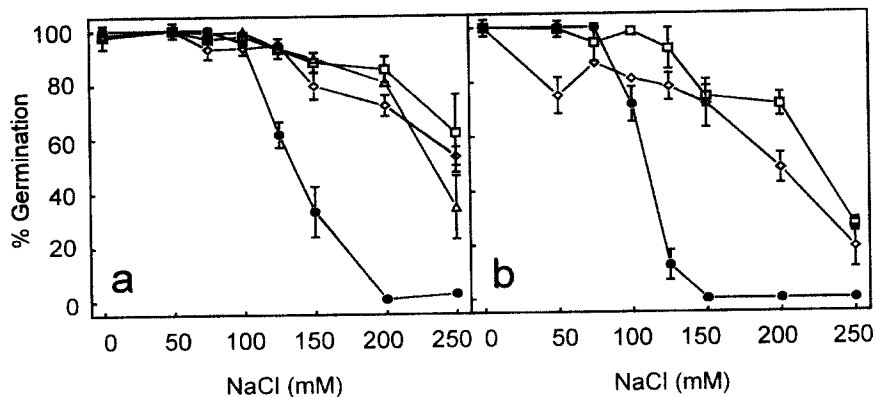


Figure 3. NaCl sensitivity of *aba* mutants. Germination of (a) wild-type Col (●), J11 (*aba1-5*) (◇), J12 (*aba2*) (△), and J25 (*aba3-1*) (□) seeds and of (b) wild-type Ler (●), A26 (*aba1-1*) (◇) and CB2a-9 (*aba3-2*) (□) seeds on different concentrations of NaCl.

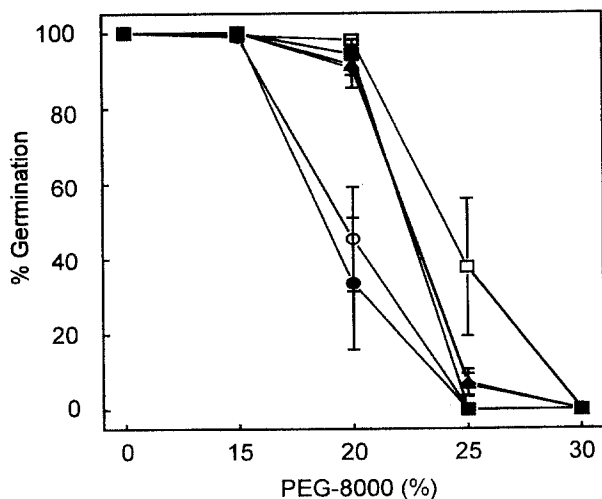


Figure 4. PEG sensitivity of *aba* mutants. Germination of wild-type Col (●), wild-type Ler (○), A26 (*aba1-1*) (◇), J12 (*aba2*) (▲), J210 (*aba3*) (■), and CB2a-9 (*aba3-2*) (□) seeds on different concentrations of PEG-8000.

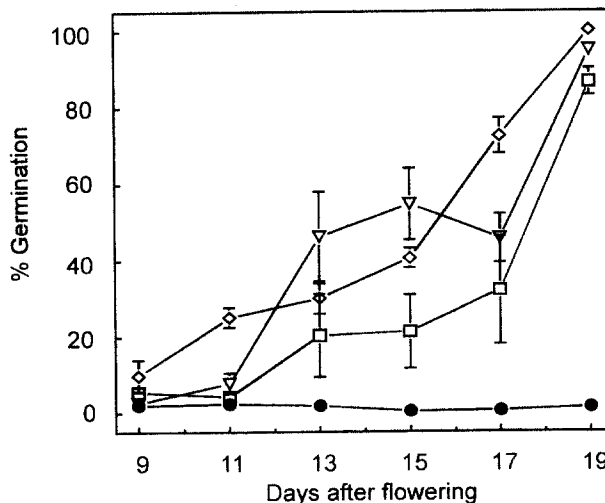


Figure 5. Germination on water of wild-type Col (●), J11 (*aba1-5*) (◇), J14 (*aba2-1*) (▽), and J25 (*aba3-1*) (□) seeds that were taken out from siliques at different stages during development.

light but did not germinate in the dark. The high dark germination of the double mutant seeds indicated that these had a lower degree of dormancy than the seeds of the single mutants.

Discussion

ABA-deficient mutants of *Arabidopsis* have been isolated on the basis of their reduced or absent GA requirement for germination; either by screening for germinating revertants from GA-deficient mutagenized seeds (Koornneef *et al.*, 1982) or by selection for germinating seeds on the GA inhibitor paclobutrazol (this report). A screen on a GA inhibitor has been used previously to isolate extreme *abi3* alleles (Nambara *et al.*, 1992). GA requirement at germination is dependent on the degree of dormancy, which is in turn determined by the amount of ABA present during seed development (Karssen and Laćka, 1986). Reduced dormancy is an important characteristic of ABA-deficient mutants also in other plant species (Koornneef, 1986).

Table 3. Abscisic acid content of turgid and partly dehydrated rosettes (data are averages of measurements of two samples)

Line	Genotype ($\mu\text{g g}^{-1}$ dry wt.)	Turgid	Stressed
Col	Wild-type	0.22	2.92
J11	<i>aba1-5</i>	0.02	0.06
J14	<i>aba2-1</i>	0.03	0.07
J12	<i>aba2</i>	0.04	0.11
J25	<i>aba3-1</i>	0.07	0.29
F ₃ (J11 × J14)	<i>aba1-5, aba2-1</i>	0.01	0.01
F ₃ (J14 × J25)	<i>aba2-1, aba3-1</i>	0.01	0.02
Ler	Wild-type	0.18	1.61
CB2a-9	<i>aba3-2</i>	0.09	0.14

ABA-deficient mutants of other species have been isolated by other criteria: in tomato, potato and pea on the basis of their wilty phenotype. ABA-deficient mutants of *N. plumbaginifolia* have been isolated by means of selection for cytokinin and auxin resistance. Furthermore these mutants express a reduced dormancy and resistance to paclobutrazol (Rousselin *et al.*, 1992). All *Arabidopsis aba*

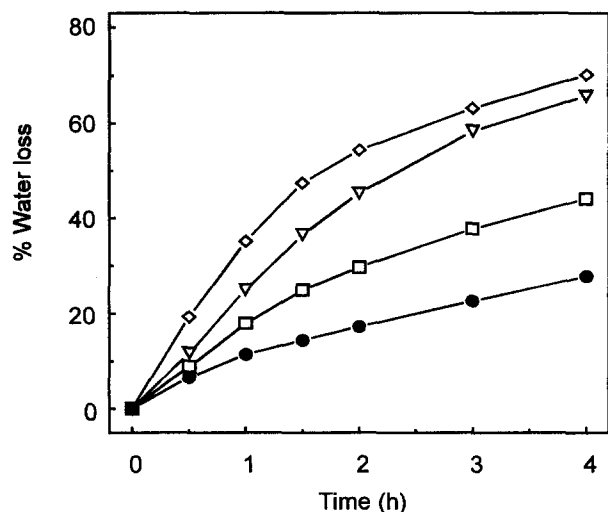


Figure 6. Rate of water loss of *aba* mutants. The rate of water loss of the wild-type Col (●), J11 (*aba1-5*) (◇), J14 (*aba2-1*) (▽), and J25 (*aba3-1*) (□) plants is expressed as percentage lost of initial weight in a time-course of detached rosettes.

mutants have the latter characteristics in common with the *N. plumbaginifolia* mutants, but are not resistant to cytokinin and auxin as was tested for *aba1* (Rousselin *et al.*, 1992), and confirmed for *aba2* and *aba3* (data not shown).

The *aba3-2* mutant was found in a screen for salt tolerance and showed a more vigorous growth on a medium containing 200 mM NaCl than the wild-type. It is difficult to understand that a plant with lowered levels of a hormone involved in stress adaptation can do so. Nevertheless, all *aba* mutants and *abi* mutants (Werner and Finkelstein, 1995) are salt tolerant and, to a lesser extent, low osmotic potential-tolerant at germination. This is in agreement with, but less pronounced than the situation in tomato, of which the *sit* mutant is able to germinate on a medium with a much lower osmotic potential than the wild-type (Groot and Karssen, 1992; Koornneef *et al.*, 1985; Ni and Bradford, 1993). The ability to germinate in adverse conditions such as low osmotic potential is considered to be a reflection of the reduced dormancy as well. *Arabidopsis* mutants, able to germinate on elevated NaCl concentrations have been isolated (Saleki *et al.*, 1993; Werner and Finkelstein, 1995), but no effect on dormancy was reported. The salt-resistant *rss* mutant was not wilty like *aba* mutants. The *RSS* gene is located on chromosome 1, but it is unlikely to be allelic to *aba2* because close linkage with the molecular marker *ADH* (located near *gl2*) and no linkage with *GAPB* (located near *ch1*) was reported (Werner and Finkelstein, 1995).

In the *aba* mutants analysed here, the ABA levels correlated with the severity of the phenotype, expressed as wiltiness (rate of water loss) and plant weight. Plant growth in *aba* mutants is less vigorous because of their disturbed water relations. The *aba1,aba2* and *aba2,aba3* double

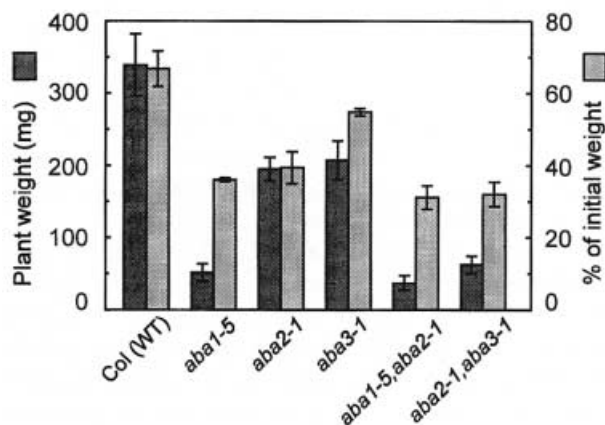


Figure 7. Effect of *aba* mutations on plant growth and transpiration. Plant weight after 21 days of culture and the percentage of initial weight 4.5 h after separating plants from the root system are given for *aba* mutants and double mutants. The data for the double mutants were compared with the data for their respective single mutants by *t*-test and were found to be significantly different ($P < 0.05$).

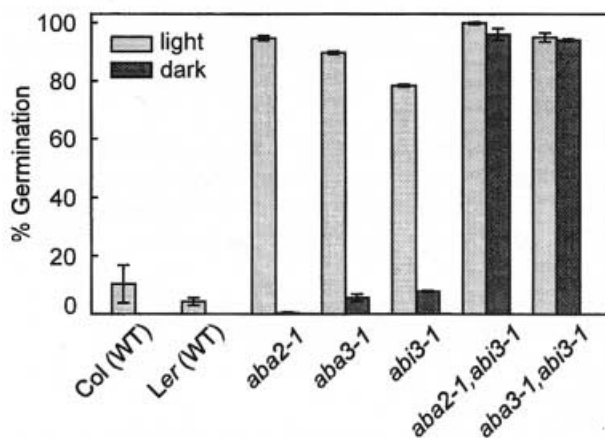


Figure 8. Germination of freshly harvested seeds of the wild-types, *aba*, *abi3-1*, and *aba*, *abi3-1* double mutants in light and darkness.

mutants showed a more severe phenotype than the single mutants with respect to plant fresh weight, transpiration rate, and ABA levels. The presence of significant amounts of ABA in the single mutants and the lower levels of ABA in the double mutants indicate that the mutants have a leaky phenotype. Most likely, the three loci are involved in separate steps of ABA biosynthesis, assuming that the C_{40} route is the main route in *Arabidopsis* (Rock and Zeevaart, 1991). It is not clear why no mutants with a stronger reduction in ABA content were found since the presently described mutants are relatively vigorous and more extreme mutants should have been viable. The presence of relatively high ABA levels in clearly ABA-deficient mutants was also described for the *N. plumbaginifolia* *aba2* mutant (an orthologue of the *Arabidopsis* *aba1* mutants) where this was found in the null mutants used to clone the gene (Marin *et al.*, 1996). This may suggest that some redundancy for the ABA pathway exists, which might be

another explanation for the additive effect of the mutants. Furthermore, it may indicate that some of the ABA produced is not physiologically active and/or that a relatively high threshold of ABA is required for processes such as stomatal closure and seed dormancy induction. It cannot be excluded that the mutations are leaky and that extreme alleles have not been recovered.

The phenotype of the *aba3-1,abi3-1* double mutant resembles that of the *aba1-1,abi3-1* double mutant (Koornneef *et al.*, 1989): a very low degree of dormancy (expressed as high dark germination) and green seeds. Nevertheless, the *aba1-1,abi3-1* double mutant is more extreme than the *aba3-1,abi3-1* double mutant with respect to desiccation intolerance. The phenotype of the *aba2-1,abi3-1* double mutant was even less extreme based on the seed colour phenotype, although the synergistic effect was visible in the high dark germination. An explanation for this can be that the ABA levels during seed development are affected differently in *aba2* and *aba3* mutants. The double mutant phenotype is probably not only a result of the ABA level present but can also be an indirect effect of the interaction specific for an *aba* locus with the *abi3-1* mutation.

Mutants at the new ABA-deficient loci *ABA2* and *ABA3* physiologically resemble the *aba1* mutant and can be useful in the studying of ABA action and the elucidation of the ABA biosynthetic pathway(s) in plants.

Experimental procedures

Mutant isolation

The mutant selection experiment using *Arabidopsis* ecotype Columbia (Col) seeds from which the mutants were isolated has been described by Jacobsen and Olszewski (1993). From this screen, mutants that germinated in the presence of 35 mg l⁻¹ paclobutrazol but whose vegetative tissues were sensitive to paclobutrazol emerged and those that were wilted were analysed further and described in this report.

Arabidopsis ecotype Landsberg *erecta* (Ler) seeds were mutagenized with γ -irradiation as described before (Léon-Kloosterziel *et al.*, 1996). M₂ seeds were sown on medium consisting of Murashige-Skoog macro- and micro-elements, 2% (w/v) sucrose, 0.7% (w/v) agar and 200 mM NaCl. Seedlings that grew more vigorously than control seedlings were transferred to soil and cultivated in the greenhouse. One of the plants selected in this way was wilted.

Genetic analysis and construction of double mutants

The mutant plants were intercrossed and crossed with the *aba1-1* (A26) mutant for complementation tests. To study the inheritance of the mutations, the mutant plants were crossed with the wild-type Col. Mutants selected from the F₂ derived from these crosses were crossed a second time with the wild-type. The F₂ from this second backcross yielded the mutant lines used for further characterization.

For mapping, both mutants were crossed with the multiple marker line W100 (Koornneef and Stam, 1992). Refinement of the map positions was achieved by crossing the *aba2* mutant with line W4 (containing the markers *ch1*, *ap1*, and *gl2*) and the *aba3* mutant with lines W122 and W143 (containing the markers *an*, *dis1*, *ga4*, *th1*, *tt1* and *alb1*, *an*, *dis1*, and *cer1*, respectively). An *aba3,an* recombinant was crossed with the wild-type Ler. F₂ and F₃ populations derived from these crosses were scored for the *aba* phenotype and the morphological markers. Recombination percentages with standard errors were determined with the computer program RECF2 (Koornneef and Stam, 1992). Map locations were determined with the computer program JOINMAP (Stam, 1993). For this purpose, the linkage data obtained in the present study were added to the data set for morphological markers used by Koornneef (1994).

Double mutants were constructed by intercrossing the mutant lines J11 (*aba1-5*), J14 (*aba2-1*) and J25 (*aba3-1*). Wilted F₂ plants derived from these crosses were testcrossed with each parent, followed by determination of F₁ plant phenotype. Double mutants were those that failed to complement both parent lines. The construction of *aba*, *abi3* double mutants was carried out by crossing the mutant lines J14 (*aba2-1*) and J25 (*aba3-1*) mutants with the *abi3-1* (line CIV) mutant. F₂ seeds that germinated on 10⁻⁵ M ABA were planted in the greenhouse; subsequently wilted plants were selected among these.

ABA determinations

ABA extractions and analysis using GC were performed as described before (Léon-Kloosterziel *et al.*, 1996).

Germination assays

All plants for seed production were grown in an airconditioned greenhouse (18°–23°C) with additional light during winter (16 h photoperiod, Philips HPI-T/400W); for each experiment all genotypes were harvested on the same day. Developing seeds were staged by tagging flowers at the day of anthesis. Immature or mature, bulk-harvested seeds were sown (50–100 seeds) in triplicate in petri dishes on filter paper (Schleicher & Schuell no. 595) saturated with water or solutions of NaCl or paclobutrazol (ICI, Bracknell, UK). The dishes were incubated in a growth room (25°C, 16 h photoperiod, Philips TL57) and germination (radicle emergence) scored after 7 days. The average germination percentages with standard error of the triplicates were calculated. In the case of incubation on NaCl or paclobutrazol a cold treatment (4 days at 4°C) was given prior to incubation in the growth room. For dark incubation the dishes were wrapped in aluminium foil and placed in a box.

Water loss assay

Plants were grown in soil in a climate chamber (23°C, 16 h photoperiod (Philips TL57 and incandescent bulbs) 70–80% RH) and after 21 days of culture transferred to the laboratory and cut from the root system. Of each genotype, five to 15 plants (0.4–1.4 g fresh weight) were placed in triplicate in plastic beakers on the bench at ambient temperature, and weighed every 30 min.

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