Dormancy, ABA content and sensitivity of a barley mutant to ABA application during seed development and after ripening

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Abstract
Assessment of dormancy inception, maintenance and release was studied for artificially dried immature seeds harvested throughout seed development in the barley cv. Triumph and its mutant line TL43. Each was grown in Spain and Scotland under low and high dormancy inducing conditions, respectively. Both TL43 and Triumph followed a similar pattern of release from dormancy across the seasons, although seeds of TL43 were able to germinate at an earlier seed development stage. Abscisic acid (ABA) content was also studied in immature grains throughout the seed development period. Total amount of ABA in seeds of Triumph and TL43 was higher in plants grown in Scotland than in Spain. However, no clear genotypic differences in ABA pattern in the course of grain development could be detected whilst significant genotypic differences were observed for germination percentage (GP). Endogenous ABA content alone throughout grain development did not explain genetic differences in GP within environments. Environmental and genetic differences in dormancy were also observed on mature seeds throughout the after-ripening period. The initial germination (GP\textsubscript{0}) played a key role in the sensitivity to ABA of post-harvest mature seeds. For the same after-ripening stage, TL43 was more insensitive to exogenous ABA than Triumph. However, ABA responses in seeds of the two genotypes with similar GP\textsubscript{0} at different after-ripening stages were comparable. Therefore, differences in exogenous ABA sensitivity of post-harvest mature grain of these two genotypes seemed to be determined by, or coincident with, the initial germination percentage.

Key words: Germination, seed dormancy, mutagenesis, barley, abscisic acid.

Introduction
Dormancy is defined as the inability of a viable seed to germinate under conditions otherwise adequate for germination. Expression of dormancy is affected by genetic and environmental factors, particularly the conditions prevailing during seed development and storage after harvest (Corbineau and Côme, 1996). Low temperature and high relative humidity during grain development are the main environmental factors inducing dormancy in barley (Zoppolo \textit{et al}., 1982; Buraas and Skinnes, 1984; Strand, 1989), and tissues peripheral to the kernel, rather than the embryo itself, may induce or maintain dormancy (Van Beckum \textit{et al}., 1993), as in most cereals (Black \textit{et al}., 1987). The seedcoat is considered to exert its influence on embryonic activity and emergence by limiting oxygen supply (Simpson, 1990; Benech-Arnold \textit{et al}., 1999).

Inception of dormancy occurs at early stages of seed development. In some species, artificially dried seeds are capable of precocious germination during the second

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Abbreviations: DDAA, degree days after anthesis; DPH, days post-harvest; G, germination; GP, germination percentage; GP\textsubscript{0}, initial germination percentage.

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week after anthesis and then dormancy gradually sets in during the third week (Bianco et al., 1994; Romagosa et al., 1999). A desiccation period is needed to proceed from seed development to germination (Kermode, 1995). The mechanisms that relieve dormancy during after-ripening are unknown, but may involve non-enzymatic oxidative reactions (Leopold et al., 1988; Esashi et al., 1993) or may result from turnover of products inhibiting germination (Dyer, 1993).

The genetics and biochemistry of dormancy are not fully understood. Mutants have been developed in Arabidopsis thaliana that, besides showing changes in ABA content and response, also show either absence of, or alteration in dormancy (Koornneef et al., 1984; reviewed in Karssen, 1995). However, despite the economic importance of germination in matling barley, dormancy is less understood in this species. Early investigations (Freistd, 1935; Moorman, 1942) were continued by Buraas and Skinnes who indicated that dormancy is highly heritable, controlled by several recessive genes and not affected by cytoplasmic factors (Buraas and Skinnes, 1984). Recent attempts to study the genetics of dormancy in barley have utilized molecular and mapping techniques for quantitative trait loci (QTL), based on a doubled-haploid line (DHL) population derived from the Steptoe (high dormancy) × Morex (low dormancy) cross. Four regions of the barley genome are associated with most of the differential genotypic expression of dormancy (Oberthur et al., 1995; Han et al., 1996; Larson et al., 1996; Romagosa et al., 1999).

Abscisic acid plays a major role in development and germination of seeds. During early seed development, ABA is involved in embryogenesis, whereas at a later stage of seed development it prevents precocious seed germination. However, the actual mechanisms of dormancy are unknown (Bewley, 1997). Some of the ABA responses are rapid and involve the modification of ion fluxes, whereas others are longer term and involve changes in gene expression (Kermode, 1995). A major action of ABA in seeds is the regulation of gene expression, particularly the induction of several different kinds of polypeptides (Skriver and Mund, 1990) and the inhibition of genes for certain reserve mobilizing enzymes (Jacobsen and Chandler, 1987). Not only ABA content, but also ABA sensitivity may influence seed dormancy and germination. The free ABA content is highest in developing seeds and is generally relatively low or even undetectable in mature seeds, although in several species considerable amounts of ABA are detected (Black, 1991). The appearance of ABA peaks during development can be manipulated by different growth conditions (Radley, 1976; Weidenhoft et al., 1988; Walker-Simmons and Sessing, 1990). In barley, ABA peaks earlier in high-temperature-grown barley than in low-temperature-grown grains, and cultivars with a lower level of dormancy have less ABA during development (Goldbach and Michael, 1976; Wang et al., 1995). It was found that ABA contents in barley embryos are up to 10-times greater than those in the endosperm (Quarrtie et al., 1988).

The role of ABA in seed dormancy has been studied in Arabidopsis using mutants defective in either ABA biosynthesis or action (Koornneef et al., 1998). ABA-insensitive (abi1-abi5) mutants were selected by germinating seeds at ABA concentrations that normally inhibit germination (Koornneef et al., 1984). These mutants either affect many ABA responses (abi1 and abi2) or, primarily, seed germination (abi3, abi4 and abi5).

The absence of barley mutants with reduced dormancy and sensitivity to ABA in germination indicates the need to develop such genotypes. Recently, mutants were induced in the barley cultivar Triumph (Molina-Cano et al., 1999). One of them, TL43, exhibits fast germination, reduced dormancy and appears to tolerate a 10-fold increase in ABA before germination is inhibited, compared to cv. Triumph. In the present study a detailed characterization of this mutant and its original genotype for dormancy and ABA sensitivity during seed development and after ripening is presented.

Materials and methods

Plant material

The mutant line TL43 was obtained from cv. Triumph following the mutagenic treatment and selection procedure described earlier (Molina-Cano et al., 1999). The near-isogenic nature of these two lines was confirmed using molecular markers (Molina-Cano et al., 1999). Both genotypes were field-grown under low- and high-dormancy-inducing conditions in 1997, 1998 and 1999 at Lleida (Spain), and in 1998 and 1999 at Dundee (Scotland). There were contrasting growing regimes between the sites, with grain autumn-sown in Spain and spring-sown in Scotland. Typical differences in temperature and rainfall during grain filling have been described previously (Swanston et al., 1997).

Inception and release from dormancy during grain development

To provide the same developmental stage for all the samples, approximately 250 spikes were tagged in the field plots at ear emergence. Starting 2 weeks after tagging, and twice a week during grain filling, 10 spikes per entry were harvested and hand threshed. Two kernels per spike were collected for determination of dry weight and moisture content. The remaining seeds were placed in a forced-ventilation oven at 37 °C for 24 h, when they reached a constant weight. These artificially dried seeds were kept at −20 °C until the last sample was taken at the end of grain filling. After thawing, germination tests for all samples were immediately conducted in the dark at 20 °C in 10 cm Petri dishes lined with three sheets of Whatman No. 1 filter paper, saturated with 3 ml distilled water. Three replications of 40 seeds were used per genotype × sampling date and the remaining seeds were kept at room temperature to enable germination tests to be repeated, 66 d post-harvest (DPH) in 1997 and 144 DPH in 1998.
1998. After 3 d, germinated seeds (those where the coleoptile had emerged through the hull) were counted and expressed as a germination percentage of the total (GP). By comparing these two assays, the initial test and the one repeated after the storage period, it was possible to distinguish dormancy from lack of viability in artificially-dried immature seed.

Quantification of ABA during grain development

Samples of 10 spikes were harvested from each genotype throughout the grain development period. They were quickly frozen in liquid nitrogen and stored at −80 °C prior to determination of endogenous ABA content. The samples were then thawed and two grains from the central part of each spike lyophilized. The analyses were carried out on the embryo-half of each grain, to avoid the dilution effect on ABA caused by the endosperm (Quarrie et al., 1988). Samples were milled and 50 mg of dry flour were extracted with water (20:1, v/w). ABA determination was by the method of Phytoabec (IDEXX Laboratories, Westbrook, ME, USA), based on previous studies (Mertens et al., 1983; Weiler, 1984), and the results were expressed as ng ABA g⁻¹ DW. Three, five and six sampling dates during grain filling were analysed in 1997, 1998 and 1999, respectively. Furthermore, ABA was also measured in 1999, in mature dry grains grown in Spain and Scotland, 1 month after harvest.

Sensitivity to ABA during germination

Germination tests on post-harvest seed stored at room temperature were conducted by adding ABA into the incubation medium according to the same germination protocol as described previously. The assays were performed 75 DPH in 1998, and 30 DPH in 1999. The experiment layout was as follows: (1) 3 replications of 40 fully matured seeds of size between 2.5 and 2.8 mm, and (2) ABA concentrations in the germination medium of 0, 10⁻⁸, 10⁻⁷, 10⁻⁶, 10⁻⁵, 10⁻⁴, and 10⁻³ M [(±)-cis, trans-abscisic acid, Sigma, Alcobendas, Madrid, Spain].

Statistical analysis

Data from grain filling and germination tests encompassing the onset, maintenance and release of dormancy during grain development were fitted to a logistic model with thermal time, measured as degree days after anthesis (DDAA) and a base temperature of 0 °C, as independent value. The following non-linear equation was fitted, using standard SAS/STAT (SAS Institute, 1991) procedures: 

\[ GP = A \left(1 + \exp(B \times \text{time} - C)\right) \]

where A represents the maximum G; B is the time needed to reach GP = 50%, i.e. the LD₅₀; and C is a coefficient without a direct biological meaning that together with A and B determines the inflexion point of the curve.

The results from the studies on ABA sensitivity were also fitted using SAS procedures to the following logistic model:

\[ GP = A \left(1 + \log(\text{ABA})/B\right) C \]

These parameters also have a direct biological meaning: A represents the germination of the untreated seeds (GP₀); B the ABA concentration that reduces germination of the untreated control to half, LD₅₀; and C together with A and B determines the inflexion point of the curve. Comparison of these parameters by means of the homogeneity test (Kimura, 1980) allows analysis of differences in the response to exogenous ABA.

Results

Dormancy during seed development

Assessment of dormancy inception and release was based on germination studies for artificially-dried seeds of Triumph and its mutant line TL43 throughout seed development (Fig. 1). TL43 showed strongly reduced dormancy compared with Triumph in 1997 and 1998 in Spain (Fig. 1A, B, C, D). Both TL43 and Triumph seemed to follow a similar pattern of release of dormancy during development, with relative differences in the stage at which the seed was able to complete germination. No precocious germination occurred prior to dormancy inception, unlike some barley genotypes (Romagosa et al., 1999), as both lines were fully dormant at the beginning of the germination test 2 weeks after anthesis. The environmental influence on dormancy inception was clearly shown by comparing the results from Spain 1997 versus Spain 1998 and Scotland 1998. In Spain 1997, TL43 started to lose dormancy progressively from over 500 DDAA while Triumph remained dormant until about 700 DDAA. The mutant showed higher germination percentage (GP) than Triumph at the end of the grain filling process (87% against 73%, respectively). In Spain 1998, TL43 showed little precocious germination at 500 DDAA, reaching 25% germination at seed maturity, while Triumph was fully dormant through the grain filling period and at seed maturity. TL43 and Triumph did not germinate throughout seed development in the 1998 Scottish conditions (Fig. 1E, F).

The germination tests were repeated to check the viability of developing seeds after dry storage at room temperature to break dormancy. The storage period required for dormancy release varied between the years. In 1997 when the seeds developed under environmental conditions not imposing high levels of dormancy, dormancy disappeared after 66 DPH, when the most fully developed samples almost reached 100% G. In 1998 the required period for dormancy release was longer than 144 d, when the fully developed grains reached around 80% G. For every genotype × season combination and by comparing both curves 1 DPH and 66 or 144 DPH it was possible to assess and distinguish between dormancy and viability effects. Germination percentage after storage at room temperature, which basically reflects seed viability, was less affected by genetic and environmental effects. Genotypic differences in the germination profiles, once dormancy was released, were not as large as in the case of seeds germinated at 1 DPH. Therefore, seed viability through seed development of TL43 and Triumph was very similar within a given environment (Fig. 1). In fact, the viability curve seems to be closely related to the dry matter deposition pattern, rather than to genotypic differences. In Spain in the two studied years, both genotypes had the highest germination rate when they
reached about 80% of their constant end dry weight at seed maturity. A similar trend was observed in Scotland 1998 (Fig. 1E, F). However, under these conditions the dormancy of these grains was not fully released after 144 DPH and, therefore, these samples did not reach 100% G. Contrasting differences in barley growing conditions exist between the two sites, particularly in relation to the phenology and length of the barley life cycle as determined mainly by daylength during the early phases of plant development, and temperature and moisture conditions at later stages.

**ABA content during seed development**

The ABA content was also studied during seed development (Fig. 2). The amount of endogenous ABA decreased through grain filling in all experiments. This decrease may be necessary to trigger the germination process since ABA content was lowest at seed maturity, when the GP was highest. However, within a given growing environment, no genotypic differences in ABA pattern through grain development could be detected. For both years in Spain, there were no differences in ABA content between Triumph and TL43, whereas significant differences were observed for GP (Fig. 2A, B, C, D). Genotypic differences for GP at seed maturity were large (TL43: 87%, Triumph: 73%, in Spain 1997 versus TL43: 25%, Triumph: 0% in Spain 1998), but the corresponding differences in ABA concentration were small and not significant (110 and 121 versus 738 and 788 ng g⁻¹ DW for TL43 and Triumph, respectively, in the two years studied). Likewise, in Scotland 1998 there were no genotypic differences in ABA pattern, but the total amounts of ABA in Triumph and TL43 were much higher than in Spain for each developmental stage (Fig. 2E, F). There were no genotypic differences in the pattern of endogenous ABA content under Scottish conditions, since both genotypes

![Diagram](image-url)
reached an ABA peak at the same developmental stage around 600 DDAA.

These values suggest that endogenous ABA content alone does not explain differences in GP throughout grain development and at the end of the grain filling period between genotypes and within environments.

**Endogenous ABA content and response to exogenous ABA in mature dry seed**

Response curves to different concentrations of ABA in the germination medium are shown in Fig. 3. Germination was carried out with mature dry grain stored at room temperature for 1 and 3 months after ripening, in 1998 and 1999. Data were fitted to a dose-response logistic model and very good levels of fit were obtained ($r^2$ ranging from 0.94 to 0.99). These curves were compared by means of the homogeneity test (Kimura, 1980).

Three different curves were compared in Fig. 3: seeds grown in Spain in 1998 and 1999 (Spain 98 and 99) and in Scotland in 1999 (Scotland 99). In Spain 1998, response to exogenous ABA in mature dry seed was determined in three seed lots: TL43 at 75 DPH, Triumph at 75 DPH and TL43 at 7 DPH. The latter had approximately the same initial germination percentage as Triumph (75 DPH) at a different after-ripening developmental stage.

The initial germination level played a key role in the sensitivity to ABA. TL43 (7 DPH) had a statistically significantly different ABA response profile ($GP_0 = 48.7\%$, $LD_{50} = 1.6 \times 10^{-2}$ M ABA) from TL43 (75 DPH) ($GP_0 = 92.8\%$, $LD_{50} = 1.1 \times 10^{-4}$ M ABA). However, TL43 (7 DPH) was not significantly different from Triumph (75 DPH) ($GP_0 = 44.4\%$, $LD_{50} = 3.1 \times 10^{-5}$ M ABA) (Fig. 3). That is, for the same developmental stage TL43 is relatively more insensitive to exogenous ABA than Triumph, but they have the same ABA response at any given common $GP_0$.

Additional experiments were performed to identify the influence of the endogenous ABA content on the sensitivity of barley grains to application of ABA. The endogenous ABA content was analysed in barley seed grown in Spain and Scotland in 1999 one month after-harvest, using the same seed lot that was used to study the
response to exogenous ABA application in germination (Table 1). At every site, the endogenous ABA content was not statistically significant between the two genotypes (2101 and 2092 ng g⁻¹ DW for Triumph and TL43 in Spain; 2255 and 1942 ng g⁻¹ DW for Triumph and TL43 in Scotland). However, as is shown in Fig. 3, significant differences in sensitivity to exogenous ABA were found (LD₅₀ Triumph = 1.5 x 10⁻⁵ versus LD₅₀ TL43 = 8.6 x 10⁻⁵ M ABA in Spain and LD₅₀ Triumph = 1.7 x 10⁻⁵ versus LD₅₀ TL43 = 4.8 x 10⁻⁵ M ABA in Scotland). Genotypes with lower initial germination (GP₀ Triumph = 66.3 versus GP₀ TL43 = 76.6% in Spain and GP₀ Triumph = 35.3 versus GP₀ TL43 = 77.6% in Scotland) were more sensitive to exogenous ABA in the germinating media. Therefore, endogenous ABA content seems not to affect the later response to different exogenous ABA concentrations. On the contrary, differences in ABA sensitivity on post-harvest mature grain seem to be mainly determined by or coincident with the initial germination percentage (Fig. 3, Spain 1998).

Table 1. ABA content, measured as ng ABA g⁻¹ DW, 1 month after harvest of Triumph and TL43 mature grain
The same letter indicates no significant differences among genotypes within a given location in 1999, according to a Duncan test (P≤0.05).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Spain</th>
<th>Scotland</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triumph</td>
<td>2101 a</td>
<td>2255 a</td>
</tr>
<tr>
<td>TL43</td>
<td>2092 a</td>
<td>1942 a</td>
</tr>
</tbody>
</table>

Discussion

In a previous paper, the phenotypic characterization of TL43 and Triumph was presented (Molina-Cano et al., 1999). The mutant TL43 appeared to be slightly later in heading, with shorter stems, smaller and lighter kernels, and lower yield than its parental genotype Triumph, when grown in Spain. TL43, however, showed a phenotypic similarity to its parental cultivar under Scottish growing conditions. TL43 consistently showed reduced dormancy under a series of environmental conditions across a number of growing years in Spain and Scotland. The objectives of this work were (1) to assess the pattern of inception, maintenance and release of dormancy during seed development and after ripening; (2) to study the role of endogenous ABA in determining these processes; and (3) to characterize the genotypic sensitivity of seed to increasing exogenous ABA concentrations.

Although dormancy was highly variable across years and sites, the two genotypes seemed to follow a relatively similar pattern of inception of dormancy. Both TL43 and Triumph were fully dormant at the first sampling date, 2 weeks after anthesis. The viability and dormancy profiles for TL43 and Triumph across seasons did not reveal differences in the time that dormancy inception took place. Release of primary dormancy during seed development took place in TL43 at an earlier point of the grain-filling process. Under Spanish growing conditions, dormancy of TL43 was reduced and seeds were able to undergo precocious germination earlier during seed development than Triumph. TL43 also reached higher GP at seed maturity. Viability of the developing seeds was much less affected by genotypic and environmental differences than dormancy.

There is considerable circumstantial evidence that ABA is involved in regulating the onset of dormancy and in maintaining the dormant state (Black, 1991; Bewley, 1997). The role of ABA content in imposing dormancy on the barley lines used in this study was equivocal. No clear correlation between ABA content of the grains and dormancy has been shown in some studies carried out in barley (Boivin et al., 1995) or in other cereals (Berrie et al., 1979; Dunwell, 1981; Walker-Simmons, 1987; Walker-Simmons and Sessing, 1990). In this study, when differences in germination percentage of
developing grains between genotypes and between environments were large, corresponding differences in endogenous ABA were small. For both lines, however, ABA concentrations of developing grains under Scottish conditions (Fig. 2E, F), which impose high levels of dormancy in the grain, were higher. The initiation of dormancy may also depend partially or wholly on seed sensitivity to ABA where the amount of hormone would be less critical (Walker-Simmons, 1987). Recently, it was shown that although ABA is required for seed maturation, active gibberellins (GA) are also present in developing maize embryos (White et al., 2000). The relative amounts of ABA and GA, rather than the concentration of ABA alone, determine whether maize developing embryos undergo precocious germination or quiescence and maturation (White and Riva, 2000).

TL43 was not similar to the ABA-deficient (aba) mutants reported in Arabidopsis thaliana (reviewed in Karssen, 1995; Koornneef et al., 1998), nor to the dnd mutants which showed reduced dormancy but ABA contents and sensitivity similar to wild-type Arabidopsis (Léon-Kloosterziel et al., 1996). TL43 showed some similarities to the abi (ABA-insensitive) mutants, defined as those able to maintain germination at ABA concentrations 10-times higher than the wild type, with similar levels of endogenous ABA concentrations (Koornneef et al., 1984). In this study, there were no clear differences in endogenous ABA concentrations between the two ABA genotypes across grain filling and after-ripening within a given environment. Therefore, genotypic differences in dormancy release are not associated with this character. Distinct sensitivity to exogenous ABA in germination was confirmed using mature grain grown under several environmental conditions. TL43 maintained the ability to germinate at exogenous ABA concentrations five to ten times higher than the wild type, across changing environmental conditions, as measured by the LD50. However, sensitivity to ABA seemed to be driven by, or at least correlated with, the initial germination percentage of the seed lot. Both traits, decreased dormancy and insensitivity to ABA, in this ‘abi-like’ mutant could be expressions of a common phenomenon rather than independent phenotypical events.

The phenotype of TL43 resembles that of abi3, one of the Arabidopsis ABA-insensitive mutants. Extreme abi3 mutants are characterized by severely disturbed seed maturation as shown by a strong reduction in storage proteins and many seed-maturation specific transcripts, desiccation sensitivity of mature seeds, lack of seed dormancy, and a very reduced sensitivity to the inhibiting effect of ABA in seed germination (Parcy et al., 1994). The amino acid sequence of ABI3 (Giraudat et al., 1992) revealed that this gene is homologous to the maize Viviparous-1 (Vp1) gene (McCarty et al., 1991). Both genes encode transcription factors with seed specific expression. Unsuccessful attempts have been made to relate the seed dormancy (SD) QTL identified in barley with the maize and rice Vp1 sequences (F Han, personal communication). Current research is under way to cross TL43 and Triumph with both Septoe and Morex to try to relate the mutation present in TL43 with the SD1 to SD4 quantitative trait loci identified in the Septoe (highly dormant) × Morex (not dormant) cross. Recently, the wheat Vp1 homologues (vplA, vplB, vplD) have been located in the long arm of chromosome 3 (Bailey et al., 1999). However, none of the barley SD QTL (Han et al., 1996) are in this genomic region. Some clues may come from cloning the Vp1 homologous gene from Triumph and TL43, which is currently under way.

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References


