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Changes in Abscisic Acid Biosynthesis and Catabolism during Dormancy Breaking in *Fagus sylvatica* Embryo

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Abstract. At harvest, embryos of *Fagus sylvatica* are dormant. A cold pretreatment without medium at 30% moisture content allowed them to germinate. A comparison of the abscisic acid (ABA) content before and after the pretreatment has no significant relevance since dormancy is expressed during the culture at 23°C. During this culture, both de novo biosynthesis and conjugate hydrolysis contributed to maintain a high level of ABA in the dormant axis. The level of conjugates and the rate of hydrolysis were not modified substantially by the cold pretreatment. In contrast, the dormancy release was associated with a strong decrease in the capacity for ABA synthesis. Moreover, feeding (+)-[^{3}H]ABA to untreated and pretreated embryos proved that the cold treatment also induced a hastening of ABA catabolism.

Key Words. Embryo dormancy—ABA synthesis—ABA catabolism—Chilling treatment

Over the past decade, work with hormone-deficient and hormone-insensitive mutants (for review, see Hilhorst 1995, Hilhorst and Karssen 1992, Kornneef and Karssen 1994) provided a considerable body of evidence for the pivotal role of abscisic acid (ABA) in the induction of primary dormancy during maturation. In sunflower, the induction and maintenance of dormancy were demonstrated to be associated with ABA synthesis in the axis (Le Page-Degivry and Garello 1992). A de novo synthesis of ABA took place in embryos isolated from dormant barley grains during incubation (Wang et al. 1995). In these two examples, the breaking of dormancy by dry storage was correlated with the loss of the capacity for ABA synthesis. In the case of psychrolabile embryo dormancy, de novo ABA biosynthesis is also necessary for the expression of dormancy during cultures of *Fagus sylvatica* embryos (Le Page-Degivry et al. 1997) and *Pseudotsuga menziesii* seeds (Bianco et al. 1996). Moreover, in *F. sylvatica*, hydrolysis of ABA conjugates also contributed to generate active ABA during culture (Le Page-Degivry et al. 1997).

The work reported here aimed to determine changes occurring during the dormancy breaking induced by a cold pretreatment, taking into account both the dual origin of ABA and its catabolism.

Abbreviations: ABA, abscisic acid; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; RIA, radioimmunoassay; PA, phaseic acid; DPA, dihydrophaseic acid.

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Materials and Methods

Plants

Two seed lots were used. The first one (1993) originated from the Tree Improvement Station, Humleback, Denmark. The second one (1994) was provided by the Office National des Forêts, Sécherie de la Joux, France.

Dormancy breakage treatment was performed in the Centre National de la Recherche Forestière, Champenoux, France, as described by Muller and Bonnet-Masimbert (1985). The nuts were prechilled at 4°C without medium at 30% moisture content (fresh weight basis).

Embryo Culture In Vitro

Twenty-four embryos isolated aseptically were cultured on water agar (6 g/liter). The cultures were maintained for 1 month under white fluorescent light (45 $W \cdot m^{-2}$, 16 h/day) at 23°C, a temperature that allowed dormancy of *Fagus* seeds to be expressed strongly. Another set of embryos, cultured in the same conditions, were treated with fluridone (1-methyl-3-phenyl 5-[3(trifluoromethyl) phenyl]-4-(1H)-pyridinone), generously provided by Dow Elanco, and applied on the axis in 10% acetone/water (v/v), in quantities of 20 μL/embryo, at a concentration of 100 μg/mL. An embryo was considered to have germinated when elongation of the radicle reached 2 mm.

ABA Extraction

Fifty axes or 50 pairs of cotyledons isolated from dry seeds or from embryos cultured on water agar for various durations (3, 6, 9, 11, or 15 days) were ground with chilled 80% methanol containing 2,6-di-*t*-butyl-4-methyl phenol as an antioxidant (100 mg/liter). The homogenate was stirred for 2 h at 4°C and centrifuged for 10 min at 2,000 ×*g*. The pellet was reextracted twice with the same volume of cold 80% methanol. The supernatants were collected and evaporated under reduced pressure at 40°C. The remaining aqueous extract was adjusted to pH 3.5 and extracted four times with diethyl ether. The organic phase contained free acids.

Hydrolysis of Esters

The remaining aqueous extract was submitted to a mild alkaline hydrolysis (pH 12, 30 min, 60°C). The acids released from esters were extracted at pH 3.5 with diethyl ether. The final aqueous phase remaining after extraction of these acids contained the non-alkali-hydrolyzable conjugates.

Endogenous ABA Purification

Free ABA was separated from its conjugates either by thin layer chromatography (TLC) or by high performance liquid chromatography (HPLC). Using toluene/ethyl acetate/acetic acid (50/30/4) as solvent, TLC on a silica gel plate (Merck 60F 254) allowed free ABA (*Rf* 0.55) to be well separated from its conjugates (*Rf* 0.05). A good separation was also obtained by HPLC on a column (Merck Lichrosorb RP 18) eluted with a gradient of ethanol/water/acetic acid (Vaughan and Milborrow 1984), which allowed free ABA to be detected at a retention time of 52 min.

Endogenous ABA Quantification

ABA was quantified by radioimmunoassay (RIA) performed exactly as described previously (Le Page-Degivry et al. 1984). Antibodies were demonstrated to be highly specific toward the ABA molecule; however, since they were prepared against an immunogen obtained by coupling ABA to human serum albumin through the carboxyl group, cross-reactivity was observed whether the acid function was free, esterified, or amide linked. Thus, the RIA allowed us to estimate not only free ABA or ABA released from conjugates by hydrolysis but also conjugates where binding involved the acid function.

Conversion of the carboxyl group of ABA into an amide inducing an increase in sensitivity of the free (+) ABA estimation allowed ABA values in an extract to be calculated by differential measurement before and after amidation (Le Page-Degivry et al. 1984).

Preparation of Tritiated (+) ABA

The mixture of the two enantiomers (±)-[^{3}H]ABA (2.46 TBq/mmol, Amersham, UK) was converted to its methyl esters with ethereal diazomethane. They were resolved on a column packed with Chiralcel OD (Daicel Chemical Industries Ltd., Japan) using isopropyl alcohol/hexane (1/9) as a mobile phase. Each ester was converted to the corresponding acid by saponification with 2 M KOH/EtOH (1/2) at 24°C for 30 min, followed by acidification and extraction (Railton 1987).

Study of (+)-[^{3}H]ABA Metabolites

(+)-[^{3}H]ABA was fed to *Fagus* dry embryos through their cotyledons for a pulse of 1 h (1670 Bq/embryo). The treated embryos were cultivated on 0.2 mL of agar medium for 6 h. Extraction, partition with diethyl ether, and purification by TLC or HPLC were performed in the same way as for endogenous compounds. The radioactivity was counted in a scintillation mixture (Aqualuma plus, Lumac) with a liquid scintillation counter (model Betamatic I, Kontron).

After separation of free acids, radioactivity was almost exclusively detected at the *Rf* or retention time described by Vaughan and Milborrow (1984) for ABA and its oxidative products (PA and DPA). For the two chromatographic systems, the distribution of the radioactivity between the different compounds was the same. Therefore, the percentage of nonmetabolized ABA and its oxidative products could be estimated.

Results

Influence of Cold Pretreatment on Physiologic Behavior

At harvest or after dry storage, isolated embryos germinated very slowly, reaching a low percentage (about 25%) after a 1-month culture (Fig. 1*A*, *a*). After a partial cold pretreatment (50 days) a high percentage of embryos (about 60%) were able to germinate during the 1st week of culture. The germination rate and the germination percentage increased with prechilling duration (Fig. 1*A*, *b* and *c*).

An application of fluridone strongly increased the germination rate and the germination percentage of embryos isolated from both untreated and partially cold-pretreated seeds (Fig. 1*B*). In both cases, almost full germination was obtained within 1 month. However, a difference between the two types of embryos appeared in germination rate. In the absence of cold pretreatment, the germination percentage increased progressively all along the culture, whereas after a 50-day cold pretreatment, full germination was obtained as soon as the 12th day of culture.

Influence of Cold Pretreatment on ABA Levels

The ABA content and its in between the different parts of the embryos were studied by RIA. ABA was present under free form, esterified forms hydrolyzable by mild alkali, and non-alkali-hydrolyzable forms. In untreated embryos, the greatest part of free ABA was localized in the cotyledons. In both axes and cotyledons, the free ABA content decreased during cold pretreatment, especially within the first 50 days. This decrease was not due to a leakage of ABA since cold pretreatment was performed without medium at 30% moisture content.

Esterified forms were detected at a high level in cotyledons of untreated embryos; quantitatively more important than free ABA, they did not decrease very significantly during cold pretreatment (50 or 85 days).

After removal of the ABA esterified forms, immuno-

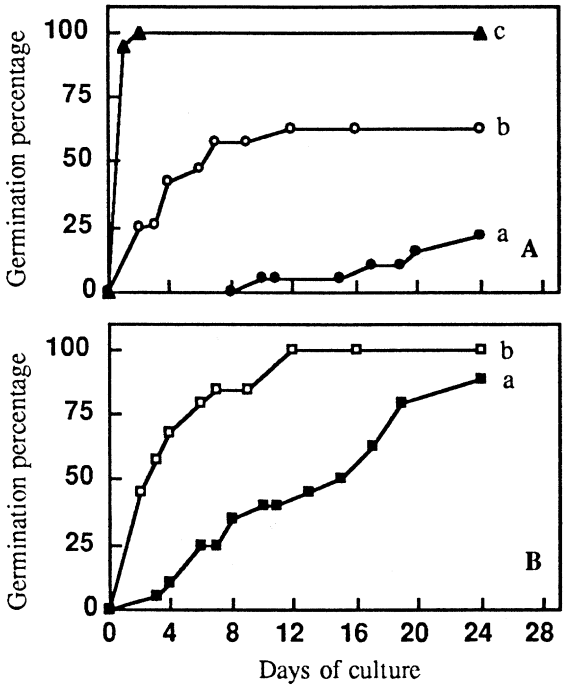

Fig. 1. Changes, during culture on water agar at 23°C in the germination percentages of *Fagus* isolated embryos, according to the prechilling treatment duration: 0 (*a*), 50 days (*b*), 85 days (*c*). *Panel A,* control conditions; *panel B,* after a fluridone treatment.

reactive material could be detected by RIA applied directly to the remaining aqueous phase. Its level, estimated in an ABA equivalent, was high in cotyledons but was not modified during cold pretreatment.

Influence of Cold Pretreatment on ABA Synthesis

Changes in the axis ABA content were studied when embryos, dormant or cold pretreated, were cultured at 23°C (Table 1).

For dormant embryos, the axis ABA level was not modified significantly during culture in control conditions; for cold-pretreated embryos, the axis ABA level decreased significantly during the same period.

To estimate the part due to de novo synthesis in this difference, ABA synthesis was suppressed by applying fluridone, an inhibitor of carotenoid biosynthesis. Compared with the value estimated after a 3-day culture in control conditions, the decrease in the axis ABA level was higher for dormant embryos (850 pg) than for cold-pretreated ones (400 pg).

Influence of Cold Pretreatment on Conjugate Hydrolysis

The level of ABA esterified forms, high in cotyledons at the moment of isolation, decreased during culture, sharply within the first 3 days and more slowly thereafter

Table 1. Free ABA levels estimated in axes after a 3-day culture at 23°C of embryos isolated from untreated seeds and from seeds cold pretreated for 50 days (harvest 1994). Results are the mean of six values (two replicate experiments and three evaluations for each replicate) ± S.E. and are expressed in pg/axis.

	Untreated	Cold pretreated
Before culture	2,500 ± 250	1,150 ± 110
After a 3-day culture		
Control	2,390 ± 240	780 ± 70
Fluridone treatment	1,540 ± 110	380 ± 40

(Table 2). The same pattern of changes occurred in untreated embryos and cold-pretreated ones.

The level of non-alkali-hydrolyzable conjugates decreased during the first 3 days of culture in both dormant and prechilled embryos (Table 2).

Influence of Cold Pretreatment on ABA Catabolism

To estimate the importance of ABA catabolism, (+)-[^{3}H]ABA was fed to embryos, and the products of its metabolism were analyzed by TLC and HPLC. ABA metabolism was much more active in cold-pretreated embryos than in dormant ones. Indeed, after a 6-h incubation, about half of the absorbed ABA was not yet metabolized in dormant embryos, whereas only 20% remained undegraded in cold-pretreated ones. This difference could be observed in cotyledons as well as in axes (Table 3). Free oxidative products were detected in a low level in both types of embryos. According to their behavior upon mild alkaline hydrolysis, two types of conjugates could be distinguished (Table 4). The conjugates resulting from an esterification process corresponded to less than 10% of the total absorbed radioactivity in both types of embryos. Among these esters, PA and DPA were always formed at a low level. However, non-alkali-hydrolyzable conjugates were present at a much higher level in cold-pretreated embryos than in dormant ones. These conjugates were analyzed by HPLC. The major fraction (70–80% of these conjugates) released DPA upon enzymatic hydrolysis by a crude extract from cotyledons of 24-h imbibed embryos of *Fagus.* This conjugate of DPA was the major product of [^{3}H]ABA catabolism; its level was much higher in cold-pretreated embryos than in dormant ones (Table 5).

Discussion

Because of the availability of hormone mutants, many studies on dormancy mechanisms were related to species, the seed dormancy of which was released by dry storage (*Arabidopsis, Lycopersicum, Nicotiana*). Very few recent studies were devoted to psychrolabile embryo

Table 2. Changes in cotyledon ABA conjugates during culture at 23°C of embryos isolated from untreated seeds or from seeds cold pretreated for 50 days (harvest 1994). Results are the mean of six values (two replicate experiments and three evaluations for each replicate) ± S.E. and are expressed in ng/embryo.

	ABA esters		Alkali-nonhydrolyzable conjugates	
	Untreated	Cold pretreated	Untreated	Cold pretreated
At isolation time	31.7 ± 3	32.5 ± 3	258 ± 26	202 ± 20
After 3-day culture	12.5 ± 1.2	6.5 ± 1.6	220 ± 22	120 ± 12
After 13-day culture	10.9 ± 1.1	8.9 ± 0.9	272 ± 27	190 ± 19

Table 3. Nonmetabolized ABA after a 6-h incubation of *Fagus* embryos, isolated from untreated seeds or from seeds cold pretreated for 50 days, following a 1-h pulse of (+)-[^{3}H]ABA. Results are expressed in percentage of the total radioactivity absorbed.

	Untreated	Cold pretreated
Axes	46.8	20.8
Cotyledons	45.4	21.4

Table 4. Distribution of radioactivity between the different pools of conjugates after a 6-h incubation of *Fagus* embryos isolated from untreated seeds or from seeds cold pretreated for 50 days, following a 1-h pulse of (+)-[^{3}H]ABA. Results are expressed in percentage of the total radioactivity absorbed.

	Untreated	Cold pretreated
Acids released from esters	5.6	8.2
Alkali-nonhydrolyzable conjugates	29.4	53.2

Table 5. Different forms of oxidative products of ABA metabolism (PA + DPA) detectable in cotyledons of *Fagus* embryos after a 6-h incubation following a 1-h pulse of (+)-[^{3}H]ABA. Results are expressed in percentage of the total radioactivity absorbed.

	Free forms	Esters	Other conjugates
Dormant	8.1	0.9	25.1
Cold pretreated	8.4	2.0	41.4

dormancy; some of them, following the questioning of Walton (1980/1981), emphasized the lack of correlation between the ABA content of the seeds and their physiologic behavior. For example, using gas chromatography-mass spectrometry/selected ion monitoring, Subbaiah and Powell (1992) demonstrated that the ABA content of dormant apple seeds was similar in three cultivars of *Malus domestica* Borkh which have substantially different chilling requirements. Moreover, in this species, the ABA content dropped rapidly upon imbibition, nearly identically under warm (20°C) and cold (5°C) temperature regimes, whereas only cold stratification promoted germination. Our results on *Fagus* are consistent with this discrepancy. The decrease in the free ABA level observed in this species during the cold pretreatment, which allowed subsequent germination of embryos, was comparable to the fall observed during culture of dormant embryo at 23°C (Le Page-Degivry et al. 1997).

However, when dormant or cold-pretreated embryos were cultured at 23°C, changes in ABA levels were observed again. Our work contributes to the evaluation of the respective involvement of conjugate hydrolysis, ABA de novo synthesis, and ABA catabolism in these changes. Conjugates present in cotyledons of untreated embryos were demonstrated previously to be hydrolyzed and therefore to constitute a potential source of ABA upon imbibition (Le Page-Degivry et al. 1997). This pool of conjugates appeared relatively large. Non-alkali-hydrolyzable conjugates seemed to be present at a very high level; however, as they were estimated by RIA directly in ng of ABA equivalent, their levels can be overestimated since the presence of an amide linkage, for example, enhanced the cross-reactivity considerably (Le Page-Degivry et al. 1984). In any case, ABA released from esters by a mild alkaline hydrolysis was detected at a level higher than free ABA. This presence of a large pool of conjugates contrasts with the low level of conjugates detected in sunflower embryos (Le Page-Degivry et al. 1992), the dormancy of which was released by dry storage. However, the cold pretreatment modified neither the level of these conjugates nor the rate of their hydrolysis after transfer at 23°C significantly.

On the contrary, the equilibrium between de novo synthesis and ABA degradation was modified by cold pretreatment. During culture at 23°C, the axis ABA level was not modified in dormant embryos, suggesting that ABA synthesis equilibrated its degradation; after cold pretreatment, the axis ABA level decreased, suggesting that ABA degradation exceeded its synthesis. The inhibition of ABA synthesis by fluridone effectively allowed a stronger decrease in the axis ABA level in dormant embryos (850 pg) than in cold-pretreated ones (400 pg). Such a reduction in ABA synthesis capacity during dormancy breaking treatment was already reported in sun-

flower embryos during dry storage or culture in liquid medium (Bianco et al. 1994). Among potential sources of ABA upon imbibition, de novo biosynthesis appeared to play a key role in the expression of dormancy.

Moreover, feeding embryos with (+)-[^{3}H]ABA allowed us to demonstrate that the dormancy release was associated with a hastening of ABA catabolism. Such a change in ABA catabolism during dormancy breaking was already suggested by Toyomasu et al. (1994) in lettuce seeds, where red light irradiation applied to break dormancy lowered the endogenous ABA content substantially. The inability of dormant barley grains to germinate was found also to be partly due to a lower turnover of ABA compared with nondormant barley grains (Visser et al. 1996). However, in this latter case, it was the turnover outside the embryo which correlated with the physiologic behavior. As demonstrated already with other plant material (Mertens et al. 1982, Vaughan and Milborrow 1984), (+)-[^{3}H]ABA was conjugated poorly by *Fagus* embryos, and ABA was catabolized mainly by the oxidative processes. The dormancy release was associated with a hastening of oxidative process, oxidative products accumulating under a conjugated form.

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