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RESEARCH PAPER

Abscisic acid in the thermoinhibition of lettuce seed germination and enhancement of its catabolism by gibberellin

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Abstract

Germination of lettuce (*Lactuca sativa* L. cv. 'Grand Rapids') seeds was inhibited at high temperatures (thermoinhibition). Thermoinhibition at 28 °C was prevented by the application of fluridone, an inhibitor of abscisic acid (ABA) biosynthesis. At 33 °C, the sensitivity of the seeds to ABA increased, and fluridone on its own was no longer effective. However, a combined application of fluridone and gibberellic acid (GA_3) was able to restore the germination. Exogenous GA_3 lowered endogenous ABA content in the seeds, enhancing catabolism of ABA and export of the catabolites from the intact seeds. The fluridone application also decreased the ABA content. Consequently, the combined application of fluridone and GA_3 decreased the ABA content to a sufficiently low level to allow germination at 33 °C. There was no significant temperature-dependent change in endogenous GA_1 contents. It is concluded that ABA is an important factor in the regulation of thermoinhibition of lettuce seed germination, and that GA affects the temperature responsiveness of the seeds through ABA metabolism.

Key words: Abscisic acid metabolism, fluridone, gibberellin, high temperature, *Lactuca sativa*, seed germination.

Introduction

Temperature is a primary factor regulating seed germination. When seeds are exposed to high temperatures, their germination is often inhibited. The seeds can germinate again by lowering the temperature. This suppression of germination at supraoptimal temperatures is called thermoinhibition, and a state of the seeds that have become dormant by exposure to high temperatures is called thermodormancy (Negm *et al*., 1972; Vidaver and Hsiao, 1975). Thermoinhibition plays an ecologically important role in the detection of the appropriate seasonal timing for germination in soil-buried seeds of winter annual plants (Baskin and Baskin, 1998), and often causes delayed or poor germination of cultivated crops, flowers, and vegetables that have relatively low optimal temperatures for seed germination.

Lettuce (*Lactuca sativa* L.) seeds show thermoinhibition at temperatures above 28–30 °C (Berrie, 1966; Thompson, 1973). Reynolds and Thompson (1971) showed that an application of abscisic acid (ABA) to the incubation medium lowers the threshold temperatures for lettuce seed germination, suggesting an involvement of ABA in thermoinhibition. In early work, however, no difference in endogenous ABA contents was observed between lettuce seeds incubated at low and high temperatures (Berrie and Robertson, 1976). Even when the ABA content in the lettuce seeds was significantly changed by an

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Abbreviations: ABA, abscisic acid; ABA-G, glucose conjugates of ABA; ABA-GE, ABA glucosyl ester; ABA-GS, ABA glucoside; DPA, dihydrophaseic acid; GA, gibberellin; *m/z*, mass-to-charge ratio; PA, phaseic acid.

increase in temperature, the changes were small and not always correlated with germination behaviour (Braun and Khan, 1975). Fong *et al*. (1983) working with maize, Xu *et al*. (1990) with alfalfa, and Le Page-Degivry and Garello (1992) with sunflower proved the requirement of ABA biosynthesis for the development and maintenance of seed dormancy using fluridone (1-methyl-3-phenyl-5-(3-trifluoromethyl-(phenyl))-4-(1*H*)-pyridinone). This chemical is a herbicide that blocks phytoene desaturase in the carotenoid synthetic pathway (Bartels and Watson, 1978). Because the carotenoids are precursors of ABA, it is also an inhibitor of ABA biosynthesis. By treating the lettuce seeds with fluridone, Yoshioka *et al*. (1998) found that continuous ABA biosynthesis is indispensable for both the maintenance of high ABA contents and the inhibition of germination at a high temperature. Roth-Bejerano *et al*. (1999) reported similar findings that showed that treatment of thermoinhibited lettuce seeds with the phytoene desaturase inhibitor prevented the accumulation of ABA and restored germination. However, the induction of seed germination by the fluridone treatment appeared only in a restricted temperature range (Yoshioka *et al*., 1998). That is, the germination of the seeds of many winter weeds and winter crops, including lettuce, was promoted by fluridone at certain temperatures, but the seeds failed to germinate at much higher temperatures even in the presence of fluridone. It is suggested, therefore, that thermoinhibition at the higher temperatures is not caused by enhanced ABA biosynthesis alone.

Gibberellin (GA) is considered to promote seed germination, acting after the ABA-mediated inhibition of germination has been overcome (Bewley, 1997*b*; Jacobsen *et al*., 2002). Recent studies, however, revealed antagonistic effects of GA on the expression of ABA-inducible genes in dormant beechnut seeds (Nicolás *et al*., 1997; Lorenzo *et al*., 2001) and on the ABA-dependent inhibition of precocious germination in maize seeds (White *et al*., 2000). In lettuce seeds, the application of a GA biosynthesis inhibitor cancelled out the restorative effect of fluridone on germination at supraoptimal temperatures (Endo *et al*., 2001), and exogenous GA_3 promotes germination with a reduction in endogenous ABA content (Toyomasu *et al*., 1994). Thus, GA might act early in the germination process of lettuce seeds, affecting their responses to temperature through its influence on ABA metabolism (biosynthesis and catabolism). In addition, there is evidence that the sensitivity to ABA of lettuce seeds is enhanced by high temperatures; 10 μM ABA inhibits germination at 30 °C but 100 μM ABA is not completely effective at 20 °C (Robertson and Berrie, 1977). Furthermore, in isolated wheat embryos, ABA was 100 times more effective at 30 °C than at 15 °C in reducing their germination (Walker-Simmons, 1988).

Therefore, in order to elucidate how seeds regulate their germination in response to temperature through the interaction of ABA and GA, the content and activities of ABA, GA, and ABA catabolites were determined using lettuce seeds supplied with these hormones and fluridone at various temperatures.

Materials and methods

Seeds and germination test

Lettuce achenes (seeds) of cultivar Grand Rapids were used. Seeds of cultivar Great Lakes were also used in experiments for the determination of ABA sensitivity. The seeds of both cultivars were purchased from the Sakata Seed Co. Ltd. (Yokohama, Japan) and stored at –30 °C until needed. Germination tests and seed incubation for hormone analyses were conducted in darkness. In each germination test, 50 seeds were sown on two layers of filter paper wetted with 1.8 ml of water, 30 μM fluridone, 2 mM GA_3, and/or (*S*)-(+)-ABA (ABA) at given concentrations in a 6 cm diameter Petri dish. After wrapping with sheets of aluminium foil to avoid light, the Petri dishes were placed at 18, 28, and 33 °C in an incubator in which temperature fluctuated within a range of 1 °C. The number of germinated seeds were scored at 24, 48, 72, and 120 h after the start of incubation. Protrusion of the radicle from the pericarp was the criterion for germination.

Determination of ABA and ABA catabolites

For ABA determination, extraction and purification were performed as in previous work (Yoshioka *et al*., 1998) with a slight modification. Seeds (1 g) were incubated at 18 °C and 33 °C in 6.0 ml of water, 30 μM fluridone, 2 mM GA_3, and 30 μM fluridone plus 2 mM GA_3 solutions in 9 cm diameter Petri dishes. They were sampled at the designated times and homogenized and extracted with 80% methanol containing 0.1 M acetic acid, followed by additions of [3′,5′,5′,7′,7′,7′-$^2H_6$]ABA as an internal standard. After filtering the slurries, the extracts were purified using LC-18 solid phase extraction (SPE) columns (Supelclean, 1 ml, Supelco), LC-NH_2 SPE columns (Supelclean, 1 ml, Supelco), and HPLC (LC-10, Shimadzu) equipped with an ODS-column (Mightysil RP-18 GP, 4.6 mm i.d. × 250 mm, Kanto Chemical Inc.). The ABA fractions separated by HPLC were converted into a methylated form (Me) with diazomethane. The putative ABA-Me analytes were identified and quantified using full-scan GC-MS (GC, GC14A, Shimadzu; MS, QP-2000 equipped with a DB-1 column, Shimadzu), showing a mass-to-charge ratio (*m/z*) spectrum identical to that of the authentic sample: 278 (M^+, 1.2), 260 (5.9), 246 (4.5), 205 (8.6), 190 (100), 162 (45.8), 147 (13.4), 134 (53.1), and 125 (40.9). Contents of ABA in the seeds were calculated from peak area ratios of base ions for ABA-Me and [$^2H_6$]ABA-Me, *m/z* 190 and 194, respectively.

For analyses of ABA catabolites, phaseic acid (PA), dihydrophaseic acid (DPA), and glucose conjugates of ABA (ABA-G) were determined. Seeds (2 g) were incubated at 18 °C in 4 ml of 29.1 mM chlormequat chloride (2-chloroethyl-trimidine methylammonium chloride) solution and 29.1 mM chlormequat chloride plus 2 mM GA_3 solution in 6 cm diameter Petri dishes. To obtain reduced endogenous GA contents that would highlight the effects of exogenous GA_3, the chlormequat chloride solution was used as a control medium; this chemical inhibits GA biosynthesis but not a cytochrome P_{450}-dependent mono-oxygenase (Rademacher, 2000), thereby not blocking ABA 8′-hydroxylase, a key enzyme in oxidative ABA catabolism. The seeds and the incubation media were sampled at 18 h of incubation. The seeds were rinsed with water, and the rinse was combined with the incubation media. These seed- and medium-samples were homogenized in 10 ml of 80% methanol containing 0.1 M acetic acid and extracted overnight at 4 °C, then [$^2H_6$]ABA, [7′,7′,7′-$^2H_3$]PA and [7′,7′,7′-$^2H_3$]DPA were added as internal

standards. After filtering the slurries, the extracts were evaporated under vacuum at 42 °C to a 5 ml aqueous solution, which were then adjusted to pH 2 and extracted with 5 ml of ethyl acetate. The ethyl acetate extracts contained free ABA, PA, and DPA.

The aqueous phases containing ABA-G were adjusted to pH 12 and hydrolysed at 60 °C for 1 h to give free ABA. The alkaline hydrolysed solutions were adjusted to pH 2 again after adding [$^2H_6$]ABA and extracted with ethyl acetate. The extracted organic phases containing ABA released from ABA-G were collected. Abscisic acid glucosyl ester (ABA-GE) has been reported as the major product of conjugation of ABA in germinating lettuce seeds (Orlandini *et al*., 1984). In these experiments, 87–98% of authentic ABA-GE was recovered as ABA in this alkaline hydrolysis process. Abscisic acid glucoside (ABA-GS) is another member of ABA-G (Loveys and Milborrow, 1981), although it had not been identified in Grand Rapids lettuce seeds (Orlandini *et al*., 1984). Hence, in this study, ABA-G possibly consisted of ABA-GE and ABA-GS.

These two organic phases were purified by a method similar to that for the ABA determination using LC-18 and LC-NH_2 SPE columns and HPLC. The ABA, PA, and DPA fractions were methylated with diazomethane, and analysed by GC-MS with selected ion monitoring (GC, HP6890, Hewlett Packard; MS, JMS-700, JEOL) equipped with a fused silica capillary column (DB-1, 0.25 mm i.d. × 15 m, 0.25 μm film thickness, J &W Scientific Inc.). The ABA-Me, PA-Me, and DPA-Me analytes (1 μl) were injected onto the column at 80 °C in a split-less mode. One minute later, the column temperature started to rise, at a rate of 30 °C min^{-1}, to 245 °C and then at a rate of 5 °C min^{-1} to 280 °C. The *m/z* of ions selected for monitoring were as follows: ABA-Me, 190 and 162; [$^2H_6$]ABA-Me, 194 and 166; PA-Me, 294 and 139; [$^2H_3$]PA, 297 and 142; DPA-Me, 296 and 278; [$^2H_3$]DPA-Me, 299 and 281. Contents of ABA, PA, and DPA were determined from peak area ratios of their main ions at *m/z* 190 to 194, 294 to 297, and 296 to 299, respectively. Contents of ABA-G in the seeds and the incubation media were denoted by the amounts of ABA released from 1 g seeds by the alkaline hydrolysis.

Determination of GA

Seeds (20 g) incubated in water in 15 cm diameter Petri dishes at 18 °C and 33 °C were collected, frozen in liquid nitrogen, and stored at –30 °C until use. The seed samples were homogenized in 100 ml of methanol and extracted overnight at 4 °C. After adding [17,17–$^2H_2$]GA_1 as an internal standard and filtering the slurries, the extracts were evaporated under vacuum at 42 °C. The remaining aqueous residues were adjusted to pH 2.5 and fractionated three times with ethyl acetate according to the method of Endo *et al*. (1989). After evaporating and dissolving with 30% methanol, the fractionated samples were submitted to the purifications using LC-18 and LC-NH_2 SPE columns and HPLC as described above. The HPLC conditions were as follows: column temperature, 40 °C; flow rate, 1.5 ml min^{-1}; step-wise linear gradient elution with methanol, 20% at 0–5 min, 20–80% at 5–40 min, 80% at 40–55 min, and 80–20% at 55–60 min. The fractions containing GA_1 were methylated with diazomethane after drying and subsequently converted into a methyl ester trimethylsilyl ether (MeTMSi) form with *N*-methyl-*N*-(trimethylsilyl)-trifluroroacetamide. The samples were analysed by GC-MS with selected ion monitoring as described above. The *m/z* of monitored ions were as follows: GA_1-MeTMSi, 506 (M^+), 448, 376, and 207; [$^2H_2$]GA_1-MeTMSi, 508 (M^+), 450, 378, and 209. The contents of GA_1 in the seeds were obtained from peak area ratios of ions at *m/z* 506 to 508.

Data analysis

Data shown in the figures are the means of three replicates with SEs. Fisher's least significant difference tests and *F*-tests were performed for multiple comparing analyses.

Chemicals

Fluridone was a gift from Mr Kazuma Yamauchi (Dow Chemical Japan, Co. Ltd., Tokyo, Japan) and chlormequat chloride (Cycocel) was purchased from Sankyo, Co. Ltd. (Tokyo, Japan). For authentic compounds, (*S*)-(+)-ABA and GA_1 were gifts from Dr Y Kamuro (BAL Planning, Ichinomiya, Japan) and Professor T Sassa (Yamagata University, Tsuruoka, Japan), respectively. Phaseic acid was prepared by the hydrolysis of the β-hydroxy-β-methylglutaryl ester of 8′-hydroxy-ABA isolated from immature seeds of *Robinia pseudoacacia* (Hirai *et al*., 1978). Dihydrophaseic acid was obtained by reduction and hydrolysis of PA methyl ester (Milborrow, 1975). Abscisic acid glucosyl ester was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). For internal standards used in the GC-MS analyses, [$^2H_2$]GA_1 was provided by Professor LN Mander (Australian National University, Canberra, Australia), [$^2H_6$]ABA was purchased from ICON Inc. (New York, USA), and [$^2H_3$]PA and [$^2H_3$]DPA were prepared according to Hirai *et al*. (2003).

Results

Promotion of germination by fluridone in combination with GA at high temperatures

The germination responses of Grand Rapids lettuce seeds to temperatures were tested in darkness (Table 1). All seeds germinated at 18 °C in water. At 28 °C, 0, 13.3, 86.0, and 100% germination occurred in water, 2 mM GA_3 solution, 30 μM fluridone solution, and a solution containing 30 μM fluridone and 2 mM GA_3, respectively. At a higher temperature, 33 °C, germination was induced by the combined treatment with fluridone and GA_3, although neither fluridone nor GA_3 alone was effective.

Levels of GA_1 and ABA at optimal and supraoptimal temperatures

The contents of GA_1 in seeds incubated at different temperatures were compared in Fig. 1A. The seeds contained 0.40 ng g^{-1} of GA_1 before incubation. When the seeds were incubated at 18 °C for 12 h, the GA_1 content slightly decreased to 0.33 ng g^{-1}. The GA_1 content in the seeds incubated at 33 °C for 12 h was 0.29 ng g^{-1}, which did not significantly differ from the value at 18 °C based on a Fisher's least significant difference test at the 5% level. The seeds contained 351 ng g^{-1} of ABA before the incubation, and the ABA content was maintained at a high

Table 1. *Effects of fluridone and GA on germination of lettuce seeds at different temperatures*

Seeds of a lettuce cultivar Grand Rapids were incubated for 120 h in darkness at designated temperatures in 30 μM fluridone solution with or without 2 mM GA_3. Data are means of three replicates with SEs.

Incubation temperature (°C)	Germination in darkness (%)			
	Water	GA_3	Fluridone	Fluridone+GA_3
18	100	100	100	100
28	0	13.3±8.2	86.0±5.2	100
33	0	0	0	95.3±2.4

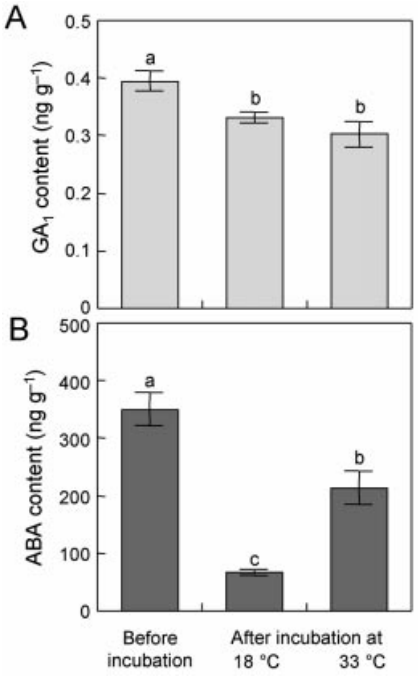

Fig. 1. Effects of temperatures on GA_1 and ABA contents in lettuce seeds. Seeds of a lettuce cultivar Grand Rapids were incubated at 18 °C and 33 °C in water in darkness. The seeds were incubated for 12 h and 24 h for GA_1 (A) and ABA (B) analyses, respectively. The seeds were also sampled before the incubation. Data are means of three replicates with SEs. For each plant hormone, the means designated with the same letter are not significantly different (P <0.05) according to Fisher's least significant difference test.

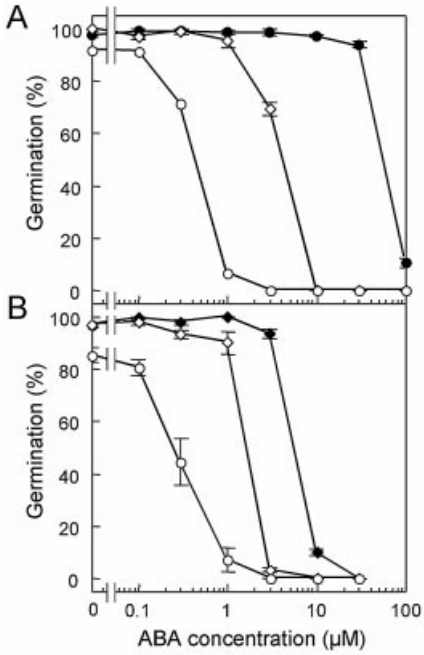

Fig. 2. Effects of temperatures on germination responses of two lettuce cultivars to exogenous ABA. Seeds of a lettuce cultivar Grand Rapids (A) were incubated for 5 d in darkness at 18 °C in water (solid circle), 28 °C in 30 μM fluridone solution (open diamond), and 33 °C in 30 μM fluridone plus 2 mM GA_3 solution (open circle), and seeds of a cultivar Great Lakes (B) at 23 °C in 30 μM fluridone solution (solid diamond), at 28 °C in 30 μM fluridone solution (open diamond), and at 33 °C in 30 μM fluridone solution (open circle), each in combination with various concentrations of ABA. Data are means of three replicates with SEs. Abscisic acid concentrations for 50% inhibition of germination obtained from dose–response curves are described on the text.

level of 214 ng g^{-1} after a 24 h incubation at 33 °C (Fig. 1B). In contrast to GA_1, the ABA content decreased to 76 ng g^{-1} at 24 h of incubation at 18 °C.

Increase in ABA responsiveness with temperature

Germination tests for Grand Rapids lettuce seeds were conducted at 18 °C in water, at 28 °C in fluridone solution, and at 33 °C in fluridone plus GA_3 solution, each in the presence of ABA at various concentrations (Fig. 2A). Abscisic acid started to inhibit germination at 18, 28, and 33 °C at concentrations of 30, 3, and 0.3 μM, respectively, almost completely inhibiting at 300, 10, and 1.0 μM. Concentrations of ABA causing 50% inhibition of germination at 18, 28, and 33 °C were 75, 4.5, and 0.5 μM, respectively. To discover whether the effects of changes in the medium compositions can be neglected, seeds of another lettuce cultivar Great Lakes were incubated at 23, 28, and 33 °C in a 30 μM fluridone solution in combination with ABA (Fig. 2B); the seeds of Great Lakes could germinate at 33 °C in fluridone alone since the maximum temperature for germination was 5 °C higher than that for Grand Rapids seeds. The pattern of germination inhibition by ABA was similar to Grand Rapids; ABA concentrations causing 50% inhibition of germination at 23, 28, and 33 °C were 6.4, 1.9, and 0.3 μM, respectively. The ratios of the ABA concentration causing 50% inhibition of germination at 28 °C to that at 33 °C were 9.0 and 6.3 for Grand Rapids and Great Lakes, respectively; this indicates that the responsiveness of lettuce seeds to exogenous ABA was more sensitive at 33 °C than at 28 °C in both cultivars, regardless of the difference in the composition of the germination media.

Time-course of ABA and germination changes

More than 60% of the seeds germinated at 24 h of incubation at 18 °C in water, whereas this occurred at 48 h of incubation at 33 °C in the solution of fluridone plus GA_3 (Fig. 3A). Less than 5% of the seeds germinated by 120 h after the start of incubation at 33 °C in water, fluridone solution, and GA_3 solution. Changes in ABA content in the seeds incubated in the solution of fluridone in combination with or without GA_3 are shown in Fig. 3B. At 33 °C, the ABA contents in the seeds incubated in GA_3 alone and fluridone alone decreased to approximately 110 ng g^{-1} at 24 h of incubation, remaining at about that content afterwards, and were constantly above 200 ng g^{-1} in the seeds incubated in water. Whereas, in the seeds applied with fluridone and GA_3, the ABA contents were 77 ng g^{-1} at 24 h of incubation, and 45 ng g^{-1} at 48 h of incubation,

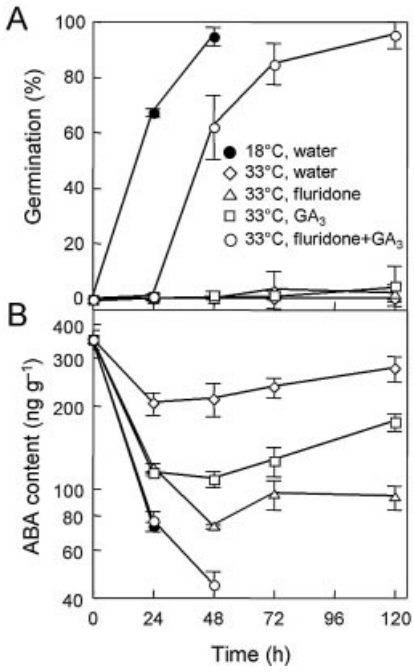

Fig. 3. Time-course of germination and decrease in ABA content of lettuce seeds in response to fluridone and GA at optimal and supraoptimal temperatures. Seeds of a lettuce cultivar Grand Rapids were incubated in darkness at 18 °C in water (solid circle), at 33 °C in water (open diamond), at 33 °C in 30 μM fluridone solution (open triangle), at 33 °C in 2 mM GA_3 solution (open square), and at 33 °C in 30 μM fluridone plus 2 mM GA_3 solution (open circle). (A) Germination. (B) ABA content. The ordinate in (B) is represented as a logarithmic scale because ABA acted exponentially in Fig. 2A, B. Data are means of three replicates with SEs.

when the seeds germinated. At the optimal temperature, 18 °C, the seeds contained 76 ng g^{-1} of ABA at 24 h of incubation at which time they germinated.

Changes in ABA catabolites in response to GA

In ABA catabolism in higher plants, ABA-GE is the major product of the conjugation pathway, and PA and DPA are those of the oxidation pathway (Zeevaart, 1999). Before incubation, seeds contained 58, 140, and 52 ng g^{-1} of PA, DPA, and ABA-G, respectively (Fig. 4). After incubation at 18 °C for 18 h in the absence of GA_3, the total amounts of PA and DPA extracted from both seeds and media were 103 and 177 ng g^{-1}, respectively, and the total amounts of PA and DPA after incubation in the presence of GA_3 were 258 and 252 ng g^{-1}, respectively. That is, increases by the incubation without GA_3 in the amounts of PA and DPA were 45 and 37 ng g^{-1}, respectively, whereas when the incubation was performed with GA_3 the increases in their amounts rose to 200 and 112 ng g^{-1}, respectively. The amounts of ABA-G were not increased by these incubations. After incubation with GA_3, the seeds contained 79 and 26 ng g^{-1} of PA and DPA, respectively, whereas 180 and 226 ng g^{-1} of PA and DPA were recovered from the media, respectively.

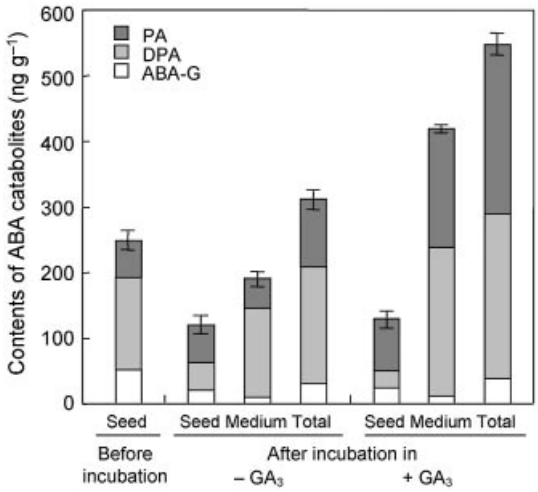

Fig. 4. Effects of exogenous GA on the contents of ABA catabolites in lettuce seeds and on their amounts exported into the incubation media. Seeds of a lettuce cultivar Grand Rapids were incubated in darkness at 18 °C in 29.1 mM chlormequat chloride solution in the presence or absence of 2 mM GA_3 solution. For determining the contents of PA, DPA, and ABA-G, the seeds and the incubation media were sampled at 18 h of incubation. The seeds were also sampled before the incubation. Contents of ABA catabolites in the media are denoted by their amounts produced by 1 g seeds. Data are means of three replicates with SEs.

Discussion

The germination of lettuce seeds was inhibited at supraoptimal temperatures, 28 °C and 33 °C, in darkness (thermoinhibition). The thermoinhibition at 28 °C was overcome by fluridone application. This is in line with previous work (Yoshioka *et al*., 1998), which showed that the fluridone application to the incubation medium enabled thermoinhibited lettuce seeds to germinate by reducing the ABA content, indicating that high temperature enhances ABA biosynthesis. Nevertheless, the combined application of fluridone and GA_3 was necessary to restore germination at 33 °C; neither fluridone nor GA_3 alone was effective. From these findings, it had been assumed that germination might be inhibited by a low endogenous GA content accompanied by a high ABA content at such a high temperature as 33 °C, and thus the endogenous contents of ABA and GA_1, which is the main endogenous bioactive GA in lettuce seeds (Toyomasu *et al*., 1993), were determined. Contrary to this assumption, no significant difference in GA_1 contents was found between the two temperatures, although ABA content was much higher at 33 °C than at 18 °C. This result indicates that the high temperature inhibits germination by maintaining high endogenous ABA contents without affecting endogenous GA_1 contents.

However, the above result does not uncover the cause of the ineffectiveness of fluridone at 33 °C. Germination responses of lettuce seeds to ABA are temperature dependent (Robertson and Berrie, 1977; Roth-Bejerano

et al., 1999). These results showed that the sensitivities of Great Lakes and Grand Rapids lettuce seeds to exogenous ABA at 33 °C were 6–9-times higher than those at 28 °C, strongly suggesting that the threshold content of endogenous ABA for the germination inhibition at 33 °C is lower than at 28 °C. In barley (Wang *et al*., 1995), yellow-cedar (Schmitz *et al*., 2000), *Nicotiana plumbaginifolia* (Grappin *et al*., 2000), and Douglas fir (Corbineau *et al*., 2002), dormant states of their seeds are maintained by high ABA sensitivities and ABA accumulations acting in concert. Thus, it is deduced that the thermoinhibition of lettuce seed germination at 33 °C is caused by both high ABA content and high ABA sensitivity and that the application of fluridone alone cannot reduce the ABA contents to a low level thus allowing germination at this high temperature.

The ABA contents were reduced not only by the fluridone application but also by the exogenous GA_3. This agrees with previous results with lettuce seeds (Toyomasu *et al*., 1994), in which endogenous ABA contents were lowered by GA_3 treatment, and with dormant *N. plumbaginifolia* seeds (Grappin *et al*., 2000), in which ABA contents do not rise in the presence of exogenous GA_3. Here, an additive effect of fluridone and exogenous GA_3 on the decrease in ABA content was found; in the seeds treated with GA_3 in combination with fluridone, the ABA content was about 50% lower than those treated with either fluridone or GA_3 alone. This suggests that GA acts on the decrease in ABA content through a process other than that of the fluridone action. The ABA content is regulated through two processes, biosynthesis and catabolism, and fluridone inhibits the former. Therefore, it is assumed that the exogenous GA enhances the ABA catabolism.

An attempt to prove this assumption was made by analysing the contents of ABA catabolites. The increase in the total amount of ABA catabolites in the presence of GA_3 was four times as large as that in the absence of GA_3, mainly depending on the accumulation of PA, which is the first stable product of the oxidative pathway in ABA catabolism. Furthermore, most of the increased ABA catabolites were recovered from the medium. It is evident from these results that exogenous GA enhances oxidative ABA catabolism in lettuce seeds and that products of the catabolism are exported from the intact seeds. This finding is supported by the work by Schmitz *et al*. (2002) who suggested, from differences in activities of germination inhibition between ABA and a metabolism-resistant ABA analogue, that dormancy-breaking treatments including GA_3 applications enhance ABA degradation in yellow-cedar seeds. Therefore, it is concluded that, at 33 °C, in association with the inhibited ABA biosynthesis by fluridone, the enhanced ABA catabolism by exogenous GA_3 reduces endogenous ABA sufficiently to allow germination of the seeds in which threshold ABA contents for their germination are lower than in the seeds suffering the milder thermoinhibition. Actually, in the solution of fluridone in combination with GA_3 at 33 °C, the seeds did not germinate at 24 h of incubation, although endogenous ABA reached a content enabling germination at 18 °C, and it was at 48 h of incubation when the ABA content further decreased by 50% that germination was observed.

Since lettuce seed germination completes when the growth potential of the embryo overcomes the constraining force of the endosperm, it is interesting to consider localizations of distribution and action of ABA between the endosperms and the embryos. Dulson *et al*. (1988) reported that lettuce seed endosperms contained a large amount of ABA, but the ABA amounts in the endosperms were not significantly different between ungerminated and germinated seeds. This implies that the endosperms do not produce ABA under unfavourable conditions for seed germination. Le Page-Degivry and Garello (1992) revealed with sunflower, a Compositae species, that the embryos of dormant seeds continuously synthesize ABA to inhibit radicle elongation. From these findings, it may be deduced that the major site of ABA accumulated in dry seeds is the endosperms and that the major site of ABA biosynthesis in imbibed seeds is the embryos. The ABA accumulated in the endosperms suppresses the production of endo-β-mannanase (Dulson *et al*., 1988), which is known to act as a key enzyme reducing the constraining force of the endosperm (Bewley, 1997*a*). However, Toorop *et al*. (1999) reported discordance between the structure–activity relationship of ABA analogues for the inhibition of lettuce seed germination and that for the inhibition of endo-β-mannanase activity in the endosperms, suggesting that the sites of ABA action on germination and on this enzyme are different. In fact, the growth potential of lettuce seed embryos is lowered by exogenous ABA (Takeba and Matsubara, 1979). It is, therefore, possible that ABA acts both in the endosperms and in the embryos. The activity of endo-β-mannanase, extracted from endosperms isolated prior to radicle protrusion, is inhibited by high temperature, and this inhibition is alleviated by exogenous GA_3 (Dutta *et al*., 1997). Similarly, decreases in growth potential of embryos at high temperatures are rescued by exogenous GA_3 (Takeba and Matsubara, 1979). Thus, it could be possible that GA also acts in both the endosperms and the embryos of thermoinhibited lettuce seeds. However, in the results by Dulson *et al*. (1988), the ABA content in the germinated seeds that had been exposed to light did not differ from that in the ungerminated seeds incubated in darkness. These germinated seeds are supposed to have had a high content of a bioactive GA, because red light irradiation drastically promotes GA_1 biosynthesis in lettuce seeds, inducing expression of GA-3β-hydroxylase within 2 h after irradiation (Toyomasu *et al*., 1993, 1998). On this supposition, GA may not reduce the ABA content in the endosperms, although GA restores the production of endo-β-mannanase

suppressed by endogenous ABA (Bewley, 1997*a*). Therefore, it may be possible that the decrease in ABA content by exogenous GA occurs mainly in the embryos. The localizations of ABA and GA sites in the seed are outside the scope of this study, thus, further investigations are needed to discover whether the site of ABA biosynthesis in response to temperature and the site of ABA catabolism enhanced by GA is the embryos.

The sensitivity of the seeds at 33 °C to exogenous ABA was 100-fold higher than that at 18 °C, whereas the ABA content in the seeds at 33 °C was lower by 50% than that at 18 °C, when the seed germination completed. If it is assumed that the action site of ABA and the accumulation site of ABA are different, one possibility of explaining the difference in the magnitude of response to temperature between the sensitivity to ABA and the ABA content is that the amounts of accumulated ABA masked the threshold ABA contents of preventing the germination at 33 °C. That is, the ABA content allowing the seeds to germinate at 33 °C might be overestimated in the present study. Further investigations into the ABA localization in the seeds will also provide information about the threshold contents of ABA in its action sites.

Among the ABA catabolites accumulated in the seeds and the media, PA and DPA were predominant and ABA-G was quite minor. This is inconsistent with the previous results by Orlandini *et al*. (1984) who reported that ABA-GE is the main ABA catabolite in Grand Rapids lettuce seeds incubated in 10^{-5} M [^{14}C]ABA for 24 h and 96 h. There is evidence in several assay systems that the rates of ABA catabolism can be changed by exogenous ABA (Zeevaart and Milborrow, 1976; Uknes and Ho, 1984; Babiano, 1995; Cutler *et al*., 1997; Windsor and Zeevaart, 1997). Hence, one possible explanation for the inconsistency is that lettuce seeds change the main pathway in their ABA catabolism from oxidation to conjugation when they are fed with an excessive amount of ABA. In addition, *epi*-DPA and glucose conjugates of PA and DPA have been identified as products of the oxidation catabolism of ABA in some plant species (Zeevaart, 1999), therefore, if these compounds are determined in lettuce seeds, the contents of oxidative catabolites may be still higher. Besides, Batge *et al*. (1999) showed that a gibberellin-deficient mutant *lh-2* of pea sets young seeds associated with a reduced content of GA_1 and with an accumulation of ABA, and Endo *et al*. (2001) reported that the restorative action of fluridone on lettuce seed germination disappears in the presence of a GA biosynthesis inhibitor. From their results and this study's findings, it may be speculated that GA_1 existing at low concentrations in lettuce seeds acts on ABA catabolism during the germination processes. These possibilities should be investigated further.

The present results are summarized below. When the seeds sense that the temperature is too high to germinate, ABA biosynthesis is enhanced and high ABA content is maintained to prevent germination. This mild thermoinhibition is avoided by the inhibition of ABA synthesis by fluridone application. If the temperature is much higher, the seeds increase their sensitivities to ABA, maintaining the enhanced ABA biosynthesis, but not affecting the bioactive GA_1 contents. Therefore, for reducing the endogenous ABA content sufficiently to rescue seed germination from severe thermoinhibition, the inhibition of ABA synthesis must be combined with the enhancement of ABA catabolism by exogenous GA.

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