Fluorescence Staining Technique for Evaluating Embryo Sensitivity to Abscisic Acid of Sprouting-Resistant Wheat Cultivars

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Embryo sensitivity to abscisic acid (ABA) of wheat cultivars "Lancer", "Horoshi" and "Lewis", which showed different sprouting resistance, was estimated in the initial stage of germination in terms of lipase-esterase activity detected by the fluorescein dibutyrate (FDB) method. Lipase-esterase activity increased in the endosperm adjacent to the epithelium of the scutellum and expanded to the inner area of the endosperm as germination proceeded. Seed germination and lipase-esterase synthesis of sprouting-resistant "Lancer" were inhibited by ABA, while sprouting-susceptible "Lewis" was not suppressed in terms of either germination or enzyme synthesis by ABA. In moderately resistant "Horoshi", germination was suppressed, but its lipase-esterase synthesis was not suppressed by ABA. Seed of "Lancer" that had been stored for six months after harvest and had lost their dormancy still maintained embryo sensitivity to ABA. The experiments showed that embryo sensitivity to ABA can be estimated with the FDB method by treating whole seeds with ABA, after seeds have lost their dormancy.

KEY WORDS: Triticum aestivum, wheat, preharvest sprouting, ABA, fluorescein dibutyrate.

Introduction

Preharvest sprouting in wheat reduces the quality of the grain for food products through the hydrolysis of starch in the endosperm by $\alpha$-amylase and proteolytic damage of grain proteins. Seed dormancy is an important factor for minimizing preharvest sprouting. Wheat cultivars vary in susceptibility to preharvest sprouting and in the duration of seed dormancy. Useful methods to select for genotypes with preharvest sprouting resistance have been therefore required.

White wheat cultivars are generally thought to lack dormancy at maturity and to be susceptible to preharvest sprouting. Red wheat cultivars, on the other hand, show a wide range of dormancy and resistance to preharvest sprouting. However, seed color is not always correlated with seed dormancy as some white wheat genotypes possess relatively high dormancy (Bhatt and Derera 1980).

The nature of seed dormancy is not clearly understood. Miyamoto et al. (1961) showed that dormancy in wheat was caused by germination inhibitors, including catechin-tannins, which were located in the seed coat and decreased as the seed aged. Stoy and Sundin (1976), comparing embryo sensitivity of mature grains to the germination inhibitors, catechin-tannins and ABA, reported that excised embryos of sprouting-resistant cultivars were sensitive to these inhibitors, while those of sprouting-susceptible cultivars were

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insensitive. McCrate et al. (1982) and Walker-Simmons (1987) also showed that variation in dormancy among wheat cultivars was primarily caused by a differential response of their embryos to an endogenous inhibitor.

Enzyme synthesis at the early stage of seed germination has been studied extensively. Endogenous GA₃ in the scutellum of embryo has been thought to stimulate synthesis of hydrolytic enzymes, mostly α-amylase, in the aleurone. Okamoto and Akanawi (1979), Akaawa and Miyata (1982), and Gibbons (1981), on the other hand, indicated that the scutellum was the initial site of activity of α-amylase and other hydrolytic enzymes at the beginning of germination. Recently, Jensen and Heltved (1982), using the FDB method, showed that lipase-esterase activity could be easily detected by fluorescence in seed tissues and that the FDB method has a potential as a detector of de novo synthesized enzymes in germination.

The objects of this research were to determine if the embryo sensitivity to ABA of sprouting resistant cultivars is reflected in reduced enzyme activity in such seed tissues as detected by the FDB method, and to examine the possibility of employing the FDB method for selecting genotypes with sprouting resistance.

Materials and Methods

Seeds of three common wheat (Triticum aestivum) cultivars—“Lancer”, “Horoshiri” and “Lewis”, which show different degrees of preharvest sprouting resistance according to Tsuchiya (1982)—were used. “Lancer” is resistant to sprouting, “Lewis” is susceptible, and “Horoshiri” is moderately resistant. In the following treatments, seeds of the three cultivars stored at room temperature for six months after harvest were used.

Treatment

GA₃ (Sigma) and ABA (Sigma) were prepared at 1.0×10⁻⁶ M and 1.0×10⁻⁵ M, respectively. Our preliminary experiments and the results of Stoy and Sundin (1976) indicated that these concentrations of the solutions induced remarkable differences in seed germination among wheat cultivars with different degrees of sprouting resistance. These reagents were dissolved in 0.5 ml of 99.5% ethanol and diluted to their final concentrations with distilled water. As a control, 0.005% (v/v) ethanol solution was used. Cycloheximide (CH, Wako) was also used at a concentration of 5×10⁻³ M to examine the relationship between protein synthesis and seed germination. Seeds of each variety were incubated at 25°C in the dark for 24 h or 48 h on a filter paper wetted with GA₃, ABA, CH, or the control solution in a Petri-dish.

Localization of lipase-esterase activity

The fluorescence staining technique used is based on the FDB method of Jensen and Heltved (1982). Seeds incubated at 25°C for 24 h or 48 h were freeze-sectioned longitudinally in the thickness of 100 μm with a microtome. Section samples obtained from the middle of seeds were placed on a slide glass and covered with a few drops of 1×10⁻³ M fluorescein dibutyrate (FDB, Research Organics Inc.) dissolved in 80% (v/v) ethanol. Where ester bonds of FDB were hydrolyzed by lipase-esterase activity, FDB emitted fluorescence (520 nm) when excited at 490 nm. After a 30-min incubation of the section samples in the dark, fluorescence intensity became stable. Localization and
intensity of FDB fluorescence in the different tissues of the seed were examined by fluorescent microscopy (BHS-RFK, Olympus). To measure fluorescence intensity numerically, a spot photometry device integrated in autophotographing controller (PM 10 ADS, Olympus), which can measure light intensity in a 1% area of the microscopic field, was used. The autophotographing controller allowed shorter exposure time for higher intensities of fluorescence. Exposure times from eight grains were averaged to estimate light intensity.

**Results and Discussion**

The pericarps over the embryos of the three varieties “Lancer”, “Horoshiri”, and “Lewis” ruptured during the initial 24-h imbibition in the control solution (Fig.1 a). There was no remarkable developmental difference in seed germination among these three varieties after 24-h or 48-h imbibition in the control (Fig.1 b), although “Lewis” tended to germinate a little faster than the other two varieties at the stage of 24-h imbibition. This early germination of seeds of the three varieties after a 24-h imbibition indicated that seeds of these varieties lacked dormancy at the time of the experiment.

With ABA treatment for 24-h, there was no apparent difference in germination among the three cultivars, although “Lancer” tended to germinate more slowly (Fig.1 c). However, a large difference in seed germination was observed after a 48-h ABA treatment (Fig.1 d). Coleoptile and root development of “Lancer” and “Horoshiri” were remarkably retarded, compared with those of “Lewis”. The preharvest sprouting-resistant variety “Lancer” and moderately resistant variety “Horoshiri” appeared to be sensitive to ABA, while “Lewis” was insensitive. This present observation agrees with the results of Stoy and Sundin (1976) and McCrate et al. (1982) that preharvest sprouting-resistant varieties were sensitive to ABA and ‘catechin–tannins’.

Walker-Simmons (1987), in a study of the embryonic sensitivity of preharvest sprouting-resistant and -susceptible wheat cultivars to ABA, reported that ABA had no effect on the germination of whole seeds collected around the harvest maturity, although germination of excised embryos from the sprouting-resistant cultivars was reduced by ABA. Miyamoto et al. (1961) indicated that germination inhibitors contained in the seed tissues except the embryo caused dormancy and decreased as the seed aged. Probably, the seeds used by Walker-Simmons were still dormant and contained endogenous germination inhibitors in the seed tissues and the embryos responded to the endogenous inhibitors rather than to exogenous ABA. The present results, on the other hand, showed that the whole seeds of sprouting-resistant varieties with six months' after-ripening responded to ABA. These seeds may have lost endogenous germination inhibitors during after-ripening and a responsiveness to exogenous ABA can now be measured.

The results from GA$_3$ treatment suggest also the disappearance of the endogenous germination inhibitors from the seeds presently used. GA$_3$ has been indicated to act antagonistically to germination inhibitors ‘catechin–tannins’ and ABA (Stoy and Sundin 1976, Paulsen and Heyne 1983). Seeds of the three varieties treated with GA$_3$ germinated as observed in the control (Fig.1 e and f). No enhancement of germination by GA$_3$ was observed in any of the three varieties. This non-effectiveness of GA$_3$ might be related
Fig. 1. Seed germination of "Lewis", "Horoshiri", and "Lancer" that had imbibed the control (0.005% ethanol), $GA_3$ ($10^{-4}$M), ABA ($10^{-4}$M), or CH ($5 \times 10^{-4}$M) solution for 24 h or 48 h.
to the lack of the endogenous inhibitors of germination.

Taken together, it appears that the embryo sensitivity to ABA can be maintained for at least six months even after the dormancy disappeared and embryos react to the exogenous ABA, while germination inhibitors contained in the seed tissues except the embryo disappear as seeds age.

Seed dormancy of wheat at harvest time might be determined by two factors: (1) embryo sensitivity to endogenous germination inhibitors (such as the presently known substances ABA and catechin-tannins), and (2) the amount of the endogenous inhibitor in the seed tissues except the embryo. Thus, strong dormancy might be attained by the cultivar with the embryo highly sensitive to endogenous inhibitor and a large amount of inhibitor in tissues surrounding the embryo. Weak dormancy would be related to

Fig. 2. Progressive appearance of lipase-esterase activity in freeze sections (100 μm) of seeds treated with fluorescein dibutyrate.

a) "Lewis" in control solution for 24 h. b) "Lewis" in control solution for 48 h. c) "Horoshiri" in GA₃ for 24 h. d) "Horoshiri" in GA₃ for 48 h. e) "Lancer" in control solution for 24 h. f) "Lancer" in control solution for 48 h. g) "Lancer" in ABA for 24 h. h) "Lancer" in ABA for 48 h.
the embryo insensitive to the inhibitors and small amount of inhibitors in seed tissues. Various degrees of intermediate dormancy might be attributed to the combination of embryo with different degrees of ABA-sensitivity and various amount of inhibitors in seed tissues.

Seed germination is not only observable as developmental changes of coleoptile and roots, but is also detectable biochemically as change in enzyme activity. Lipase–esterase activity was examined by the FDB method in terms of fluorescent area and intensity in the different seed tissues. In seeds that had imibed the control solution for 24-h, lipase–esterase activity was detected only in the area of endosperm adjacent to the epithelium (Fig. 2a). After a 48-h imbibition, fluorescence intensity increased and the fluorescent area expanded to the inner area of the endosperm (Fig. 2b). CH treated seeds of “Lewis”, “Horoshiri”, and “Lancer”, did not germinate (Fig. 1g and h), and no remarkable fluorescent area was observed in any seed tissues Therefore, lipase–esterase appears not to be stored in the mature seed, but rather to be synthesized after water imbibition.

In their study of lipase activity in the germinating wheat grain, Tavener and Laidman (1972) reported that lipase activity was induced by factors emanating from the embryo. Akazawa and Miyata (1982) showed that α-amylase and other hydrolytic enzymes were synthesized in the epithelium of the scutellum at the initial stage of germination. Jensen and Heltved (1982), using the same FDB techniques, suggested that the scutellum played a major role in the synthesis of hydrolytic enzymes. The present results also agree that the epithelium of the scutellum plays an important role in early germination.

The increase of fluorescent area during germination was almost the same in seeds of “Lewis” and “Horoshiri”, when they imbibed the control, GA₃, or ABA solution. Seeds of “Lancer” also showed the same pattern of fluorescent area increase as “Horoshiri” and “Lewis”, when they imbibed the control or GA₃ solution (Fig. 2c, d, e, and f). However, “Lancer” that had imbibed the ABA solution showed restricted fluorescent area, compared with the seeds imbibing the control or GA₃ solution. In the case of 24-h ABA imbibition, fluorescence was observed only in a small area adjacent to the epithelium (Fig. 2g). After 48-h, the fluorescent area was almost the same as that observed after a 24-h imbibition by “Horoshiri” or “Lewis” (Fig. 2h). These results indicate that “Lancer” was more sensitive to ABA than “Lewis” or “Horoshiri”.

Fluorescence intensity in the area with lipase–esterase activity was measured in the

Table 1. Relative intensity (exposure time, sec) of FDB fluorescent light activated by lipase–esterase in the endosperm area adjacent to the scutellum of seeds incubated in control, GA₃, or ABA solution.

<table>
<thead>
<tr>
<th></th>
<th>Lewis</th>
<th>Horoshiri</th>
<th>Lancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Cont.</td>
<td>6.23 (2.79)</td>
<td>1.71 (1.59)</td>
<td>7.88 (1.06)</td>
</tr>
<tr>
<td>GA</td>
<td>3.76 (1.01)</td>
<td>2.43 (1.02)</td>
<td>6.59 (2.70)</td>
</tr>
<tr>
<td>ABA</td>
<td>7.83 (3.93)</td>
<td>1.99 (0.89)</td>
<td>11.22 (4.44)</td>
</tr>
</tbody>
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( ) : standard deviation
three varieties that had imbibed the control, ABA or GA$_3$ solution (Table 1). "Lancer" showed the lowest activity of lipase-esterase after 24-h imbibition in the control solution. After a 48-h imbibition, however, there was no difference in fluorescence intensity among the three varieties. This slow enzyme synthesis of "Lancer" at 24-h imbibition was not detected clearly in morphological terms of germination and fluorescent area of the seed tissues.

With GA$_3$ treatment, fluorescence intensity increased at the early stage of germination in the three varieties, although the difference between the control and GA$_3$ treatments was not significant statistically at the 5% level. It is possible that GA$_3$ reacted antagonistically with germination inhibitors remained a little in the seed tissues, and enhanced lipase-esterase synthesis.

In seeds treated with ABA solution, fluorescence intensity was reduced in the three varieties. However, the reduction of fluorescence intensity by ABA was not significant statistically at the 5% level except in the case of "Lancer" after a 48-h ABA imbibition. This result suggests again that "Lancer" is sensitive to ABA.

"Horoshiri" was ABA-sensitive in terms of coleoptile and root development, but not so sensitive in the case of lipase-esterase activity. The reason for the difference between morphological and biochemical response to ABA is not clear at present. However, it appears that there is a case in which enzyme activity is high even when root and coleoptile development is inhibited.

For the practical selection of genotypes with preharvest sprouting resistance, it might be better to select for ABA-sensitive genotypes by applying ABA to whole seeds which almost lacked seed dormancy, because embryo sensitivity to ABA of sprouting-resistant cultivar is maintained even after its dormancy disappear and can be estimated without the disturbance by the endogenous germination inhibitors. Also, embryo sensitivity estimated by the FDB method appears to be sensitive and can be used to differentiate a sprouting-resistant genotype from moderately sprouting-resistant one.

Acknowledgments

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Literature Cited

コムギ穀物帯抗性品種選育のアブサイシン酸感受性の蛻光染色法による識別

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ヒメスオガサト帯抗性品種“Lancer”，中程度帯抗性品種“ホロシリ”，感受性品種“Lewis”的種子を用いて，ABA感受性の有無と感受性程度の違いをFluorescein dibutyrate (FDB) 蛍光染色法によって酵素レベルで明らかにしようとした。

“Lancer”、“ホロシリ”、“Lewis”の種子をコントロール（0.005％エタノール），GA₃（10⁻⁷M），ABA（10⁻⁴M），Cycloheximide（5×10⁻²M）に24, 48時間浸し，発芽反応を調査した。“Lancer”，“ホロシリ”はABAに感受性を示したが，“Lewis”は不感受性であった（Fig.1）。一方，コントロールにおいて，いずれの品種も24時間後に発芽したことから，何れの種子も休眠性を失っていることは明らかである。以上の結果は，穂発芽抵抗性品種の種子は休眠性をなくしても，ABA感受性を維持していることが示唆された。また，種子休眠性に関わっている発芽抑制物質がABAに拮抗的に働くGA₃によって，いずれの品種でも発芽促進作用がみられなかったことは，休眠性をなくした種子では発芽抑制物質やABAがすでに失われたか，発芽を抑制するほど十分に存在しないことを示唆する。

24, 48時間浸し後，コムギ種子を100μmの厚さに凍結切片し，FDB法によりlipase-esterase活性変化を観察した結果，時間経過と共にlipase-esterase活性は胚乳部付近より第2に胚乳部に広まり，また蛻光点数も増加した（Fig.2）。

ABA処理された“Lancer”，“ホロシリ”，“Lewis”種子におけるlipase-esterase活性は，“Lancer”で明らかに低く，lipase-esterase合成がABAによって抑制されていることが示唆された。一方，“ホロシリ”，“Lewis”では，明らかに抑制がみられなかった。以上，ABAに対し“Lewis”は，発芽およびlipase-esterase活性とともに感受性でなく，“ホロシリ”では，発芽が感受性であるのに対し酵素活性では感受性でない。一方，“Lancer”は発芽，酵素活性とともに感受性であることから，穂発芽抵抗性遺伝子型をABA感受性とFDB法を組合せることにより選抜することが可能と思われる。