Relation of Seed Structures to Soybean Cultivar Difference in Pre-germination Flooding Tolerance

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Abstract: Flooding during germination inhibits the sprouting and emergence of soybean [Glycine max (L.) Merr.], reducing subsequent growth and yield. Previous studies revealed that cultivars tolerant to pre-germination flooding have a mechanism of reducing water absorption speed (WAS) during the initial stage of inundation; however, seed structures involved in WAS have not been fully clarified to date. The objectives of this study were to identify possible seed structures responsible for cultivar difference in WAS and pre-germination flooding tolerance. WAS of two tolerant cultivars (Peking and Williams) and two susceptible cultivars (Nakasennari and Enrei), which were identified in our previous study, was compared in relation to seed structures. In Peking, WAS was markedly lower than that of the other cultivars, either in intact seed or seed with the seed coat removed, suggesting that both the seed coat and the embryo have a mechanism of reducing WAS in this cultivar. WAS of the hilum side tended to be higher than that of the back side, and sealing of the micropyle significantly lowered WAS, showing that the micropyle rather than the hilum appeared to be responsible for the higher WAS of the hilum side regardless of cultivar. A comparison of cross section area of the hilum revealed that the tolerant cultivars tended to have a larger area than the susceptible cultivars, suggesting that an inner space of the hilum can act as a reservoir at the initial stage of inundation, thereby reducing WAS in tolerant cultivars.

Key words: Aleurone layer, Flooding tolerance, Germination, Hilum, Micropyle, Seed structure, Soybean, Water absorption.

In Japan, the climate in late spring to early summer is characterized by continuous rainfall for several days. Under such climatic conditions, crop fields are prone to flooding, and germination and emergence of crops can be adversely affected. Soybean is especially vulnerable to such pre-germination flooding (Arihara et al., 2000). The majority of soybean cultivation in Japan is conducted in paddy fields, which are now being converted for growing upland crops including soybean. In converted fields that are not well drained, flooding during the pre-germination stage of soybean often leads to poor seedling emergence.

Agronomical countermeasures include improvement of drainage by setting up drains in the field, and adjustment of seed moisture prior to sowing (Obendorf and Hobbs, 1970; Hobbs and Obendorf, 1972; Nakayama et al., 2004; Nakayama et al., 2005). Screening cultivars or germplasms for tolerance to pre-germination flooding is another option for addressing this problem. Hou and Thseng (1992) attempted to develop a method for selecting varieties tolerant to pre-germination flooding, and found that continuous flooding of seeds for four days at 25°C allowed identification of tolerant genotypes. Both additive and dominant genes were found to control tolerance to pre-germination flooding, and selection for tolerant genotypes was considered feasible (Hou et al., 1995). Despite these findings, specific seed traits responsible for such tolerance in soybean have not been clarified to date.

Previous studies indicated that rapid water uptake is a major cause of damage to the embryo, thereby reducing the germination percentage of soybean seed (Bramlage et al., 1978; Powell and Mathews, 1978; Leopold, 1980; McDonald et al., 1988; Wuebker et al., 2001). In this regard, Kannenberg and Allard (1964) found that the pigmented seed of the lima bean, which contains almost 15 times more lignin in the seed coat than non-pigmented seeds, germinated more vigorously, probably because of lesser mechanical injuries sustained during harvesting. Raymond et al. (1981) shared the same view on soybean. On the other hand, McDonald et al. (1988) demonstrated the roles of soybean seed coat in retarding water uptake. In our previous study (Tian et al., 2005), however, removal of the seed coat did not increase WAS in 3 out of 4 cultivars tested, and the presence of the seed coat markedly reduced WAS only in Peking, the most tolerant among the cultivars examined.

Previously, we investigated differences in flooding...
tolerance of soybean cultivars during the germination stage, and found that tolerant cultivars have the capacity of lowering WAS during the initial stage of inundation (ca. 30 min) and that the aleurone layer acts as a barrier against water penetration into the seed (Tian et al., 2005). In addition, the presence of the seed coat retarded WAS only in Peking. However, details of seed structures involved in cultivar differences in WAS remain unclear. The objectives of this study were to identify possible seed structures responsible for cultivar difference in WAS and pre-germination flooding tolerance. Specifically, we attempted to clarify structural characteristics of the tolerant cultivar Peking responsible for regulating WAS.

Materials and Methods

1. Plant materials

Seeds of four soybean cultivars (i.e., Peking, Williams, Enrei, Nakasennari) were used in this study. Flooding tolerance of these cultivars was evaluated in our previous study; Peking and Williams were tolerant, whereas Enrei and Nakasennari were susceptible (Tian et al., 2005). They were harvested from the same field in 2004, and the seeds were stored at ca. 5°C until analyses.

2. Examination of germination percentage

Germination percentage under optimum condition (GPo) was measured according to the International Seed Testing Association (ISTA) rule (1985). Briefly, seeds folded in two layers of paper towels (230×218 mm, double-layer type, Crecia Company Ltd., Japan) saturated with de-ionized water and placed in an incubator. The temperature inside the incubator was controlled at 25°C. Seeds were considered to have germinated when the radicle was visible. All germinated seeds were counted daily after the seeds had been placed into the incubator. The test was repeated three times, using 30 seeds for each replicate.

Germination percentage after flooding treatment (GPf) was also measured. A glass box measuring 35×85×85 mm was used as the seed container. Thirty seeds of each cultivar were flooded for 1 and 2 days by adding 100 mL of de-ionized water to the container. After this treatment, seeds were allowed to germinate in Petri dishes (11 cm in diameter) under optimum condition as described in the measurement of GPo. Germination percentage was monitored daily after the end of the respective flooding period for 7 days. No additional water was added to the Petri dishes after the transfer of seeds.

For the measurements in this study, healthy seeds with no injury on the surface were used, and seed moisture was measured using a moisture tester (PM-830-2, Kett Electric Lab., Tokyo).

3. Measurements of WAS from various seed parts

To examine the effects of the seed coat on WAS, we carefully peeled off the seed coat with the edge of a razor, and flooded the seeds as well as intact seeds for 30 min. The container for WAS measurement was the same as that used for the germination experiments. Thirty seeds for each replicate were weighed and consequently flooded with 100 mL of de-ionized water. The temperature of the container was controlled at 25°C under ambient light conditions. Seeds were periodically taken out of the water (every 5 min until 30 min of flooding), blotted on a filter paper and weighed; blotting removed excess surface water. WAS for each interval was calculated as the ratio of the increase in seed weight to the original weight.

Next, WAS from the hilum side and back side was compared. Twenty seeds were placed in a Petri dish with either the hilum side or back side facing down and soaked in de-ionized water up to half the seed width, and then WAS was monitored in the same manner as described above at 10-min intervals. For comparison between WAS from the hilum and that from the micropyle, either the hilum or micropyle was sealed using an adhesive (Aron alpha, Toagosei Co., Ltd., Tokyo), and WAS was monitored in the same manner as described above. Twenty seeds were used in either treatment and the experiment was repeated three times.

![A](image1.png)

![B](image2.png)

Fig. 1. Photographs of cross-sectioned seeds contained in a holder (A) and cross section of the hilum with surrounding tissues (B). Area of hilum (Ah) in the cross section was estimated using the following equation: \[ Ah = (a + b) \times h / 2 \]. Bar = 100 µm.
4. Observation of seed structures

To observe water penetration into the embryo after flooding treatment, we soaked seeds (Enrei) in 0.5 mg L⁻¹ (w/v) toluidine blue O (E. Merck, Darmstadt, Germany) solution for 25 min, and observed the cut section with an optical microscope equipped with a digital microscope camera (Polaroid DMC le, Nippon Polaroid Co., Ltd., Tokyo).

For comparison of the cross section of seeds among the four cultivars, seeds with a moisture of ca. 12% were used in this experiment. As shown in Fig. 1, seeds were placed in a handcrafted seed holder, the size of which was adjusted to that of seeds of respective cultivar, and then the seeds were adhered tightly to the holder using adhesive. Seeds contained in the holder were cross-sectioned with a diamond disc equipped with a grain cutting machine (designed and crafted by A. Horigane). Dimensions of the tissues in cross sections were measured using a 3D measurement system (Quick Vision Pro, Mitutoyo Corp., Kawasaki). The area of the hilum (Ah) in a cross section was estimated using the following equation: \( Ah = (a + b) \times h / 2 \), where \( a \) and \( b \) are the length of the upper and lower bases, respectively, and \( h \) is the height if the hilum shape resembles a trapezoid. Twenty seeds for each cultivar were used.

Results

1. Germination percentage under optimum condition and after flooding treatment

The GPo of the four cultivars ranged from 96.7 to 100.0% (Table 1). The GPF for 1 or 2 days decreased significantly except in Peking, where it remained very high (97.8%) even after a 2-day flooding treatment. Cultivar differences between GPo and GPF for 2 days ranged from 2.2 to 61.1%, enabling identification of tolerant and susceptible cultivars; Peking was the most tolerant, Nakasennari was the most susceptible, and Enrei and Williams were intermediate.

2. Comparison of WAS from various seed parts

When seeds were subjected to flooding treatment, WAS was higher in intact seeds than in seeds with the seed coat removed regardless of cultivar, indicating that the seed coat absorbs water during the initial stage of inundation (Fig. 2).

There was a significant difference in WAS of intact seeds among the cultivars during the first 30 min of flooding, with that in Peking being significantly slower than that in the other cultivars (Fig. 2). WAS of seeds
without the seed coat was also slower in Peking than that in the other cultivars, suggesting that both the seed coat and the embryo (including the aleurone layer) have a mechanism of reducing WAS in this cultivar. The difference in WAS among the other three cultivars was not conspicuous in the seed either with or without the seed coat, although WAS in Nakasennari tended to be higher than that in the other cultivars during the initial 20 min under flooding.

When seeds were soaked up to half the seed width with either the hilum side or the back side facing down, WAS was generally higher in the hilum side than in the back side, and the difference in WAS between the two sides was significant after 20 min of flooding (P<0.05) irrespective of cultivar (Fig. 3). Among cultivars, WAS from the hilum side was in the following order: Williams>Nakasennari, Enrei>Peking, while WAS from the back side was the lowest in Peking with no significant difference among the other three cultivars (Fig. 3).

In the following experiment using two cultivars (Enrei and Peking), we compared WAS of intact seeds with that of seeds with the hilum or micropyle sealed (Fig. 4). In both cultivars, WAS tended to decrease when the micropyle was sealed, while WAS of seeds with the hilum sealed showed no difference from that of intact seeds. These results reveal that the micropyle acted as a major area for the entry of water into the embryo irrespective of cultivar.

3. Seed structures and water entry into seed

Fig. 5 shows micrograph of the hilum and surrounding tissues soaked in toluidine blue O solution for 25 min. The micropyle and its adjacent inner tissues (radicle and embryo) appeared darker,
indicating that the micropyle allowed water to enter and diffuse rapidly in the seed during the initial stage of flooding. Although the hilum looked very dark, the inner tissues adjacent to the hilum were lighter, suggesting that water accumulated in the hilum thereby blocking water entry into the embryo.

Estimation of percentage of cross section area of the hilum aperture to that of whole seed revealed a cultivar difference; the percentage in Nakasennari was the lowest among the four cultivars, whereas that in Peking was the highest although the difference among the other three cultivars (Peking, Williams and Enrei) was not significant ($P < 0.05$, Fig. 6).

**Discussion**

In this study, we confirmed our previous findings that soybean cultivars tolerant to flooding during germination exhibit reduced WAS of seeds during the initial stage of inundation, and that Peking is the most tolerant among the cultivars tested. However, Williams, previously identified to be tolerant (Tian et al., 2005), was found to be relatively susceptible. In our previous study, we used seeds harvested at several locations and years, therefore, different environmental conditions during seed formation between the previous and present studies might have resulted in the inconsistent evaluation of this cultivar. Because we used seeds harvested from the same field in the same year, we believe that the evaluation of cultivar tolerance in the present study reflects genetic tolerance of cultivars more reliably than in our previous study.

The outer portion of soybean seed consists of several layers: epidermis, hypodermis, inner parenchyma cells, an aleurone layer and remnants of parenchyma cells of endosperm (Esau, 1997; Miller et al., 1999; Carlson and Lersten, 2004). Anatomical characteristics might explain why cultivars differ in WAS, thereby in flooding tolerance. In our previous study, we found that the aleurone layer, a complete single-cell layer lying adjacent to the remnants of parenchyma cells of endosperm (Yaklich et al., 1992), acts as a barrier to water entering embryo tissues (Tian et al., 2005). The same line of evidence was reported by other studies. For example, Matsui et al. (1996), using seeds without a seed coat or the aleurone layer, demonstrated that the aleurone layer rather than the seed coat significantly alleviated the extent of flooding damage in soybean. Nordin (1984) demonstrated that the seed coat acted as a semi-permeable membrane, permitting the entry of water and certain solutes while restricting others. Duke and Kakefuda (1981) examined the seeds of four legumes with or without the seed coat and revealed that embryonic cells of soybean and navy bean seeds without a seed coat ruptured when flooded. A common observation in our previous study is that the aleurone layer blocked rapid entry of water into the embryo irrespective of cultivar. On the other hand, the seed coat appeared to be associated with tolerance only in Peking.

A significant difference was observed in WAS of intact seeds among the cultivars during the first 30 min of flooding, with that of Peking seeds being significantly slower than that of the other cultivars (Fig. 2). When the seed coat was removed, WAS was also slower in Peking than that of the other cultivars, suggesting that both the seed coat and the embryo (including the aleurone layer) have a mechanism of reducing WAS in this cultivar. Therefore, in the present study, we attempted to identify which parts of the seed surface are associated with tolerance or
enhancement of retardation of water entry into the embryo.

A comparison of WAS between the hilum and back sides showed that WAS was significantly higher in the hilum side than in the back side (Fig. 3), similarly to the observation by Hagiwara et al. (2005). In Peking, WAS of either side was consistently lower than that in other cultivars, indicating that there is a mechanism of retarding WAS on both sides of the seed coat in this cultivar.

When the micropyle was sealed, WAS was substantially reduced, showing that the micropyle acted as a major entry point of water in both cultivars (Fig. 4). In contrast, the sealing of the hilum did not markedly reduce WAS compared with that of intact seeds in either tolerant (Peking) or susceptible (Enrei) cultivar. This result suggests that the hilum does not act as a major entry point of water, as we expected based on its large aperture on the seed surface.

Microscopic observation of seeds soaked in toluidine blue O solution demonstrated that water rapidly entered from the micropyle and distributed into adjacent inner tissues. In contrast, water appeared to be accumulated in a space inside the hilum, thereby blocking the rapid entry of water into adjacent inner tissues at 25 min of flooding (Fig. 5).

To examine the role of the hilum in blocking the entry of water, we compared the percentage of the hilum area to that of the whole seed area. As for the result, cultivar ranking of the percentage of the hilum area coincided with that of flooding tolerance; Peking exhibited the highest and Nakasennari the lowest, although the difference between Peking, Williams and Enrei was not significant (P<0.05, Fig. 6). This result raises a hypothesis that the hilum might act as a reservoir during the initial stage of inundation, and blocks the rapid entry of water into the embryo. This hypothesis and precise mechanisms underlying the tolerance of Peking to flooding need verification through additional studies.

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