Role of Abscisic Acid in Flood-Induced Secondary Aerenchyma Formation in Soybean (*Glycine max*) Hypocotyls

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Abstract: Phellogen (cork cambium) usually produces cork tissue, but when flooded it produces secondary aerenchyma, comprising living cells with non-suberized walls in the stems, roots, and root nodules of some Fabaceae. In the cell walls of cork tissues, the plant hormone abscisic acid (ABA), promotes suberin deposition. Thus, ABA may decrease in flooded tissues, where secondary aerenchyma cells are developing. Here, we investigated whether ABA is involved in the formation of aerenchyma in soybean (*Glycine max*) hypocotyls when flooded. Hypocotyls flooded with water produced a large amount of secondary aerenchyma, and were highly porous. On the other hand, application of 1.0 \(\mu\)M ABA suppressed the enlargement of phellogen-derived cells, thereby suppressing subsequent gas space formation, and then almost completely inhibited aerenchyma development. Berberine-aniline blue staining indicated that not only elongated cells in the secondary aerenchyma but also packed cells, which were formed under flooding with ABA, contained no suberized cell walls. Compared to non-flooded plants, the endogenous ABA concentration in the flooded hypocotyls was decreased to 50\% within 24 hr, and the low level was maintained for at least 72 hr. In addition, phellogen developed at 48 hr after flooding and secondary aerenchyma was observed at 72 hr. These results indicate that secondary aerenchyma formation requires a decrease in negative regulator ABA in soybean plants, that is, ABA inhibits elongation of cells derived from phellogen in secondary aerenchyma formation such as internodal cell elongation of floating rice stems.

Key words: Abscisic acid (ABA), Aerenchyma, Flooding, Hormonal regulation, Phellogen, Soybean (*Glycine max*).

A shortage of oxygen due to soil flooding restricts the respiration of roots in many mesophytes, and consequently causes damage to the roots within a few days. Hence, for plants to survive long-term flooding, aerobic respiration must be maintained via oxygen transport to the root tips to enhance internal gas diffusion. For example, wetland plant species survive soil flooding because atmospheric oxygen that enters the shoot is efficiently transported through the aerenchyma to the root tips (Evans, 2003). Therefore, aerenchyma formation is considered an important strategy by which plants can tolerate prolonged flooding. Generally, the term “aerenchyma” is used to describe “cortical aerenchyma,” whereby schizogenous and lysigenous aerenchyma are produced by cell separation and cell death, respectively, in the roots of flood-tolerant plants, such as rice (*Oryza sativa*) (Kawai et al., 1998), *Phragmites australis* (Armstrong et al., 2000), and some *Rumex* species (Laan et al., 1989). These aerenchyma tissues are formed in a primary tissue that is directly derived from the root apical meristem.

Phellogen (cork cambium) is a secondary meristem, and it develops cork tissue that usually has no intercellular space and consists of dead cells with suberized walls. Secondary growth occurs in many dicot plants, especially
woody plants, whereby the cork usually functions as a protective tissue after the collapse of the cortex in the roots and stems. Under flooded conditions, the phellogen also develops a homologous tissue, secondary aerenchyma, in the stems, roots, and root nodules in some plant species. Examples include some Fabaceae plants such as Sesbania aculeata (Scott and Wager, 1888), Sesbania rostrata (Saraswati et al., 1992; Shibata and Daimon, 2003), Neptunia oleracea (Metcalfe, 1931), Melilotus siccus (Teakle et al., 2011; Verbeken et al., 2012), and Viminaria juncea (Walker et al., 1983). In addition, this phenomenon is observed in Onagraceae, e.g., Ludwigia spp. (Schenck, 1890; Angeles, 1992) and Lythraceae, e.g., Decodon spp. and Lythrum spp. (Stevens et al., 1997, 2002; Lempe et al., 2001; Little and Stockey, 2003, 2006). This form of aerenchyma is a white spongy tissue that consists of living cells with non-suberized walls and is referred to as “secondary aerenchyma” (Arber, 1920; Fraser, 1931; Williams and Barber, 1961; Jackson and Armstrong, 1999). Observation of the plant species listed here revealed that the process of secondary aerenchyma formation comprises the following 3 steps: the first step is phellogen development, the second step is aerenchyma cell division from the phellogen, and the third step is aerenchyma cell elongation to develop porous tissues.

In the soybean, which is a major crop used for food and forage worldwide, the secondary aerenchyma is found in the stems, roots, and root nodules when the plant is subject to flooding for a few weeks, and it contributes to the maintenance of root growth and root nodule activity (Shimamura et al., 2002, 2003, 2010; Thomas et al., 2005). However, soybeans are generally susceptible to flooding during its vegetative stages (Sojka, 1985; Griffin and Saxton, 1988; Scott et al., 1989; Linkemer et al., 1998). Therefore, it is important to improve the flooding tolerance of soybeans; this requires an analysis of the potential to improve aerenchyma characteristics, such as oxygen diffusion and porosity, as well as the rate and quantity of formation.

Several studies on the physiological mechanism of cork tissue formation and suberin deposition have been reported. When cork tissue develops in the outer cell layers of tumors in Arabidopsis thaliana that are induced by Agrobacterium tumefaciens, abscisic acid (ABA) promotes suberin deposition in cell walls (Efetova et al., 2007). In addition, ABA induces suberin biosynthesis in the roots of A. thaliana. Ginzberg et al. (2009) indicated the possible integrated action of the transcription factors involved in the response to stress, cell division and differentiation using transcriptome analysis of the heat-stress response in the potato periderm, which is composed of phellogen, suberized phellem cells (the cork) and phelloderm. In contrast, there is limited information on the physiological mechanism of secondary aerenchyma formation. The walls of secondary aerenchymatous cells are not suberized under flooding (Sculthorpe, 1967), which probably enables cell elongation and separation, so we predict that secondary aerenchyma formation requires a decrease in ABA, which is a suberin deposition accelerator, in soybean plants.

The morphological and anatomical changes in root tissues are easily affected by soil moisture conditions, while the hypocotyls above the soil surface are not affected by the conditions. Therefore, the hypocotyls enable detailed comparison between the control and flooded soybean plants in the development of phellogen and secondary aerenchyma. Here, we investigated the mechanisms of secondary aerenchyma development in soybean hypocotyls under flooded conditions and focused on the role of the plant hormone ABA.

Materials and Methods

1. Plant material and experimental design

Soybean (Glycine max (L.) Merril cv. Asoaogari) seeds were sown in 200 mL of silica sand (that had been passed through an 18 – 26 mesh), without chemical or liquid fertilizer, in 400-mL plastic pots (8.5 cm top and 6.5 cm bottom diam. and 10 cm height) with a hole (1 cm diam.) in the bottom (one seed per pot). The plants were grown in a growth cabinet under artificial light (25°C, 14 hr light period and 10 hr dark period, 590 μmol m² s⁻¹ PAR). All experiments were performed when the primary leaves had fully expanded (10 – 11 d after sowing). In non-flooded plants, which were used as the control plants, water was poured into the pot as needed and excess water dripped from the bottom drainage hole. The flooding treatment was started at 1 hr after the onset of the light period in the growth cabinet. For the flooding treatment, the planted pot was put on a new one without a hole in the bottom, but previously each ABA solution was poured into the planted pot and excess solution dripped from the bottom drainage hole not to be diluted by the water held in the sand (ABA treatment only). Then water or ABA solution was poured into the pots, and the plants were maintained under continuously flooded conditions, in which the level of water or ABA solution was maintained at 3 cm above the sand surface. Because of evaporation from the pots, the pots were watered daily to maintain the level of water or ABA solution. The solutions were renewed after 3 d.

2. Measurement of porosity

The plants were grown with or without flooding in solutions containing 0 – 1.0 μM ABA. The hypocotyl segments from 0.5 to 2.5 cm above the sand surface were excised after 6 d of flooding. Immediately after sampling, the porosities of the segments were measured using a modified version of the pycnometer method used by Jensen et al. (1969). This method quantifies porosity as the percentage of gas space in the hypocotyl, thus indicating
the extent of secondary aerenchyma development. Internal gas within the tissues was removed by evacuation under low pressure in deionized water, rather than by grinding the plant material in liquid N$_2$ to degas it (Sojka, 1988; Bacanamwo and Purcell, 1999; Fan et al., 2003). Hypocotyl porosity was calculated as follows:

Porosity ($\%$, v/v) = 100 × [(FB – FA)/(FW + TW – FA)]

where FB is the weight of the pycnometer flask and water with hypocotyl material after vacuum infiltration, FA is the weight of the pycnometer flask and water with hypocotyl material before vacuum infiltration, FW is the weight of the pycnometer flask when full of water without hypocotyl material, and TW is the fresh weight of the hypocotyl material.

3. Effect of exogenous ABA on secondary aerenchyma formation

The plants were flooded with 0 – 1.0 $\mu$M ABA solutions. After 6 d of flooding, the surface of each hypocotyl at 1.5 cm above the sand surface was observed with a stereoscopic microscope (MZ16, Leica Microsystems, Wetzlar, Germany). Transverse sections (100 – 300 $\mu$m in thickness) of fresh hypocotyls at the same position were cut using a plant microtome (MTH-1, Nippon Medical & Chemical Instruments Co. Ltd., Osaka, Japan). Some cross-sections were stained with 0.05% (w/v) toluidine blue O and then viewed using a light microscope (Eclipse 80i, Nikon Co. Ltd., Tokyo, Japan). To detect suberin in the cell walls, some sections were stained for 1 hr with 0.1% (w/v) berberine hemisulphate and subsequently for 30 min with 0.5% (w/v) aniline blue and finally for several minutes with 0.1% (w/v) FeCl$_3$ in 50% (v/v) glycerine at room temperature (Brundrett et al., 1988). The sections were observed using a fluorescence microscope (Axioskop2 plus, Zeiss, Oberkochen, Germany) with an ultraviolet filter set (excitation filter BP 365, dichroic mirror FT 395, and barrier filter LP 397; Zeiss, Oberkochen, Germany).

4. Measurement of endogenous ABA concentrations in the hypocotyls

The plants were flooded with water without ABA or grown without flooding. The hypocotyl sections were excised 1 to 2 cm above the sand surface at 0, 24, 48 and 72 hr after flooding. Some transverse cross sections of the flooded hypocotyls were stained with 0.05% (w/v) toluidine blue O, and viewed using a light microscope (see above).

The ABA contents were quantified according to the method used by Gonai et al. (2004). The frozen samples (1 g air-dried weight) were disrupted and homogenized in 80% methanol containing 0.1 M acetic acid, followed by the addition of [3',5',5',7',7',7'-H$_6$]ABA as an internal standard. The extracts were purified using the LC-18 column (Supelclean, 1 mL, Supelco, Bellefonte, PA, USA), LC-NH$_2$ column (Supelclean, 1 mL, Supelco, Bellefonte, PA, USA), and high-performance liquid chromatography (HPLC) (LC-10; Shimadzu, Kyoto, Japan) equipped with an ODS-column (Mightysil RP-18 GP, 4.6-mm i.d. × 250 mm, Kanto Chemical Inc., Tokyo, Japan). The ABA fractions separated by HPLC were subjected to methylation with diazomethane. The methylated ABA was identified and quantified using full-scanning and selected ion monitoring GC-MS (GC, HP-6890, Hewlett Packard; MS, HP-5973 equipped with a DB-1 column; Hewlett Packard, Palo Alto, CA, USA). The amount of ABA in the hypocotyls was determined from the ratio of peak area of base ions for methylated ABA to that for methylated [3'H$_6$]ABA (m/z 190 and 194, respectively).

Results

1. Effects of exogenous ABA on porosity and secondary aerenchyma formation

Flooding with water for 6 d induced secondary aerenchyma development in the hypocotyls, with a 3-fold increase in porosity, compared to that in the controls (Fig. 1). In the control hypocotyls, phellogen development was minimal, and secondary aerenchyma was not observed in any of the cross-sections (Fig. 2A). Thus, the epidermis and cortex were clearly observed without collapse, and lenticels did not develop on the hypocotyl surfaces (Fig. 2F). In contrast, the fully developed phellogen produced aerenchyma with loose and elongated cells in the hypocotyls subjected to flooding (Fig. 2B). The aerenchyma developed rapidly in a radial direction, and...
Fig. 2. Transverse sections and surfaces of soybean hypocotyls at 6 d after flooding.

The sections were excised from hypocotyls at 1.5 cm above the sand surface, stained with toluidine blue O, and then photographed under a light microscope. The surfaces were photographed under a stereoscopic microscope. Five plants were used in each treatment. The photographs are typical sections and surfaces from soybean plants grown without flooding (control; A and F) and under flooding with water (B and G), 0.1 μM ABA (C), 0.5 μM ABA (D), and 1.0 μM ABA (E and H). ae: aerenchyma, co: cortex, hl: hypertrophic lenticels, pc: packed cell, pf: phloem fiber, ph: phellogen, xy: xylem. Scale bar = 0.25 mm (black) or 3 mm (white).

Fig. 3. Difference between the sites of suberin deposition and tightly packed cells derived from phellogen in the hypocotyls.

Transverse sections were prepared from the hypocotyls of soybean plants flooded with water (A and B) or 1.0 μM ABA (C and D) for 6 d and stained with berberine-aniline blue. Five plants were used in each treatment. The photographs show typical sections observed under a light microscope (A and C) or a fluorescence microscope (B and D). ae: aerenchyma, co: cortex, pc: packed cell, pf: phloem fiber, ph: phellogen, xy: xylem. Scale bar = 0.15 mm.

Fig. 5. Transverse sections of soybean hypocotyls at 24, 48 and 72 hr after flooding.

The sections were excised from hypocotyls at 1.5 cm above the sand surface, stained with toluidine blue O, and then photographed under a light microscope. Three plants were used in each stage. The photographs are typical sections from soybean plants grown under flooding with water at 24 (A), 48 (B) and 72 hr (C) after the onset of flooding treatment. Single arrowhead indicates elongated aerenchymatous cell (C). ae: aerenchyma, co: cortex, pf: phloem fiber, ph: phellogen, xy: xylem. Scale bar = 0.25 mm.
the epidermis and cortex were pushed out and collapsed, so hypertrophic lenticels were observed (Fig. 2G). Although no significant difference was observed in hypocotyl porosity or in the development of aerenchyma between hypocotyls flooded with water and those flooded with 0.1 μM ABA (Figs. 1, 2B and 2C), 0.5 μM ABA suppressed aerenchyma development and decreased the porosity of the hypocotyl sections (Figs. 1 and 2D). Although 1.0 μM ABA almost completely inhibited the formation of secondary aerenchyma and decreased the porosity of the hypocotyl sections to the same level as observed in the controls (Figs. 1 and 2E), hypertrophic lenticels developed on the hypocotyl surfaces (Fig. 2H).

Although phellogen development and subsequent cell division were noticeable in the sections of hypocotyls flooded with 1.0 μM ABA, the proliferated cells did not elongate but remained tightly packed, and no aerenchyma cells were observed (Fig. 2E). This result indicated that ABA suppresses secondary aerenchyma development by inhibiting cell elongation rather than inhibiting phellogen differentiation. We could not detect suberized walls in the aerenchymatous cells (Figs. 3A and B) or the packed cells (Figs. 3C and D) by berberine-aniline blue staining. However, xylem and phloem fibers were clearly stained in the same section, indicating that the aerenchymatous cells and the packed cells were different from the cork cells.

2. Endogenous ABA concentrations in the flooded hypocotyls and secondary aerenchyma formation

The endogenous ABA levels were quantified in the hypocotyls for 72 hr after flooding (Fig. 4). At 24 hr after flooding, the ABA concentration quickly decreased from 38.3 to 18.1 ng gFW⁻¹, and the level was nearly half of that in the controls. At 72 hr after flooding, the concentration further decreased to 11.8 ng gFW⁻¹. The range of ABA concentration during the experiment was 32 – 40 ng gFW⁻¹ without flooding, whereas flooding increased it to 12 – 18 ng gFW⁻¹. Significant differences in ABA concentration between the control and flooded conditions were observed from 24 to at least 72 hr after the start of treatment.

At the same time, these hypocotyls showed rapid morphological changes within 72 hr after flooding. Although well-developed phellogen was not observed at 24 hr after flooding, the cortical tissues were somewhat loose (Fig. 5A). After 48 hr, a few phellogen layers completely girdled the stele (Fig. 5B). Some cells derived from phellogen began to elongate, and the loosely packed cells constructed secondary aerenchymatous tissues after 72 hr (Fig. 5C).

Discussion

Since flooding causes major stress in upland crops, it is important to improve their flood tolerance. Aerenchyma formation is considered the most suitable morphological strategy to address flooding stress in many plant species, because aerenchyma provide an internal pathway for oxygen transfer to the root tips (Evans, 2003). Lysigenous aerenchyma is formed through programmed cell death in the root cortex under hypoxic conditions, and it has been shown that lysigenous aerenchyma formation is triggered by ethylene in maize (*Zea mays*) (Drew et al., 1979, 2000) and rice (*O. sativa*) (Shiono et al., 2008). Although flooded soybeans develop phellogen-derived secondary aerenchyma, which also functions as an oxygen transfer pathway to the roots (Shimamura et al., 2002, 2003, 2010; Thomas et al., 2005), there is limited knowledge about the physiological mechanism(s) of secondary aerenchyma formation. In this study, we referred to the information on the development of a homologous cork tissue in which ABA plays an important role, as it does in the induction of suberin biosynthesis and deposition (Efetova et al., 2007). Since living cells with non-suberized walls, which are derived from phellogen, are necessary for secondary aerenchyma formation, we examined the ABA concentration that would prevent suberin deposition in aerenchyma-developing portions and studied the involvement of ABA in the formation of secondary aerenchyma.

We found that ABA is involved in secondary aerenchyma formation in soybean hypocotyls. Exogenous ABA (1.0 μM) suppressed neither phellogen formation (first step) nor the division of phellogen-derived cells (second step), but it did inhibit the elongation of these cells (third step) in the hypocotyls of the flooded soybean plants (Figs. 1 and 2). In addition, endogenous ABA concentrations decreased within 24 hr after flooding (Fig. 4). Furthermore,
rapid morphological responses in the hypocotyls were observed within 72 hr after flooding (Fig. 5). The phellogen developed at 48 hr and produced secondary aerenchymatous cells at 72 hr after flooding. Especially, elongation of some phellogen-derived cells was observed and ABA concentration in flooded hypocotyls was decreased to 30% of control at 72 hr. These initial responses of phellogen and secondary aerenchyma formations were similar to the results reported by Shimamura et al. (2003), and cell elongation was accompanied by a rapid decrease in endogenous ABA concentration. It is well known that deepwater rice can lengthen the submerged shoot by cell elongation and that this response is regulated by ABA (Hoffmann-Benning and Kende, 1992). Namely, the reduction in endogenous ABA levels is important to trigger cell elongation in submerged shoot tissues (Fukao and Bailey-Serres, 2008). Our results indicate that a decrease in endogenous ABA concentration might be required for the elongation of phellogen-derived cells.

In potato tubers, ABA promotes the biosynthesis of suberin (Cottle and Kollattukudy, 1982) as a signal compound for wound-healing (Kumar et al., 2010). In soybean roots, cork tissue is also produced from phellogen as a protective tissue instead of collapsed cortex under non-flooded conditions (Lersten and Carlson, 1987). Hence, we assumed that suberization in the development of cork tissue is also regulated by ABA in soybeans. The decrease in the ABA level after flooding probably suppresses suberin biosynthesis and might permit the formation of aerenchyma cells without suberized walls. However, exogenous ABA (1.0 μM) did not induce suberin deposition in the walls of packed aerenchyma cells (Figs. 3C and D). These findings indicate that flood-induced changes in factors other than ABA may be involved in the suppression of suberin biosynthesis in these cells.

Flooding influences not only aerenchyma formation but also the root system. In soybean plants, flooded stems and hypocotyls easily develop new adventitious roots (Bacanamwo and Purcell, 1999; Shimamura et al., 2003; Thomas et al., 2005; Henshaw et al., 2007), and the exogenous auxins promote adventitious rooting from hypocotyls in cuttings from 7-day-old plants without root systems (Chen et al., 2002; Chou et al., 2010). These findings suggest that auxin accumulates in the flooded hypocotyls and stems of soybeans. Although we focused on the involvement of ABA in secondary aerenchyma formation in flooded soybeans, active cell division and elongation are necessary for secondary aerenchyma formation (Scott and Wagner, 1888; Schenck, 1890; Metcalfe, 1931; Shiba and Daimon, 2003; Shimamura et al., 2003), so auxin might also play an important role in this process.

In conclusion, the rapid decrease in the endogenous ABA concentration in the hypocotyls of flooded soybean plants and the inhibition of aerenchymatous cell development by exogenous ABA indicate that secondary aerenchyma formation requires a decrease in the level of the negative regulator ABA. Exogenous ABA did not enhance suberin deposition in the walls of aerenchymatous cells (packed cells) derived from phellogen under flooded conditions. Further investigation of the mechanisms controlling phellogen development, aerenchyma cell division, and cell elongation is necessary to better understand and improve the flooding tolerance of soybeans.

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