Triterpenoids in aerenchymatous phellem contribute to internal root aeration and waterlogging adaptability in soybean

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Mikio Nakazono
Abstract

Aerenchymatous phellem (AP) is important for internal aeration and adaptation to waterlogging in plants. Herein, the extensive accumulation of triterpenoids such as lupeol and betulinic acid was identified in AP. However, the biological and physiological roles of these triterpenoids in plants are largely unknown. Lupeol is converted from 2,3-oxidosqualene by lupeol synthase (LUS) and oxidized to betulinic acid. Functional analysis of LUS genes in soybean revealed that GmLUS1 is crucial for triterpenoid biosynthesis in AP. Lupeol and betulinic acid were found to be the major components of epicuticular wax on the surface of AP cells, and they contributed to tissue hydrophobicity and oxygen transport to roots. Additionally, the lus1 mutant produced a shallow root system due to less oxygen transport via AP under waterlogged conditions. In conclusion, triterpenoid accumulation in AP aids internal aeration and root development for adaptation to waterlogging.

Introduction

Waterlogging is one of the most significant environmental pressures on highland crops. Under waterlogged conditions, soil pores are filled with water, and plant roots and microbes consume dissolved oxygen through respiration. Moreover, oxygen diffusion in water is 10,000 times slower than in air. Therefore, roots experience oxygen deprivation in waterlogged environments\textsuperscript{1,2}. Wetland plants feature a variety of adaptive characteristics that allow them to escape from being affected by waterlogging\textsuperscript{3,4}. Aerenchyma is one of the most important tissues that creates interior air gaps for gaseous transport to sustain internal aeration\textsuperscript{3,5}. It is largely classified into primary and secondary aerenchyma\textsuperscript{6}. The formation of primary aerenchyma occurs in primary tissues such as root and shoot cortical tissue. However, secondary aerenchyma is newly differentiated from secondary meristem (i.e., phellogen or cork cambium)\textsuperscript{7}. Secondary aerenchyma is classified into the aerenchymatous phellem (AP), differentiated inward from phellogen, and the porous secondary cortex, differentiated outward from phellogen\textsuperscript{7}. AP is a white, spongy, and highly porous tissue, and transports oxygen to roots under waterlogged conditions\textsuperscript{8,9}. Soybean (\textit{Glycine max}) develops AP in hypocotyl, adventitious roots, tap root, and nodules under waterlogged soil conditions\textsuperscript{8,10,11}. In soybean, the formation of phellogen and AP are induced in response to waterlogging conditions\textsuperscript{8,10,11}, possibly triggered by a reduction in abscisic acid concentration\textsuperscript{12}. Under waterlogged conditions, the formation of phellogen and AP necessitates a sugar supply from photosynthesis as an energy source\textsuperscript{13}. There are various physiological and morphological studies on AP, but there is limited molecular evidence on their structure and functions.

Triterpenoids are a class of plant metabolites derived from 2,3-oxidosqualene, a 30-carbon compound consisting of six isoprene units. In the triterpenoid biosynthesis pathway, 2,3-oxidosqualene is mainly converted to \(\alpha\)-amyrin, \(\beta\)-amyrin, and lupeol, by \(\alpha\)-amyrin synthase (\(\alpha\)AS), \(\beta\)-amyrin synthase (\(\beta\)AS), and lupeol synthase (LUS), respectively, which are oxidosqualene cyclases (OSCs). These compounds are catalyzed by cytochrome P450 monooxygenases (P450s), producing a wide variety of oxygenated triterpenoids\textsuperscript{14,15}. Several legume plants reportedly produce abundant glycosylated triterpenoids, called
Saponins, which are catalyzed by UDP-dependent glycosyltransferases (UGTs) and cellulose synthase superfamily-derived glycosyltransferases (CSyGTs)\textsuperscript{15,16}. *Glycine max*, *Medicago* spp., *Glycyrrhiza* spp., and *Lotus japonicus*, in particular, accumulate soyasaponins\textsuperscript{17–19}. Different organs such as leaves, stems, and roots of *Lotus japonicus* reportedly accumulate different types of glycosylated and non-glycosylated triterpenoids\textsuperscript{20}. The roles of these triterpenoids and their glycosides have been extensively studied in animal cells as some molecules exhibit anticancer, antibacterial, antifungal, or anti-inflammatory effects\textsuperscript{21}. It has been suggested that certain triterpenoids contribute to the plant's defense mechanism against pathogens\textsuperscript{22,23}, whereas β-amyrin or lupeol has an adverse effect on cell division or elongation in plants\textsuperscript{24,25}. However, their physiological functions in plants remain unclear.

AP production in soybean involves four key phases (Extended Data Fig. 1): (1) Phellogen cells are differentiated from cortical cells; (2) Phellogen cells undergo repeated cell division and create multiple cell layers; (3) AP cells are differentiated from the outermost phellogen cell, and (4) AP cells are elongated to form the porous tissue for internal aeration. To understand the development of AP cells, we focused on tissue-specific microarray analysis by laser capture microdissection (LCM). As a result, we identified that the triterpenoid biosynthesis pathway is activated in AP and that lupeol and betulinic acid are abundant in AP cells. In order to uncover the biological and physiological functions of triterpenoids in AP, we obtained a mutant of the *lupeol synthase 1* (*LUS1*) gene that lacked lupeol and betulinic acid accumulation potential in AP. We examined the surface structure of AP cells, along with their porosity and activity. In addition, oxygen transfer to roots and root development in the *lus1* mutant under waterlogged soil conditions were investigated, and the physiological function of triterpenoids in AP was explored.

**Results**

**Upregulation of triterpenoid biosynthesis and its accumulation in AP**

In response to the waterlogging stress, soybean formed phellogon and AP (Fig. 1a and Extended Data Fig. 1). Oxygen transport was visualized by methylene blue staining (Fig. 1b). The blue color was almost not visible in soybean growing under drained soil conditions because AP was not produced, indicating that oxygen was not transferred from the shoot to the roots. When soybean was grown under waterlogged conditions and developed AP partially above the water surface, the roots were colored blue. However, when the highly developed AP in the hypocotyl was entirely submerged, roots did not show the blue color (Fig. 1b). In addition to internal aeration, these data indicated that the AP serves as an oxygen uptake entrance point. These outcomes were consistent with a prior study involving oxygen electrodes\textsuperscript{8}. As we previously described, AP has a physiological role in internal aeration in several plant species\textsuperscript{8,9,40}. However, the molecular evidence associated with the AP has not been reported; hence, we performed tissue-specific microarray analysis. Because AP was adjacent to the phellogen and cortex, LCM was used to separate each tissue (Fig. 1c). AP and phellogen gene expression was compared with the cortex using microarrays and RNA extracted from each tissue. In phellogen, 1,760 and 1,366 genes were significantly
upregulated and downregulated, respectively ($p < 0.05$, fold change (FC) $\geq 2$ and FC $\leq 0.5$, Fig. 1d, Supplementary Tables 1–2). In AP, 284 and 468 genes were upregulated and downregulated, respectively ($p < 0.05$, FC $\geq 2$ and FC $\leq 0.5$, Fig. 1d, Supplementary Tables 3–4).

To understand the mechanism that occurred in AP cells, we focused on the 207 genes that were uniquely upregulated in AP cells (Fig. 1d, Supplementary Table 5), and performed Gene Ontology (GO) enrichment analysis. As a result, 19 GO terms with enrichment false discovery rate (FDR) < 0.01 were selected (Extended Data Fig. 2). Isopentenyl diphosphate, acetyl-CoA, sterol biosynthetic, and metabolic pathway-related genes were enriched (Extended Data Fig. 2), and several mevalonates, sterol biosynthesis, triterpene biosynthesis pathway-related genes were upregulated in AP cells (Fig. 2a).

Three different types of OSCs catalyze 2,3-oxidosqualene, but only $LUS$ genes were upregulated in AP cells (Fig. 2a). Accumulation of lupeol, betulin, and betulinic acid was observed in the soybean hypocotyls grown in waterlogged soils, but not in those grown in drained soil (Fig. 2b, Table 1). The amount of lupeol, betulin, and betulinic acid in waterlogged soil-grown hypocotyl was $74.8 \pm 14.0$, $1.8 \pm 0.2$, $171.2 \pm 27.2$ mg/g dry weight (DW), respectively (Table 1).

<table>
<thead>
<tr>
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<th>Lupeol</th>
<th>Betulin</th>
<th>Betulinic acid</th>
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<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>Waterlogged soil conditions</td>
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<td>$1.8 \pm 0.2$</td>
<td>$171.2 \pm 27.2$</td>
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<td>EnT-6693 (WT)*</td>
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<td>$2.2 \pm 0.2$</td>
<td>$234.7 \pm 18.1$</td>
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<tr>
<td>EnT-6693 ($lus1$)*</td>
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<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>CRP_WT*</td>
<td>$55.5 \pm 7.3$</td>
<td>$1.7 \pm 0.2$</td>
<td>$149.9 \pm 18.2$</td>
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<tr>
<td>CRP_$lus1*$</td>
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Values represent mean $\pm$ S.E. ($n = 3$)

*These plants were grown under waterlogged soil conditions.

N.D.: not detected.

**Lupeol synthase 1 (GmLUS1) is a key gene for triterpenoid accumulation in AP cells**
Soybean had two genes encoding the enzyme lupeol synthase (i.e., \textit{GmLUS1} and \textit{GmLUS2}, Extended Data Fig. 3). Both \textit{GmLUS1} and \textit{GmLUS2} exhibited catalytic activity for lupeol synthesis (Extended Data Fig. 4). However, only the \textit{GmLUS1} gene showed elevated expression in response to waterlogging stress (Extended Data Fig. 5). In addition, \textit{GmLUS1} was only expressed in AP and some cortical cells (Fig. 2c and d). We identified three mutants of the lupeol synthase gene to identify the gene responsible for triterpenoid accumulation in AP cells. EnT-6693 (the line name in mutant population) was the mutant derived from ethyl methanesulfonate (EMS) treatment\textsuperscript{27}. EnT-6693 has a heterogeneous base substitution from C to T in the 17th exon (Fig. 3a). Therefore, we obtained wild-type (WT) and homozygous mutant offspring, which we designated EnT-6693 (WT) and EnT-6693 (\textit{lus1}), respectively. In addition, we produced a single mutant of \textit{GmLUS1} (named CRP\_lus1) and a double mutant of \textit{GmLUS1} and \textit{GmLUS2} (named CRP\_lus1/lus2). We could not identify lupeol, betulin, and betulinic acid accumulation in the hypocotyl of these mutants in response to waterlogging (Extended Data Fig. 6, Table 1), indicating that \textit{GmLUS1} is a critical gene for the accumulation of lupeol, betulin, and betulinic acid in AP cells.

\textbf{\textit{GmLUS1} contributed to the epicuticular wax in AP cells and porosity in AP}

It was reported that lupeol and betulinic acid are major components of epicuticular wax in caster bean and white birch, respectively\textsuperscript{28,29}. To evaluate whether these triterpenoids are also involved in the epicuticular wax of soybean AP cells, we observed the cell surface of AP using cryo-scanning electron microscopy (SEM). Crystal structures were observed in EnT-6693(WT) and CRP\_WT, but not in EnT-6693(\textit{lus1}), CRP\_lus1, CRP\_lus1/lus2 (Fig. 3b). This result was consistent with the negative accumulation of lupeol and betulinic acid in these mutants (Extended Data Fig. 6, Table 1). These results suggested that these crystals were the structures of epicuticular wax derived from lupeol or betulinic acid. AP maintains air in the intercellular space even when submerged in water for internal aeration. Lupeol and betulinic acid are hydrophobic molecules; therefore, these compounds may play a role in water resistance. We examined the tissue porosity of the well-developed hypocotyl AP. It was observed that the tissue porosity of EnT-6693 (\textit{lus1}) decreased by 50% and CRP\_lus1 and CRP\_lus1/lus2 by 20% (Fig. 3c). Tissue porosity may have decreased because the intercellular space was filled with water and/or the volume of intercellular space was decreased. We used an X-ray micro computed tomography (CT) scanner on soybean hypocotyls to determine the volume of intercellular space in AP (Fig. 3d and Extended Data Fig. 7). Because AP is distinguished from phellogen, the intercellular space of AP steadily rises during the development process, and such volumes differ between “inside”, which is near the phellogen, and “outside”, which is far from the phellogen. As a result, we compared the AP intercellular space on the inside and exterior of CRP\_WT and CRP\_lus1 (Fig. 3d and Extended Data Fig. 7). The percentages of intercellular space of “inside” AP to the volume of the whole segment were 56.3 ± 5.3% in CRP\_WT and 51.0 ± 8.0% in CRP\_lus1 and those of “outside” were 84.7 ± 7.7% in CRP\_WT and 83.5 ± 2.9% in CRP\_lus1 (Fig. 3d). The volume of intercellular space was greater in “outside” than in “inside”, but there was no significant difference between CRP\_WT and CRP\_lus1 (Fig. 3d).
Triterpenoid accumulation leads to reduced cell activity in AP

The *lus* mutant lacked epicuticular wax on the surface of AP cells (Fig. 3b) and had lower porosity than the WT (Fig. 3c). Porosity shows the volume of air in AP, and low porosity leads to a low amount of oxygen in AP. Therefore, we hypothesized that cell activities would be reduced in *lus* mutants due to oxygen deficiency because AP cells comprise live cells. 2,3,5-triphenyl tetrazolium chloride (TTC) staining revealed that the mitochondrial activities of EnT-6693 (*lus1*), CRP_1lus1, and CRP_1lus1/lus2 were considerably higher than those of EnT-6693 (WT) and CRP_WT, respectively (Fig. 3e, f and Extended Data Fig. 8b). AP cells in the WT showed a significant red hue in TTC staining at 5 and 7 days following waterlogging treatment but were less stained at 9 and 14 days (Extended Data Fig. 8a). Even 9 and 14 days after treatment, phellogen and AP cells showed a significant red hue in TTC staining immediately after differentiating from phellogen (Extended Data Fig. 8a and b). These findings suggested that triterpene buildup reduces mitochondrial activity in AP cells of the WT but not in *lus* mutants.

Lack of triterpenoid accumulation in AP affected oxygen transport and the pattern of root system development

The *lus* mutants exhibited significant levels of mitochondrial activity in AP cells, indicating that they use more oxygen than the WT. In addition, AP in *lus* mutants had a lower porosity than that in WT plants. We expected that oxygen delivery to the root would be restricted in *lus* mutants as a result. In fact, radial oxygen loss in the root tip of adventitious roots that sprouted from AP was significantly decreased in CRP_1lus1 plants (Fig. 4a). Although internal aeration from the shoot to root was limited, no significant differences in plant height, number of nodes, shoot DW, root DW, and total root length were observed between CRP_WT and CRP_1lus1 under waterlogged soil conditions (Supplementary Table 6). However, we found that many surface roots, which were present between the water surface and soil surface, emerged in CRP_1lus1 (Fig. 4b). In fact, the ratio of root length and DW in Region (above the soil surface) was increased in CRP_1lus1. However, we discovered that many surface roots between the water surface and the soil surface had emerged in CRP_1lus1 (Fig. 4b). CRP_1lus1 had an increased the ratio of root to total root (length and DW) in region I (above the soil surface). However, these ratios in region II (0–40 mm below the soil surface) were reduced in CRP_1lus1 plants (Fig. 4c and d).

Discussion

Accumulation of lupeol, betulin, and betulinic acid in AP cells due to GmLUS1

In the soybean, two putative *LUS* genes (*GmLUS1* and *GmLUS2*) were identified with the catalytic activity to convert 2,3-oxidosqualene to lupeol (Extended Data Figs. 3 and 4). However, only *GmLUS1* expression was observed to have been induced and mainly expressed in AP in response to waterlogging (Extended
Data Fig. 5, Fig. 2c and d). In addition, triterpenoid accumulation in the hypocotyl was observed not only in a double mutant of *GmLUS1* and *GmLUS2* but also in a single mutant of *GmLUS1* under waterlogged soil conditions (Table 1 and Extended Data Fig. 6). Triterpenoids were measured using hypocotyl segments; however, it was believed that lupeol, betulin, and betulinic acid were primarily accumulated in AP since *GmLUS1* was significantly expressed in AP cells and slightly expressed in cortical cells (Fig. 2c and d). These findings suggest that *GmLUS1* is a crucial gene for triterpenoid accumulation in AP cells.

**Physiological and biological functions of triterpenoids in soybean AP**

Several triterpenoids are involved in pathogen defense, cell division, or cell elongation in plants$^{22-24}$. In *Lotus japonicus*, nodule formation is influenced by the silencing of the *LUS* gene$^{25}$. In contrast, the physiological and biological activities of triterpenoids mostly remain unknown. In this study, we demonstrated that epicuticular wax was present on the surface of AP cells but absent in *lus* mutants, which lacked the accumulation of lupeol, betulin, and betulinic acid in AP (Fig. 3b, Table 1, and Extended Data Fig. 6). Lupeol is a significant constituent of epicuticular wax in the stem and hypocotyl of *Ricinus communis*$^{28}$. Similarly, our data indicated that lupeol and betulinic acid were prominent components of the epicuticular wax of AP cells, as the amount of betulin was drastically lower than that of lupeol and betulinic acid (Table 1). Hypocotyl surfaces split during the formation of phellogen and AP, and lenticels were formed. It is thought that water could quickly enter the intercellular space of well-developed AP because AP is exposed to the water from lenticels under waterlogged soil conditions. However, AP is hydrophobic and retains the porous tissue even when submerged in water, and it was unclear how tissue hydrophobicity was retained. The porosity of the *lus1* mutant was significantly lower than that of the WT (Fig. 3c), but there was no significant change in the volume of intercellular space between the *lus1* mutant and the WT (Fig. 3d). These findings indicated that water was partially immersed in the intercellular space of the *lus1* mutant, whereas triterpenoids contributed to tissue hydrophobicity and prevented water penetration into the AP as epicuticular wax.

Initially, we hypothesized that cell activities were diminished in the *lus1* mutant due to the low oxygen content in AP cells resulting from their low porosity. However, TTC labeling of AP cells from *lus1* and *lus1/lus2* mutants revealed higher cell activity than expected (Fig. 3e and f). TTC is a proton acceptor molecule generated from dehydrogenases in living cells that functions as an indicator of mitochondrial enzyme activity. The intensity of TTC staining is correlated to the number of functional mitochondria$^{29}$. High mitochondrial activity in the *lus1* mutant would cause AP cells to lose oxygen through respiration. Consequently, the amount of oxygen delivered to adventitious root tips via AP was decreased in the *lus1* mutant (Fig. 4a). The oxygen transport ability of the *lus1* mutant was less than that of the WT, but the *lus1* mutant adapted to waterlogged soil conditions by increasing surface roots and decreasing roots below the surface (Fig. 4b-d). There is no significant difference in total root growth between the *lus1* mutant and the WT (Supplementary Table 6), but the increase in surface roots and the decrease in roots under the soil would be detrimental to the waterlogging adaptation due to the risk of lodging associated with this shallow root system.
The present study demonstrated that the triterpenoids accumulated in the AP cells were a major component of the epicuticular wax, contributing to the maintenance of air space underwater and preventing the loss of oxygen through the reduction of cell activity. In addition, these actions in AP cells result in the efficient delivery of oxygen to roots via the AP and the development of the root system in soil submerged in water. However, triterpenoid accumulation in AP cells is not completely understood. Both lupeol and betulinic acid were found at high concentrations in AP-developed hypocotyls (Fig. 2b and Table 1), but whether either or both triterpenoids are essential is unknown. It has been reported that CYP716A12 converts lupeol to betulin and betulinic acid. The expression of the gene encoding CYP716A was also upregulated in AP (Fig. 2a). By analyzing the functionality of the CYP716A gene, it would be evident which triterpenoids are crucial for the AP. In addition, it was recognized that triterpenoids function through glycosylation; however, it is uncertain whether or not lupeol and betulinic acid are glycosylated in AP. Furthermore, it is primarily unknown how triterpenoids reduced the activity of AP cells. Lupeol and betulinic acid are well-studied anticancer agents in animal cells. These compounds activate the mitochondria-dependent apoptotic pathway in cancer cells while negatively affecting normal cells. Further studies should be required to answer these questions and reveal the triterpenoid functions in AP.

**Online Methods**

**Plant materials and growth conditions**

Soybean (*Glycine max* (L.) Merr. ‘Enrei’) seeds used in this study were collected from field-grown plants. The mutant line EnT-6693 was isolated by amplicon sequencing from an EMS-treated mutant population as previously described using primer pair LUS-Gln729stop-053 (Supplementary Table 7). We identified EnT-6693(WT) and EnT-6693 (lus1) from the progeny of EnT-6693 because the mutation of *GmLUS1* in EnT-6693 was a heterozygote. Genome editing of *GmLUS1* and *GmLUS2* in soybean (*G. max* (L.) Merr. ‘Williams 82’) using the CRISPR/Cas9 system was performed by BIOGLE GeneTech (http://www.biogle.cn/, Hangzhou, China). Target sequences are shown in Extended Data Fig. 9; T1 seeds were purchased from BIOGLE GeneTech. Before the start of the experiments, we extracted DNA from each individual plant and checked the genotype. PCR was performed using PrimeSTAR® MAX DNA polymerase (Takara Bio, Shiga, Japan) and specific primers are listed in Supplementary Table 7. Sequencing of amplified DNA was performed by Eurofins Genomics (https://eurofinsgenomics.jp/jp/home.aspx, Tokyo, Japan).

Soybean seedlings were grown following a previously established method. Plastic pots (300 mL) were filled with 200 mL silica sand (18–26 mesh). A 2 cm deep hole was dug in each pot, into which a single soybean seed was sown and covered with a small amount of the silica sand. Seedlings were grown at 25°C under 14 h light/10 h dark conditions for approximately 10 d (light conditions [photosynthetically active radiation, PAR]: 180–200 µmol/m²/s) until unifoliate leaves were fully expanded (approximately 10 days old). The soil was waterlogged by raising the deionized water (DI) levels, which were maintained...
at 3 cm above the soil surface for 0 d, 1 d, 3 d, 5 d, 7 d, 9 d, and 14 d. Hypocotyls from 0.5–1.5 cm above the soil surface were harvested for further analysis.

For radial oxygen loss (ROL) measurements, seeds were sterilized using CruiserMaxx (Syngenta Japan, Tokyo, Japan) and imbibed in vermiculite by wetting with water for 3 days. Seedlings were then transferred to an aerated 25% nutrient solution and grown hydroponically with aeration for 4 days (14 h light, 28 °C/10 h dark, 17 °C). The nutrient solution at full concentration consisted of 0.5 mM KH$_2$PO$_4$, 3.0 mM KNO$_3$, 4.0 mM Ca(NO$_3$)$_2$ mM, 1.0 MgSO$_4$ mM, 37.5 µM FeNa$_3$EDTA, 23.0 µM H$_3$BO$_3$, 4.5 µM MnCl$_2$, 4.0 µM ZnSO$_4$, 1.5 µM CuSO$_4$, and 0.05 µM MoO$_3$. The pH of the solution was buffered with 2.5 mM MES (2-(N-morpholino) ethanesulfonic acid) adjusted with KOH to a pH of 6.3. One week after germination, the seedlings were transplanted into pots containing 5 L of stagnant deoxygenated nutrient solution, which contained the same full-strength nutrient solution as described above and 0.1% (w/v) agar; this solution was deoxygenated by bubbling with nitrogen gas$^{34}$. The seedlings were grown under a stagnant deoxygenated nutrient solution for 14 days, and the solution was replenished every 7 days.

**Methylene blue staining**

After the unifoliate leaves were fully expanded, the seedlings were grown under drained and waterlogged soil conditions for 7 days. The seedlings were dug out from the soil and immersed in methylene blue solution containing 13 mg/L methylene blue (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), 0.1% agar, and 135 mg/L sodium hydrosulfite (Fujifilm Wako Pure Chemical Corporation).

**Preparation for LCM**

Hypocotyl segments from 0.5–1.5 cm above the soil surface were fixed with 100% ethanol. Fixed segments were embedded in paraffin using an H2850 Microwave Tissue Processor (Energy Beam Sciences, East Granby, CT, USA) as previously described$^{35}$; 14 µm-thick cross sections of the hypostyle were prepared and mounted on the PEN Membrane Frame Slides (Thermo Fisher Scientific, Waltham, MA, USA). Then, phellogen, AP, cortex, and stele were isolated by Veritas Laser Microdissection System LCC1704 (Molecular Devices, San Jose, CA, USA), as previously described$^{35}$.

**RNA extraction**

Total RNA was extracted from each LM-isolated tissue using the PicoPure RNA Isolation Kit (Thermo Fisher Scientific) and RNA quality was evaluated using the Agilent RNA 6000 Pico Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer’s instructions. RNA quantity was determined using the RiboGreen RNA Quantification Kit (Thermo Fisher Scientific) and an FP-6500 Spectrofluorometer (Jasco Inc., Tokyo, Japan).

Total RNA was extracted from hypocotyls using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

**Microarray analysis**
Total RNA samples (10 ng each) were labeled with a Quick Amp Labeling Kit (Agilent Technologies) according to the manufacturer’s instructions. Aliquots of Cy5-labeled and Cy3-labeled cRNA (825 ng each) were used for hybridization in a 4 × 44k soybean Gene Expression Microarray (Agilent Technologies) that contains 42,748 oligo probes to soybean genes.

Six biological replicates were used, and the labels of Cy3 and Cy5 were interchanged in three of the six replicates of the experiment. Microarray signal intensities were digitized, and the log₂ ratio and p-values were obtained by Feature Extraction software v.10.5.1.1 (Agilent Technologies). A complete set of microarray data was deposited in the Gene Expression Omnibus repository under accession no. GSE216072. We selected genes that showed FC ≥ 2.0 or ≤ 0.5 in expression between phellogen and cortex or AP and cortex, and p-values < 0.05 in all six replicates (Supplementary Tables 1–4). The FC of each probe was calculated using the average of six replicates. Phytozome IDs were obtained by the BLAST sequence of each probe. TAIR accession and description were obtained from Phytozome v12.1 (https://phytozome.jgi.doe.gov/pz/portal.html).

**Extraction and determination of lupeol, betulin, and betulinic acid**

Hypocotyl segments from 0.5–3 cm from the soil surface were cut from seedlings grown under drained or waterlogged soil conditions and immediately frozen with liquid nitrogen. Segments were freeze-dried using FreeZone 1 Liter Benchtop Freeze Dry System (Labconco, MO, USA). Freeze-dried segments were ground using Multi Beads Shocker (Yasui-Kikai, Osaka, Japan). Triterpenoids were extracted and analyzed following a previously described method with minor modifications.

**Quantitative real-time PCR (qRT-PCR) analysis**

Relative mRNA levels were investigated with qRT-PCR using a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). First-strand cDNA was synthesized using Superscript III (Thermo Fisher Scientific) from 20 ng of total RNA extracted from LM-isolated tissues and 2 µg from hypocotyls. SYBR Premix Ex Taq II (Takara Bio) was used for subsequent PCR amplification with appropriate primers (Supplementary Table 7): initial denaturation (95 °C for 20 s), 40 cycles of denaturation (95 °C for 3 s), annealing, and extension (60 °C for 30 s).

**In situ hybridization**

Hypocotyl segments from 0.5–1.5 cm above the soil surface were fixed with fixative solution (4% paraformaldehyde, 2.5% glutaraldehyde, 50 mM Na-P buffer pH 7.2, 0.8 mM NaOH). Fixed segments were embedded in paraffin using an H2850 Microwave processor (Energy Beam Sciences) as previously described. Sections (10 µm thickness) were cut with a rotary microtome. In situ hybridization was performed as described previously. The coding region of *GmLUS1* (Glyma.20G192700) was amplified from soybean cDNA by PCR using appropriate primers (Supplementary Table 7). PCR fragment was cloned to the pCR 4Blunt-TOPO vector (Thermo Fisher Scientific). To produce digoxigenin-labeled *GmLUS1* sense and antisense probes, a 2,263 bp DNA fragment was amplified by PCR from a cDNA
clone and used as a template for *in vitro* transcription with a Maxi Script *in vitro* transcription kit (Thermo Fisher Scientific). Hybridization was conducted at 50°C overnight.

**LUS activity assay in GmLUS1 and GmLUS2**

Coding regions of *GmLUS1* and *GmLUS2* (Glyma.08G027000) were amplified from soybean cDNA by PCR using appropriate primers (Supplementary Table 7). The PCR products were cloned into the pENTR/D-TOPO vector using pENTR Directional TOPO Cloning Kits (Thermo Fisher Scientific) following the manufacturer’s instructions. To obtain the expression vectors, pYES3-ADH-GW was used for the Gateway LR reactions by LR Clonase II Enzyme Mix (Thermo Fisher Scientific). We performed yeast *in vivo* assay as described previously with minor modifications. pYES3-ADH-GmLUS1 and pYES3-ADH-GmLUS2 were transformed to *Saccharomyces cerevisiae* INVSc1 (Thermo Fisher Scientific).

Yeast culture extracts (starting volume: 5 mL) were prepared using 3 mL ethyl acetate (Wako, Osaka, Japan), followed by 1 min of vortexing and 30 min of sonication (70% intensity; Sharp Co., Osaka, Japan). After centrifuging at 2,000 rpm for 5 min, the organic phase was transferred to a silica-gel column (6 cc; Waters Corp., Milford, MA, USA) and washed with 10 mL ethyl acetate. The samples were then placed into an evaporator for 60 min. After resuspending the obtained pellet in 300 µL chloroform–methanol, 100 µL of the mixture was transferred to a vial and placed in an evaporator for 30 min. Finally, the pellet was trimethylsilylated with 50 µL of N-methyl-N-(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 80°C. The evaporated samples were stored at 4°C until needed.

GC-MS was performed using a JMS-AMSUN200 mass spectrometer (JEOL Ltd., Tokyo, Japan) connected to a gas chromatograph (6890A; Agilent Technologies) with a DB1-HT (30 m × 0.25 mm, 0.1 µm film thickness; J&W Scientific, Folsom, CA, USA) capillary column. The injection temperature was 250°C. The column temperature program was as follows: 80°C for 1 min, followed by an increase to 300°C at a rate of 20°C/min, and a hold at 300°C for 20 or 28 min. The carrier gas was He, and the flow rate was 1.2 or 1.0 mL/min, respectively; the interface temperature was 300°C with a spitless injection. Peaks were identified by comparing the Rt and mass spectrum with that of the lupeol authentic standard.

**Cryo-SEM**

After 14 days of waterlogging treatment, hypocotyl segments from 1.0 – 1.5 cm above the soil surface were isolated using a razor blade. The segment was mounted on the metal stage with Tissue-Tek O.C.T. Compound (Sakura Finetek, Tokyo, Japan) and immediately frozen with liquid nitrogen. The surfaces of the AP cells were observed with a field emission scanning electron microscope (S-4300K, Hitachi, Tokyo, Japan) equipped with a cryo-stage (-150 to -120°C). The accelerating voltage was 3 kV.

**X-ray CT**

After 14 days of waterlogging treatment, 0.4 cm hypocotyl segments were isolated using a razor blade. The segment was inserted into the 5 cm plastic straw (φ10 mm), and both ends were closed with wet absorbent cotton. This straw was longitudinally mounted on the stage by sealing putty, and scanned with
a micro-focus X-ray CT (ScanXmate-L090T, ComscanTecno, Kanagawa, Japan). Micro-CT scans were taken at 35 keV and 200 µA; the resolution was 7 µm/pixel, and the rotation step was 0.15°. The total scan time was approximately 20 min, resulting in 1,200 cross-sectional image slices of 1,296 × 1,152 pixels each. Volume renderings and quantitative calculation of the intercellular space on the sample were performed by 3D image segmentation and isosurface representations with Image-Pro 3D (ver.10, Media Cybernetics, USA). The cuboid region (96 × 96 × 296 pixels), which was 30 pixels and 150–180 pixels far from the stele, was extracted as “Inside” and “Outside,” respectively (Extended Data Fig. 7).

**Porosity measurement**

After 14 days of waterlogging treatment, AP-well-developed hypocotyl segments from 0.5–1.5 cm above the soil surface were isolated using a razor blade. The porosity of hypocotyl segments was measured and calculated using the buoyancy method\(^{39,40}\).

**TTC staining**

After 14 days of waterlogging treatment, AP-well-developed hypocotyl segments from 0.5–1.5 cm from the soil surface were isolated using a razor blade. The segments were immersed in the TTC solution (0.06% TTC, 0.05% Tween 20, 100 mM Na-P buffer pH 7.0), vacuumed for 10 min, and incubated for 1 h at 42 °C. After washing the segments using deionized water, 200 µm cross sections were prepared with a plant microtome (MTH-1, Nippon Medical & Chemical Instruments co., Osaka, Japan). Under stereo microscopy, the area of AP was isolated with a razor blade. For the extraction of pigment, isolated sections were incubated in the 200 µL 95%(v/v) ethanol. The reduction of TTC was expressed as the absorbance of the extracted solutions at 520 nm in a spectrophotometer (DU800, Beckman Coulter Inc., CA, USA).

**Measurement of oxygen leakage from the adventitious roots**

ROL measurement was performed using a root-sleeving oxygen electrode. Root-sleeving (i.e., cylindrical platinum) oxygen electrodes enable quantification of ROL at selected positions along roots in an oxygen-free medium\(^{41}\). Twenty-one-day-old plants were immersed until 2 cm below cotyledonary node in deoxygenated solution containing 0.1% (w/v) agar, 5.0 mM KCl and 0.50 mM CaSO\(_4\)\(^{42}\). The cylindrical platinum electrode was polarized relative to a silver/silver-chloride reference. Voltage and current were monitored and digitalized as previously described\(^{43}\); 12–15 cm adventitious roots, which emerged from 1 cm below the water surface, were used for the ROL measurement. The ROL measurements were taken along each root by positioning the center of the electrode at distances of 20 mm behind the root tip.

**Root box -pin board root sampling method**

To evaluate the root system development, the root box pinboard root sampling method, which minimized the loss and destruction of roots, was applied to the soybean root\(^{44,45}\). Seeds were sterilized using CruiserMaxx (Syngenta Japan) and were imbibed in vermiculite wetted with water for 3 d and 25 °C as
described. Then, seedlings were transferred to the root box (40 cm × 24 cm × 2 cm) containing the granular soil (Nihon Hiryo Co., Ltd., Gunma, Japan; pH 5.8–6.5; 0.2 g N, 2.5 g P₂O₅, 0.2 g K₂O, 0.2 g MgSO₄ per 1 L soil; soil density 0.8 – 0.9 kg/L; granule diameter 0.5–3.0 mm). Plants were grown in a temperature-controlled, naturally lite phytotron (30°C during the day, 20°C at night) for 5 weeks in Nagoya, Japan between September and November 2021. The seedlings were grown under the drained soil condition for 2 weeks. After 2 weeks, seedlings in root boxes were moved to the large container for the waterlogging treatment and grown for 3 weeks. During waterlogging, the water level was maintained at 3 cm above the soil surface. After 3 weeks of treatment, the roots were collected by pinboard with 936 (39 × 24) nails. We separated the root system into three regions (Region : from water level to soil surface, Region : from the soil surface to 4 cm below the soil surface, and Region : more than 4 cm below the soil surface). The root length of each region was measured by a root scanner (Expression 12000XL 6.2, Epson, Nagoya, Japan) and WinRHIZO Pro LA2400 (Regent Instruments Inc, Quebec, Canada). After measurement, the roots were dried at 70°C for 3 days to determine the DW of the roots (Extended Data Fig. 10).

Declarations

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Conflicts of Interest

The authors have no conflict of interest to declare.

Author Contributions

H.T., C.A., A.Y., T.G., and M.N. contributed to the study's overall design. H.T., H.S., J.R., Y.T., M.J., H.M., H.S., and T.M. contributed to the analysis of triterpenoids. H.T., C.A., and T.O. contributed to the SEM and X-ray CT scanning. H.T., S.S., and A.K., M.I., prepared soybean seeds. The writing of the manuscript was led by H.T., with all authors contributing through comments and revisions. All authors have read and approved the final manuscript.

Data Availability

All data used during this study are provided in the article, Expanded Data figures, and supplementary tables, and are also available on reasonable request to the corresponding author. Source data are provided in this paper.

Supplementary Information
Extended Data Fig. 1. Important phases in the development of phellogen and aerenchymatous phellem (AP) in response to the waterlogging stress in soybean.

Extended Data Fig. 2. Induction of triterpenoid biosynthesis pathway in aerenchymatous phellem.

Extended Data Fig. 3. Phylogenic tree of lupeol synthase in plants. Its evolutionary history was inferred using the Neighbor-Joining method.

Extended Data Fig. 4. Enzymatic activities of lupeol synthases GmLUS1 and GmLUS2 in yeast cells.

Extended Data Fig. 5. Time-dependent gene expression of GmLUS1 and GmLUS2 in hypocotyl under drained and waterlogged soil conditions.

Extended Data Fig. 6. TIC of GC-MS analysis in the hypocotyls of EnT-6693(WT), EnT-6693(lus1), CRP_WT, CRP_lus1, and CRP_lus1/lus2 grown under waterlogged conditions for 2 weeks.

Extended Data Fig. 7. Constructed images of soybean hypocotyls by micro-X-ray CT scanning and “Inside” and “Outside” areas of AP.

Extended Data Fig. 8. Cross-section after 2,3,5-triphenyl tetrazolium chloride (TTC) staining in hypocotyl during aerenchymatous phellem formation and EnT-6693(WT), EnT-6693(lus1), CRP_WT, CRP_lus1 and CRP_lus1/lus2.

Extended Data Fig. 9. Target sequences of GmLUS1 and GmLUS2 for CRISPR/CAS9, and mutation in CRP_lus1 and CRP_lus1/lus2.

Extended Data Fig. 10. Total root length and dry root weight in different soil depths in CRP_lus1.

Supplementary Table 1. Upregulated genes in PH.

Supplementary Table 2. Downregulated genes in PH.

Supplementary Table 3. Upregulated genes in AP.

Supplementary Table 4. Downregulated genes in AP.

Supplementary Table 5. AP specifically upregulated genes.

Supplementary Table 6. Shoot and root growth in root box experiments.

Supplementary Table 7. Primer list.

References


**Figures**
Figure 1

Induction and function of aerenchymatous phellem (AP) formation under waterlogged soil conditions and phellogen (PH), AP, and cortical cells specific microarray analysis using LM.

a, Soybean did not form PH and AP under drained soil conditions (upper left picture). However, AP was observed as white spongy tissues after 7 days of waterlogging treatment (upper right picture, red arrow). Scale bar: 1 cm. PH was observed inside of AP (lower picture). Scale bar: 250 µm. b, Soybeans grown under drained and waterlogged soil conditions were immersed in methylene blue solution. c, Isolation of cortex, AP, and PH using LCM. d, Venn diagram of microarray analysis. Upregulated genes in AP and/or PH compared with the cortex (upper diagram). Downregulated genes in AP and/or PH compared with the cortex (lower diagram).

Figure 2

Accumulation of lupeol, betulin, and betulinic acid in the hypocotyl under waterlogged soil conditions via activation of the triterpenoid biosynthesis pathway and tissue expression pattern of LUS genes.

a, Triterpenoid biosynthesis genes that were upregulated in AP. b, lupeol, betulin, and betulinic acid accumulation in the hypocotyl under waterlogged soil conditions. Triterpenoid accumulation was not observed under drained soil conditions. c, the tissue expression pattern of GmLUS1 and GmLUS2 genes using LM (Mean ± S.E., n = 3). d, in situ hybridization of GmLUS1. Scale bar: 200 µm.
Figure 3

Functional analysis of *GmLUS1* in AP.

a, Mutation site of EnT6693 isolated from EMS-treated mutant panel, CRP-*lus1* and CRP-*lus1/lus2* obtained by genome editing (CRISPR/Cas9 system). b, Observation of the surface structure on the AP cells in *lus1* and *lus1/lus2* mutants by cryo-scanning electron microscopy (SEM) analysis. c, the porosity
measurement of AP well-developed hypocotyls in *lus1* and *lus1/lus2* mutants (Mean ± S.E., n = 3–9). Asterisks indicate significant differences in EnT-6693 (*p* < 0.01, two samples, t-test). Different letters indicate significant differences among CRPs (*p* < 0.01, one-way ANOVA, followed by Tukey’s HSD post-hoc test for multiple comparisons). d, Measurement of intercellular space of the *lus1* mutant in AP using X-ray micro computed tomography (CT) scanning. Cross-section images of the hypocotyl reconstructed from CT scanning (left pictures). The square described by white line and light-green dots were the area in which intercellular space was measured. Inside and outside parts of AP were differentially analyzed. e, Evaluation of cell activities by TTC staining in *lus1* and *lus1/lus2* mutants. The left panel shows EnT-6693(WT) and EnT-6693(*lus1*), and the right panel shows CRP_WT, CRP_ *lus1*, and CRP_ *lus1/lus2*. f, AP cells were isolated after TTC staining, and the pigment was extracted and quantified by absorbance at 520 nm (Mean ± S.E., n = 4). Asterisks indicate significant differences in EnT-6693 (*p* < 0.01, two samples, t-test). Different letters indicate significant differences among CRPs (*p* < 0.01, one-way ANOVA followed by Tukey’s HSD post-hoc test for multiple comparisons).
Figure 4

Effect on the ROL in adventitious roots and the root system development by lacking triterpenoid accumulation in AP:

a, Amount of ROL in adventitious roots of CRP_lus1 (Mean ± S.E., n = 8–9, p < 0.05, two samples, t-test).

b, the root system of CRP_WT and CRP_lus1 grown under waterlogged soil conditions. Region refers to
the area between water level and soil surface. Region  refers to the area between the soil surface and 40 mm below the soil surface. Region  refers to the area below 40 mm from the soil surface. c, Ratio of root length to total root length in each region (Mean ± S.E., n = 3, t-test, *: $p < 0.05$, **: $p < 0.01$). d, Ratio of root dry weight (DW) to total root DW in each region (Mean ± S.E., n = 3, t-test, **: $p < 0.01$).

**Supplementary Files**

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