Long-distance transport of the phytohormone abscisic acid (ABA) has been studied for ~50 years, yet its mechanistic basis and biological significance remain very poorly understood. Here, we show that leaf-derived ABA controls rice seed development in a temperature-dependent manner and is regulated by defective grain-filling 1 (DG1), a multidrug and toxic compound extrusion transporter that effluxes ABA at nodes and rachilla. Specifically, ABA is biosynthesized in both WT and dg1 leaves, but only WT caryopses accumulate leaf-derived ABA. Our demonstration that leaf-derived ABA activates starch synthesis genes explains the incompletely filled and floury seed phenotypes in dg1. Both the DG1-mediated long-distance ABA transport efficiency and grain-filling phenotypes are temperature sensitive. Moreover, we extended these mechanistic insights to other cereals by observing similar grain-filling defects in a maize DG1 ortholog mutant. Our study demonstrates that rice uses a leaf-to-caryopsis ABA transport-based mechanism to ensure normal seed development in response to variable temperatures.

INTRODUCTION

Hormones are endogenous chemicals that participate, at very low concentrations, in various developmental and stress response processes in both plants and animals. Generally, three steps are required for hormone function: synthesis in specific cell types, transport to distant cells, and triggering of signal transduction (1). The plant hormone abscisic acid (ABA) is essential for various physiological processes during plant growth and stress responses. Since its first identification as a growth inhibitor in the early 1960s, tremendous efforts have been invested in studying the molecular mechanisms of ABA biosynthesis and signal transduction (2), and these are now mature research areas. However, despite the fact that several ABA transporters involved in cellular level transport have been identified in Arabidopsis and rice (3–7), no report has addressed the molecular mechanism(s) of long-distance transport of ABA in plants.

To the best of our knowledge, long-distance ABA transport was first observed in Coleus rheneltianus in 1968 (8). Over the course of the next half century, several physiological approaches have been adopted to study long-distance ABA transport and its biological significance in a number of species (9–13), and these studies indicated that ABA can be translocated among organs in plants. However, the flow direction and the biological significance of long-distance ABA transport in plants remain poorly understood. This has led to long-standing debates, likely owing to the lack of genetic resources for specifically experimentally manipulating long-distance ABA transport processes among various organs. For example, it has long been widely accepted that root-derived ABA regulates leaf stomatal closure under drought stress (14); however, this concept has been challenged (15, 16) in light of evidence that ABA accumulation in roots is dependent on basipetal ABA transport from aerial organs (17). Moreover, the extent of ABA bulk that was translocated among organs also remains unknown. Using the defective grain-filling 1 (dg1) rice mutant, we discovered that the majority of the ABA present in the rice caryopses has been transported from leaves and that leaf-to-caryopsis ABA transport is regulated by a multidrug and toxic compound extrusion (MATE) transporter in a temperature-sensitive manner that functions to ensure normal seed development in response to variable temperatures.

RESULTS

The DG1 regulates seed development by promoting grain filling

Seeking deeper understanding of the molecular mechanisms regulating seed development, we screened for mutants with defective grain-filling phenotypes from an ethylmethane sulfonate–mutagenized library of an elite indica rice parent (Shuhui527). For one such mutant, dg1, 24.68% of seeds were incompletely filled; in contrast, nearly all wild-type (WT) seeds were completely filled (Fig. 1A). Further suggesting deficient seed filling in the dg1 plants, the remaining ~75% of the “fully filled” dg1 seeds also showed defective filling phenotypes, including floury seeds (Fig. 1B) and aberrantly loose starch granules (Fig. 1, C and D). The defective grain-filling of the dg1 plants resulted in a >21% overall decrease in 1000-grain weight (Fig. 1E) compared to WT plants.
Closer examination found that WT caryopses took about 27 days to finish grain filling, whereas the \(dg1\) plants had an extremely extended grain-filling duration (54 days) (fig. S1, A and B). Monitoring of ongoing grain filling over 3-day windows showed that the \(dg1\) caryopses had a significantly slower grain-filling rate than WT before 15 days after pollination (DAP) but had a greater grain-filling rate than WT after 18 DAP because of the extended grain-filling duration of \(dg1\) (fig. S1B). We also discovered that the \(dg1\) caryopses accumulated significantly higher levels of sucrose, fructose, and glucose than the WT caryopses at all of the time points tested (fig. S2, A to C). Conversely, the \(dg1\) caryopses had significantly reduced starch content at all tested time points (fig. S2D), indicating that starch synthesis is somehow disrupted in \(dg1\) caryopses.

**DG1 encodes a multidrug and toxic compound extrusion protein**

MutMap-based gene mapping (18) showed that only one single-nucleotide polymorphism (located at LOC\(_{Os03g12790}\)) co-segregated with all 40 examined individuals with the \(dg1\) phenotype (fig. S3). \(LOC_{Os03g12790}\) encodes a MATE protein. The mutation from G to A at position 786 generates a premature stop codon predicted to result in a truncated protein lacking a MATE domain in \(dg1\) (Fig. 1F).

Transgenic complementation of \(dg1\) plants with a construct harboring

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**Fig. 1. DG1 encoding a MATE transporter regulates seed development.** (A) Comparison of WT and the \(dg1\) mutant seeds showing various degrees of grain-filling defects. Scale bars, 1 cm. (B) Transverse sections of representative fully filled mature seeds. Scale bar, 1 mm. (C and D) The starch granules observed via scanning electron microscopy in magnified regions of the WT and \(dg1\) black squares in (B), respectively. Scale bars, 10 \(\mu\)m. (E) One thousand–grain weight comparison of filled seeds (including incompletely filled seeds [IFS]) between WT and \(dg1\). Data are presented as means ± SD (\(n = 5; **P < 0.01, \) Student’s t test). (F) Diagram of the DG1 gene and the mutation causing the \(dg1\) phenotype. The positions of ko-1 and ko-2 represent the two knockout sites targeted using a CRISPR-Cas9 approach. (G and H) Phenotypic comparison of mature seeds (G) and transverse sections of fully filled seeds (H) from WT Nipponbare (Nip) and the two DG1 knockout lines. Scale bars, 1 cm (G) and 1 mm (H). (I) One thousand–grain weight comparison of filled seeds (including IFS) from two knockout lines. Data are means ± SD (\(n = 5; **P < 0.01, \) Student’s t test). FFS, fully filled seeds; Photo credit: Binhua Hu.
the LOC_Os03g12790 genomic sequence completely rescued the dg1 phenotypes (fig. S4, A to D). We also used a CRISPR-Cas9 approach to knock out the LOC_Os03g12790 in the Japonica background (Nipponbare). Two knockout lines (ko-1 and ko-2) bearing edits that caused premature stop codons were obtained (Fig. 1F and fig. S5, A and B). As expected, ~20% of the seeds were incompletely filled in the ko-1 and ko-2 mutants, and this resulted in ~27% decreases in 1000-grain weight and floury seed phenotype (Fig. 1, G to I). The extended grain-filling duration was also observed in two knockout lines (fig. S4E). Therefore, we have unambiguously demonstrated that the mutation in LOC_Os03g12790 is responsible for the defective grain-filling phenotype in dg1.

**DG1 functions as an ABA efflux transporter**

In light of reports from the literature that ABA can accelerate grain-filling rates and shorten grain-filling durations in rice (19), we tentatively speculated that the DG1 protein may function to transport ABA. We pursued the potential ABA transport activity of DG1 in experiments with *Xenopus* oocytes, which revealed that oocytes expressing DG1 released ABA significantly faster and more abundantly than negative control oocytes (water-injected) (Fig. 2A). Furthermore, these experiments included oocytes expressing the well-known ABA transport protein AtDTX50 (7) as a positive control, and the fact that DG1 transported the same amount of ABA as AtDTX50 in a substantially shorter time suggests that DG1 has high ABA efflux efficiency.

We further used rice protoplasts to verify the ABA transport activity of DG1. Protoplasts prepared from a transgenic rice line overexpressing a DG1–enhanced green fluorescent protein (EGFP) fusion protein released $^3$H-ABA much faster than WT protoplasts. Again consistent with an ABA efflux function for DG1, we also found that WT protoplasts released $^3$H-ABA much faster than protoplasts prepared from dg1 seedlings (Fig. 2B). Moreover, the plasma membrane localization that we detected for the DG1-EGFP fusion also supports DG1’s function as an ABA efflux transporter (Fig. 2, C to H). These ABA transport activity results raise the fascinating possibility that the dg1 grain-filling phenotypes may result from a reduction of ABA transport.

**Long-distance leaf-to-caryopsis ABA transport is regulated by DG1**

To confirm whether the defective grain-filling phenotypes of dg1 were caused by the reduction of ABA content in caryopsis, we measured the ABA content of WT and dg1 caryopses at 3 and 5 DAP. As
anticipated, the ABA content of \( dg1 \) caryopses was significantly decreased to only ~50 and ~16% of the WT level at 3 DAP and 5 DAP, respectively (Fig. 3A). However, when we measured the ABA levels in stems and leaves, we discovered that the ABA content of \( dg1 \) leaves and stems at both 3 and 5 DAP was significantly higher than that in WT leaves and stems (Fig. 3, B and C). These unexpected results indicated that the ABA molecules present in caryopsis may not be de novo synthesized there; rather, they may be delivered into caryopses via long-distance transport from leaves.

To test this hypothesis, we first measured the content of the ABA precursors 9′-cis-neoxanthin and 9-cis-violaxanthin in WT and \( dg1 \) leaves, stems, and caryopses. We detected no significant difference between the WT and \( dg1 \) plants. Notably, both of the ABA precursor compounds of WT and \( dg1 \) were present at very low levels in caryopses and stems but were present at very high levels in leaves (Fig. 3, D and E). Conversely, the ABA content in the WT caryopses at 3 and 5 DAP was 10 and 40 times higher, respectively, than that we detected in WT leaves and stems (Fig. 3, B and C). These results strongly suggested that most, if not all, of the ABA present in caryopses and stems was not de novo synthesized but rather was transported there after being synthesized in leaves.

To directly confirm that ABA is transported from rice leaves to caryopses and to assess whether such transport is disrupted in the \( dg1 \) mutant, we conducted isotope labeling experiments where we fed leaves of WT, \( dg1 \), and the complementation line with \(^3\)H-ABA. We then measured the radioactivity signal in leaves, stems, and caryopses (Fig. 3, F and G) and found that WT caryopses and stems had significantly higher radioactivity compared to the corresponding organs in \( dg1 \) plants and that the radioactivity level in \( dg1 \) leaves was 43% higher compared to WT leaves (Fig. 3F). We also measured ABA content in different tissues after feeding stems with unlabeled (fig. S6). The differences in the distribution of ABA between WT and \( dg1 \) plants (sampled at 3- to 6-hour intervals) (fig. S6B) were similar to the differences detected on the basis of

![Fig. 3. Long-distance leaf-to-caryopsis ABA transport is regulated by DG1.](http://advances.sciencemag.org/)
presumed ³H-ABA radioactivity signals, indicating that the radioactivity level could represent ³H-ABA content and further supporting the presence of an ABA transport defect in dg1 plants. In addition, the fact that we detected some radioactivity in dg1 stems and caryopses (Fig. 3F) indicates that there are potentially other minor ABA transporters, which can partially compensate for the dominant role of DG1 in this grain-filling–regulating ABA leaf-to-caryopsis transport process.

In rice, the node has an intervascular transfer system that facilitates the long-distance transport of various minerals and sugars from leaves to stems (20–22), and a recent study demonstrated that a transpiration-independent process at the rachilla functions to distribute minerals to the pistil and stamen (23). In addition, ABA biosynthesis has been proposed to occur mainly in phloem companion cells (24). Considering the functions of phloem in signal transport from source to sink tissues (25), leaf-to-caryopsis transport of ABA should first be loaded into phloem in leaves before intervascular transfer and later unloading into caryopses. Expression pattern analysis in various organs using quantitative polymerase chain reaction (qPCR) and ProDG1::GUS transgenic plants showed that DG1 was expressed in the node, the basal region of caryopses (including rachilla), and leaves at the grain-filling stage (fig. S7, A to E), suggesting that DG1 could regulate ABA transport at these three tissues.

Further immunofluorescence analyses showed that DG1 was expressed in the parenchyma cells between diffusion vascular bundles (DVBs) and enlarged vascular bundles (EVBs) (Fig. 4, A to G, and fig. S7, I to K). Coupled with the observation of more ABA accumulation in the dg1 node (fig. S6B), the specific expression pattern of DG1 in nodes suggested that DG1 functions in the regulation of ABA intervascular transfer. The observation of DG1 expression in the leaf vascular bundle, particularly in phloem (Fig. 4, H to K; and

**Fig. 4. Expression pattern analysis of DG1 using immunofluorescence.** (A to O) Expression pattern of DG1 in node, leaf, and rachilla. Node I (A to G), flag leaf (H to K), or rachilla (L to O) at 3 DAP of representative proDG1::GUS lines were used for immunofluorescence. (E to G) Respectively represent magnified regions of white squares in (B to D). The arrow in (E) indicates the parenchyma cells positioned between the DVBs and EVBs of the node; the arrows in (I) and (M) indicate the phloem of leaf vascular bundles and the central vascular bundle of rachilla, respectively. The red dash lines in (A), (H), and (L) indicate the transverse section position for immunofluorescence in node I, flag leaf, and rachilla, respectively. Scale bars, 1 cm (A, H, and L), 200 µm (B to G), and 100 µm (I to K and M to O). Photo credit: Guohua Zhang and Peng Qin.
fig. S7, E and L to N), and the central vascular bundle of rachilla (Fig. 4, L to O, and fig. S7, O to Q) further suggested the function of DG1 in ABA loading into leaf phloem and unloading into caryopses, respectively. In addition, the expression of DG1 in the stelle of root and the vascular bundle of hull (fig. S7, F to H) suggests possible ABA unloading in these organs. Thus, DG1 mediates the long-distance transport of ABA from leaves to caryopses.

**DG1-mediated long-distance ABA transport and seed development is sensitive to temperature**

Beyond uncovering a previously unknown natural physiological process of long-distance leaf-to-caryopsis ABA transport, we discovered a dg1-specific phenotype based on unexpected observations of plants grown in field conditions at Hainan (~24°C/day at anthesis) and Sichuan (~30°C/day at anthesis) provinces. We found that the incomplete filling phenotype of the dg1 plants was only evident for the plants grown at ~30°C. Thus, we hypothesized that the DG1-mediated regulation of leaf-to-caryopsis ABA transport may be a mechanism through which rice plants regulate their seed development in response to variable temperature.

Pursuing this line of inquiry, we conducted experiments in temperature-controlled greenhouses in which WT and plants were grown at ~24° and ~30°C/day, which confirmed the dg1-specific incomplete filling phenotype from the field experiments (Fig. 5, A and B, and table S1). We further conducted experiments with short-term exposure of WT plant (grown at ~24°C/day) at 35°C for 2 hours, which revealed that DG1 expression in caryopsis was significantly increased at 35°C (Fig. 5C). To assess whether this observed temperature-sensitive expression of DG1 affects the efficiency of long-distance ABA transport, we fed the stems of WT, dg1, and complementation line plants with 3H-ABA at both 24° and 30°C. We then measured stem-to-caryopsis ABA transport efficiency by quantifying differences in the radioactivity signal intensity between caryopsis samples and combined stem/caryopsis samples. For WT plants, intensity of the radioactivity signal at 30°C (assessed over three different feeding intervals) was significantly higher than at 24°C (Fig. 5, D and E), indicating that more 3H-ABA was transported from stem to caryopsis at the 30°C growth temperature.

When we examined differences in radioactivity signal in WT versus dg1 plants, we found that the 57% radioactivity increase in...
WT plants at 30°C was much higher than the ~10% radioactivity increase in WT plants at 24°C (Fig. 5D). We observed no such temperature-dependent radioactivity increases in comparisons of WT versus complementation line plants (Fig. 5D). Considering the representativeness of radioactivity as 3H-ABA, these results genetically confirm a specific function for DG1 in regulating temperature-dependent ABA long-distance transport-mediated seed development in rice.

**ABA directly activates genes essential for starch synthesis and grain filling**

To characterize the molecular network through which the long-distance transported ABA ultimately regulates seed development in rice, we performed RNA sequencing of caryopses at 5 DAP grown in rice, we performed RNA sequencing of caryopses at 5 DAP grown in rice. 0 h, before treatment; W−2 h, after 2 hours of water treatment; ABA−2 h, after 2 hours of ABA treatment. Data are means ± SD (n = 7; **P < 0.01, Student’s t test). (K) Gene model of maize DG1 ortholog (ZmDG1) showing the site of Mu insertion (Zmdg1). (L to N) Comparison of grain-filling phenotypes between WT and Zmdg1 mutant that were pollinated on the same day, highlighting the obviously slower grain-filling rate and incompletely filled kernels [red arrows in (M)] in the mutant. (M) and (N) respectively represent the magnified regions of WT and the Zmdg1 mutant in (L). Scale bars, 3 cm (L) and 1 cm (M and N). Photo credit: Guohua Zhang.

Fig. 6. ABA induces expression of starch synthesis–related genes, and disruption of the DG1 ortholog mutant in maize causes grain filling phenotypes. (A to J) Exogenous application of ABA to WT and dg1 stems induces expression of starch synthesis–related transcription factors (A to E) and enzymes (F to J) in WT caryopses but not in dg1 caryopses. 0 h, before treatment; W−2 h, after 2 hours of water treatment; ABA−2 h, after 2 hours of ABA treatment. Data are means ± SD (n = 7; **P < 0.01, Student’s t test). (K) Gene model of maize DG1 ortholog (ZmDG1) showing the site of Mu insertion (Zmdg1). (L to N) Comparison of grain-filling phenotypes between WT and Zmdg1 mutant that were pollinated on the same day, highlighting the obviously slower grain-filling rate and incompletely filled kernels [red arrows in (M)] in the mutant. (M) and (N) respectively represent the magnified regions of WT and the Zmdg1 mutant in (L). Scale bars, 3 cm (L) and 1 cm (M and N). Photo credit: Guohua Zhang.
modules for starch synthesis were activated by the long distantly transported ABA.

**DG1 is functionally conserved in cereals**

To explore whether the mechanism of DG1-mediated ABA long-distance transport is conserved across species, we first analyzed the sequence conservation of the DG1 protein among angiosperm species. DG1 shared ~80% protein sequence identity among grass species and ~50% identity with species in other plant families (fig. S8). To test whether DG1 orthologs in the grass family are functionally conserved, we analyzed the sequence conservation of the DG1 protein among angiosperm species. DG1 shared ~80% protein sequence identity among grass species and ~50% identity with species in other plant families (fig. S8). To test whether DG1 orthologs in the grass family are functionally conserved, we analyzed a maize mutant line (mu1004348) that harbors a Mu insertion in the DG1 ortholog (ZmDG1) (Fig. 6K). We first confirmed this Mu insertion in ZmDG1 by PCR validation (fig. S9, A and B). Similar to the grain-filling phenotypes of the dg1 mutant in rice, the Zmdg1 mutant showed incompletely filled kernels and an obviously slower grain-filling rate than the WT plants (W22) (Fig. 6, L to N). In addition, analysis of publicly available transcriptomics data for heat stress–treated maize plants [Xiantian 5 (XT), Zhefengtian 2 (ZF), CB25, CM1, and An’nong 591 background] (31, 32) revealed that ZmDG1 expression levels were notably increased by high temperature (fig. S9C). Our observation of similar defective grain-filling phenotypes between the Zmdg1 and dg1 mutant and this similar temperature-mediated impact on ZmDG1 and DG1 expression support a conserved biological function of DG1 orthologs between rice and maize and suggested that DG1 is functionally conserved in cereals.

**DISCUSSION**

Our study establishes that rice plants are equipped with a sophisticated leaf-to-caryopsis ABA transport system that ensures normal seed development under variable temperature conditions (Fig. 7). DG1 initially functions in ABA loading into leaf phloem. After that and given that, in rice, the vascular bundle is not continuous from leaves to caryopses, any transport of leaf-derived ABA to the caryopsis must proceed via intervascular transfer, specifically from leaves into nodes through the EVB and then into the stem through the DVB (20, 21). When leaf-derived ABA arrives at the EVB, DG1 facilitates ABA transport at the plasma membranes of parenchyma cells between EVB and DVB. After arriving at the basal region of caryopses, DG1 facilitates ABA transport from the central vascular bundle of rachilla into the dorsal vascular bundle of the caryopsis; the presence of this transporter helps overcome the known weak transpiration-dependent transport between these two organs (23). Once inside the caryopsis, ABA directly activates the expression of genes essential for starch synthesis to regulate seed development.
Understanding the mechanism of seed development at higher temperature should be very helpful for meeting the future prospect of crop yield and quality decreases resulting from global temperature raise (33, 34). Our confirmation that DG1-mediated leaf-to-caryopsis ABA transport increases along with rising temperatures establishes that crop plants use such transport to integrate temperature information with seed development to appropriately regulate seed development under variable temperature conditions. Given the strong phenotype of the dg1 mutant and high ABA transport efficiency of DG1 at relatively high temperatures (Fig. 5, B and D), it appears that DG1 plays a dominative role among transporters involved in leaf-to-caryopsis ABA transport and seed development at higher temperature. Therefore, DG1 (and its orthologs in other crops) should be understood as an excellent potential targets for mining of favorable natural variants or editing using CRISPR-Cas9 to resolve crop productivity loss caused by globally increasing temperatures. In thinking about strategies to experimentally dissect the temperature-dependent function(s) of DG1, it is important to remember that dg1 plants grown under lower temperatures did show reduced ABA transport activity compared to WT plants and displayed a seed developmental phenotype (chalky grain). These effects were not as pronounced as for dg1 plants grown at the high temperature, but these detectable readouts of dg1 dysfunction at both temperatures should support the further experimental elucidation of DG1’s specific functional roles.

Our discoveries will promote future studies seeking the molecular machineries of ABA long-distance transport and how long-distance ABA transport helps plants integrate environmental and development signals to regulate plant development and response to stress. Our findings are also likely to motivate future studies on the different impacts of locally synthesized versus long-distance transported ABA on seed development or in responses to various stresses. Moreover, in light of studies reporting long-distance transport of other phytohormones like Gibberellic acid (GA) (35), Cytokinin (CK) (36), and Jasmonic acid (JA) (37), our discovery may also help guide studies investigating regulation mechanisms and the biological significance of long-distance transport of other phytohormones.

**MATERIALS AND METHODS**

**Plant material and culture conditions**

The dg1 mutant, WT, the F2 population for gene mapping, complemented line (proDG1:gDNA\textsuperscript{DG1}), overexpression line (35S:cDNA\textsuperscript{DG1}-eGFP), knockout lines (ko-1 and ko-2), and their background parent Nipponbare were grown in rice paddies of Sichuan Agricultural University in Chengdu, Sichuan, China. Plants used for \textsuperscript{3}H-ABA feeding assay were grown in rice paddies of the University of Science and Technology of China, Hefei, Anhui, China. The maize UniformMu insertion lines (mu1004348) was obtained from www.maizegdb.org and grown in the field of Sichuan Agricultural University in Chengdu, Sichuan, China.

**Phenotypic analyses**

To make sure that the data of dry weight, sugars, and ABA and ABA precursor contents of leaf, stem, and caryopsis were comparable between WT and dg1, we marked equivalent developmental stages for caryopsis, flag leaf, and stem. First, we marked the hull of WT and dg1 spikelets that were pollinated at the same day using marker pen and defined the marking day as 0 DAP and so on. Second, the spikelets chosen for marking were at the middle of panicle to make sure that leaves and stems used for measurement were at the same developmental stages as caryopsis and were comparable between WT and dg1. Third, we marked all spikelets used for measurements of WT and dg1 on the same day to make sure that all contents of tissues at different stages were comparable between WT and dg1.

For the dry weight measurement of caryopses at different developmental stages, we collected marked caryopses every 3 days starting with 3 DAP, dried them out at 60°C for 48 hours, and then measured their dry weight. Three biological replicates were conducted for each measurement, and two-tailed Student’s t test was used for comparison between WT and dg1.

Scanning transmission electron microscopy (TEM) of mature seeds of WT and dg1 was performed as described. Simply, seeds were cut with a razor blade; samples were processed for critical point drying using liquid CO\textsubscript{2} and coated with gold. The samples were examined with a JEM 1200 EX scanning electron microscope (Hitachi).

**Determination of sugar content**

The measurement of sugar content in flag leaf, stem, and caryopsis was performed using kits for sucrose, fructose, and glucose measurement (Megazyme, K-SUFRG) and for starch measurement (Megazyme, K-TSTA-100A). Five biological replicates were performed for each sugar content measurement, and two-tailed Student’s t test was used for comparison.

**Determination of ABA content**

The determination of ABA content in flag leaf, stem, and caryopsis was performed as described by Park et al. (38) with modification. Briefly, 50 mg of fresh sample were ground to powder in liquid nitrogen, dissolved in 1 ml of ethyl acetate with 10 μl of internal standard [chloromycetin (600 ng/ml)], and then incubated with shaking overnight at 4°C. After that, the samples were centrifuged at 15,000g for 15 min at 4°C, and the supernatants were transferred to a 2-ml fresh tube. The pellets were reextracted by adding 0.5 ml of ethyl acetate without internal standards and incubating at 4°C for 5 hours in the dark and then centrifuged at 15,000g for 15 min at 4°C. The supernatants of two extractions were combined and dried using a Thermovap sample concentrator at room temperature. The dried extracts were dissolved in 200 μl of 70% methanol, vortexed for 25 min, and then centrifuged at 15,000g for 10 min at 4°C. The supernatants were respectively transferred to 1.5-ml liquid chromatography (LC) vials and then injected into the LC/mass spectrometry (MS) system.

High-performance LC (HPLC) (1100, Agilent, USA) coupled to a triple four-pole tandem mass spectrometer (API3000, AB Science, USA) were used for ABA quantification. For HPLC, the chromatographic separation was performed on an Inertsil ODS-3 column (2.1 mm by 100 mm, 5 μm), protected by a Phenomenex C18 guard column [4 mm by 3 mm inner diameter (i.d.); Torrance, CA, USA]. The temperature of column was 30°C. The mobile phases and gradient elution are as follows: (i) solvent A (0.1% formic acid), 45 to 65% for 0 to 5 min and 65 to 45% for 5 to 6 min; (ii) solvent B (methanol), 55 to 35% for 0 to 5 min and 35 to 55% for 5 to 6 min and then equilibrated for 2 min in 45% solvent A and 55% solvent B. The injection volume and flow rate were 10 and 0.3 ml/min, respectively. The retention times were 4.53 min for ABA and 3.23 min for chloromycetin. MS was performed in the negative ion mode with electrospray ionization.
tandem MS. The main working parameters of mass spectrometer were optimized as follows: nebulizer gas, 8 liter·min⁻¹; curtain gas, 10 liter·min⁻¹; collision activated dissociation gas, 4 liter·min⁻¹; turbo ion spray voltage IS, −4000 V; and source temperature TEM, 450°C. The compound parameters such as declustering potential, focusing potential, entrance potential, collision energy, and collision cell exit potential were optimized and set to −57.58, −157.3, −5.97, −18.91, and −16.9 for ABA and to −52.96, −198, −9.2, −25.75, and −6.87 for chloromycetin, respectively. Analyses were detected by tandem MS using multiple reaction monitoring of the most intensive precursor/fragment transitions with 200-ms dwell time, at a mass/charge ratio (m/z) of 263.1/152.9 for ABA and m/z of 321.0/151.8 for chloromycetin. The data were analyzed using Analyst 1.4.1 software. ABA measurement was performed with five biological replicates, and two-tailed Student’s t test was used for comparison.

**Determination of 9′-cis-neoxanthin and 9-cis-violaxanthin content**

The determination of ABA precursor (9′-cis-neoxanthin and 9-cis-violaxanthin) content in flag leaf, stem, and caryopsis was performed as described by Zhang et al. (39) with modification. Total 50-mg fresh weight samples were ground to powder in liquid nitrogen, then dissolved in 1 ml of cold (−20°C) 80% acetone, and incubated overnight in the dark at 4°C. After that, samples were centrifuged at 15000g; the supernatant was transferred into a fresh tube, and the pellets were reextracted by suspending and adding 0.5 ml of cold (−20°C) 80% acetone for 5 hours in the dark at 4°C. The supernatant of two extractions were mixed together and filtered into LC vials using mini filter with 0.22-μm polycarbonate membrane (Millipore). ABA precursor (including 9′-cis-neoxanthin and 9-cis-violaxanthin) content was analyzed using ACQUITY H-Class UPLC (Waters, USA) coupled with a photodiode array (Waters, USA) detector. The mobile phase consisted of two solvents: acetonitrile (solvent A) and water (solvent B).

For HPLC, the chromatographic separation was performed on an Analytical DB C18 column (50 mm by 2.1 mm, 1.9 μm). The temperature of column was 35°C. The mobile phases and gradient elution are as follows: (i) solvent A (acetonitrile), 50% to 100% for 0 to 8 min, 100 to 80% for 8 to 8.5 min, and 80 to 50% for 8.5 to 9 min and (ii) solvent B (water), 50% to 0% for 0 to 8 min, 0% to 20% for 8 to 8.5 min, and 20 to 50% for 8.5 to 9 min and then equilibrated for 3 min in 50% solvent A and 50% solvent B. The flow rate was 0.4 ml/min.

The 9′-cis-neoxanthin and 9-cis-violaxanthin (Sigma-Aldrich) were used for the standard curve. The wavelength for detecting two precursors was 440 nm. The content of 9′-cis-neoxanthin and 9-cis-violaxanthin in all samples were quantified by comparing the peak areas of their relative standard substance. All experiments were conducted with five biological replicates.

**Gene mapping**

First, we generated backcross F2 segregation population by crossing WT and dg1. The DNA of 40 individuals with extremely long grain-filling duration of F2 population were pooled, and next-generation sequencing was performed. MutMap (18) was used for the mutation identification linked with the dg1 phenotype.

**Knockout and complementation assay**

For knocking out DG1, two targets were designed, and the oligos for generating single guide RNA (sgRNA) of two targets are listed in table S3. The CRISPR-Cas9 expression vector for DG1 was introduced into Agrobacterium and transformed into Nipponbare calli. The CRISPR-Cas9 vector backbone was from the laboratory of Y. Liu at South China Agricultural University (40). Sequencing was performed using the primer pairs flanking target sites (table S3) to identify mutations in positive transgenic lines. For complementation of dg1, pCambia1300-proDG1: gDNA<sup>Δch</sup> expression vector was generated by recombination between DG1 genomic DNA with native 2k promoter upstream ATG and pCambia1300 backbone, then introduced into Agrobacterium, and transformed into dg1 calli. The primers for generating complementary vector were listed in table S3.

**ABA transport activity**

The method of testing ABA transport activity of DG1 in Xenopus oocytes was performed as by Zhang et al. (7) where DG1 and AtDTX50 cDNA were cloned into the pGEMHE expression vector used for Xenopus oocytes. Complementary RNA (cRNA) was prepared using the mMESSAGE mMACHINE High Yield Capped RNA Transcription Kit (Ambion). A total of 32 nl of DG1 and AtDTX50 (positive control) cRNA and nuclease-free water (negative control) were injected into oocyte cells and then incubated at 18°C for 3 days. After that, each oocyte cell was injected with 46 nl of ABA (1 mM), then recovered on ice for 15 min, and transferred into 110 μl of tris-buffered saline buffer (pH 5.5) for ABA releasing. The ABA in buffer containing three oocytes for each sample was tested using a Phytodetek ABA immunoassay kit (Agdia, Elkhart, IN) after incubation times of 0, 10, 20, and 30 min.

For testing ABA transport activity of DG1 in protoplasts, we generated DG1 overexpression line by transforming 35S::cDNA<sup>DG1</sup>-eGFP into Nipponbare. The protoplasts of Nipponbare, ko-1, and DG1-<sup>OE</sup> were suspended in W5 buffer [154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, and 2 mM 2-(N-morpholino)ethane sulfonic acid (pH 5.8)]. For each sample, the protoplasts were transferred into four tubes; each tube contained 1 ml of protoplasts; then, 0.2 μl of (+)-ABA [³H(G)] (American Radiolabeled Chemicals Inc.) was added into each tube, and it was incubated for 1 hour at room temperature to load [³H]-ABA into protoplast. After that, the protoplasts of each tube were washed three times using cold W5 buffer, suspended into 1 ml of W5 buffer at room temperature, and then transferred into three tubes with 200 μl for releasing ABA. Radioactivity in buffer of each replicate was measured using liquid-scintillation counter (PerkinElmer, Tri-Carb 2910 TR) after 0, 10, 20, and 30 min incubation (three biological replicates for each time point). The transport activity of each sample was determined by the increased percentage of radioactivity. All experiments were conducted at least two times and with three biological replicates, and two-tailed Student’s t test was used for comparison.

**³H-ABA and regular ABA feeding**

We marked spikelets of WT and dg1 as described above and used panicles after 5 days of marking for [³H]-ABA ([±]-cis ABA [³H(G)]) (ART 1186, American Radiolabeled Chemicals) feeding experiments. For [³H]-ABA feeding of stem, panicles with the two uppermost internodes of WT and dg1 were inserted into 10 ml of water with 5 μl of [³H]-ABA. After 1, 2, and 4 hours of feeding, the WT and dg1 caryopses with same developmental stage were ground and then mixed with 5 ml of scintillation solution for monitoring radioactivity using liquid scintillation counter (PerkinElmer, Tri-Carb 2910 TR). For regular ABA feeding of stem, panicles with the two
uppermost internodes of WT and dg1 at the same developmental stage were inserted into 10 ml of water and 10 ml of water with 50 μmol/ml (±)-cis ABA (Sigma-Aldrich, A1049). After 3 and 6 hours feeding at ~34°C, the node, stem, and marked cyropays at 3 DAP were collected for ABA content determination. For 2-H-ABA feeding of flag leaf, panicles with the second uppermost internode were inserted into water, and leaves were inserted into 10 ml of water with 5 μl of 2-H-ABA; then, radioactivity was monitored in cyropays after 12 and 24 hours feeding, as above. All feeding assays were conducted with three biological replicates, and two-tailed Student’s t test was used for comparison.

Expression pattern and protein localization
To investigate expression pattern, we initially performed qPCR using various tissues at different stages and then generated the construct ProDG1::GUS (β-glucuronidase) harboring 2 kb sequence upstream of the ATG of DG1. Primer pairs are listed in table S3. Positive lines were used for GUS staining and immunofluorescence. GUS staining and sectioning were performed according to a standard protocol. Immunofluorescence was performed using flag leaf, node, and rachilla at the flowering stage and GUS antibody (Abcam, AB50148) as described (41); a confocal laser scanning microscope (LSM880, Carl Zeiss) was used for observing signal. For protein localization, the seedling root tip of 35S::CDS dg1-eGFP positive line was used for investigating DG1 protein localization. PMF-64 was used as plasma membrane marker.

Exogenous ABA induction and reverse transcription qPCR
We marked spikelets of WT and dg1 as described above. At the fifth day after marking, panicles of WT and dg1 were inserted into water (negative control) or water with (±)-cis ABA (50 μmol/ml; Sigma-Aldrich, A1049). After 2 hours of feeding, the marked cyropays were used for extracting RNA. Total RNA of cyropays was isolated using a RNA extraction kit with DNA digestion on column (QIAGEN). Total RNA (500 ng) was reverse transcribed by PrimeScript reverse transcription reagent kit with DNA geraser (Takara). cDNA (200 ng/μl) was used for qPCR assay with gene-specific primers and SYBR FAST (KAPA, KK-4601) on real-time system (qTOWER 3G, Analytik, Jena, Germany). ACTIN1 was used as internal control. Four biological replicates were applied for each gene. All primers for qPCR are listed in the table S2.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/3/eabc8873/DC1

View/request a protocol for this paper from Bio-protocol.

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