Identification of the Regulatory Region Responsible for Vascular Tissue–Specific Expression in the Rice *Hd3a* Promoter

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http://dx.doi.org/10.14348/molcells.2018.2320
www.molcells.org

Flowering time is determined by florigens. These genes include, *Heading date 3a* (*Hd3a*) and *Rice FT 1* (*RFT1*) in rice, which are specifically expressed in the vascular tissues of leaves at the floral transition stage. To study the *cis*-regulatory elements present in the promoter region of *Hd3a*, we generated transgenic plants carrying the 1.75-kb promoter fragment of *Hd3a* that was fused to the *β-glucuronidase* (*GUS*) reporter gene. Plants expressing this construct conferred a vascular cell-specific expression pattern for the reporter gene. However, *GUS* was expressed in leaves at all developmental stages, including the early seedling stage when *Hd3a* was not detected. Furthermore, the reporter was expressed in roots at all stages. This suggests that the 1.75-kb region lackings *cis*-elements that regulate leaf-specific expression at the appropriate developmental stages. Deletion analyses of the promoter region indicated that regulatory elements determining vascular cell-specific expression are present in the 200-bp region between -245 bp and -45 bp from the transcription initiation site. By transforming the *Hd3a–GUS* construct to rice cultivar 'Taichung 65' which is defective in *Ehd1*, we observed that Ehd1 is the major regulatory element that controls *Hd3a* promoter activity.

Keywords: *β-glucuronidase (GUS)*, *cis*- elements, Ehd1, Hd3a, promoter, vascular bundle

INTRODUCTION

Flowering is an important biological process in plant reproduction. The responsible molecular mechanisms have been broadly studied in a long-day (LD) flowering plant, Arabidopsis (*Arabidopsis thaliana*) (Imaizumi and Kay, 2006; Tsuji et al., 2011), and in a short-day (SD) flowering plant, rice (*Oryza sativa*) (Imaizumi and Kay, 2006; Lee and An, 2015; Cho et al., 2017). In Arabidopsis, flowering time is determined by the florigen, FLOWERING LOCUS T (FT) (Takada and Goto, 2003; Notaguchi et al., 2008). Its ortholog, *Hd3a*, has been identified in rice through QTL mapping of a cross between 'Nipponbare' and 'Kasalath' cultivars (Yano and Sasaki, 1997). Whereas RNAi lines of *Hd3a* do not flower until 300 days after germination (DAG) (Komiya et al., 2008), overexpression of this gene causes flowering at the callus induction stage (Monna et al., 2002; Hori et al., 2013). This indicates that it has a major role in controlling flowering time. *Hd3a* is preferentially functional under SD conditions and its mRNA is diurnally expressed, showing a peak at approximately 2 h after sunrise (Izawa et al., 2002; Ryu et al., 2009; Lee et al., 2010; Yang et al., 2013).

Florigens are expressed in the leaf phloem and transported to the shoot apical meristem (SAM) to provide a flowering

signal (Corbesier et al., 2007; Jaeger and Wigge, 2007; Lin et al., 2007; Tamaki et al., 2007). Hd3a protein interacts with 14-3-3 protein, generating a complex that moves to the nucleus and binds with OsFD1. This 'florigen activation complex' of Hd3a, OsFD1, and 14-3-3 activates expression of *OsMADS15,* a rice *APETALA1* ortholog (Taoka et al., 2011).

Hd3a and florigens from other species have diverse roles in plant development (Pin and Nilsson, 2012). For example, FT stimulates the opening of stomata in Arabidopsis (Kinoshita et al., 2011). SINGLE FLOWER TRUSS (SFT), a florigen in *Lycopersicon esculentum*, regulates leaf complexity and inflorescence pattern (Lifschitz et al., 2006; Krieger et al., 2010). In rice, *Hd3a* promotes lateral branching (Tsuji et al., 2015), and its expression is induced by Ehd1 (Doi et al., 2004). Phosphorylation of the receiver domain of Ehd1 induces dimerization of the protein, which is required for Ehd1 to stimulate *Hd3a* expression (Cho et al., 2016). In addition, *Hd1* appears to induce *Hd3a* expression directly (Yano et al., 2000).

Ehd1 is controlled by several upstream regulatory factors (Lee and An, 2015). During the early vegetative stages, its expression is inhibited by several repressors, including three CO-like proteins: Hd1, OsCOL4, and Ghd7 (Yano et al., 2000; Lee et al., 2010; Zhao et al., 2012). OsLFL1, SNB, and OsIDS1 are other repressors that also function upstream of *Ehd1* (Lee et al., 2014). At later stages of development, expression of those repressors is reduced due to the function of regulatory genes farther upstream. For example, *Ghd7* expression is inhibited by a chromatin remodeling factor, OsTrx1, and its interacting partner, Ehd3 (Matsubara et al., 2011; Choi et al., 2014). Another chromatin remodeling factor, OsVIL2, inhibits expression of *OsLFL1* by forming a complex with polycomb repressive complex 2 (Yang et al., 2013). Expression of *SNB* and *OsIDS1* is suppressed by increased expression of *microRNA172* at the later stage of vegetative development (Lee et al., 2014).

Several genes involved in controlling flowering time are preferentially expressed in the vascular bundle cells. Another rice florigen gene, *RFT1*, is also expressed in vascular tissues (Komiya et al., 2009). An FT homolog from maize (*Zea mays*), *CENTRORADIALIS8*, is also specifically expressed in the leaf vascular cells (Meng et al., 2011). Similar to these florigen genes, *Ehd1* is specifically expressed in the vascular bundle (An et al., 2004; Endo et al., 2005; Giakountis and Coupland, 2008). *Ghd7* expression is high in the vascular cells of leaf blades but low in the cells of roots and leaf sheaths (Xue et al., 2008).

In addition to flowering genes, numerous others are preferentially expressed in vascular tissues (Hernandez-Garcia and Finer, 2014). They include various genes from viruses and bacteria that are preferentially expressed in the phloem. Genes from rice tungro bacilliform virus (RTBV) are specifically expressed in phloem cells (Yin and Beachy, 1995), while genes from commelina yellow mottle virus are expressed in vascular bundles (Medberry et al., 1992). The activity of a promoter from wheat dwarf geminivirus is specific to vascular cells (Dinant et al., 2004). Moreover, promoters from *Agrobacterium* genes *rolB* and *rolC* are expressed only in the vasculature (Schmülling et al., 1989).

Genes involved in nutrient transport show vascular specificity. For example, *Arabidopsis thaliana Sucrose Transporter 2* (*AtSUC2*), which encodes a sucrose-H$^+$ symporter needed for long-distance sucrose transport, is expressed in the phloem cells of photosynthetic leaves (Stadler and Sauer, 1996; Gottwald et al., 2000). In rice, *OsSUT1* is expressed in the phloem cells of leaf blades and scutellar vascular bundles (Scofield et al., 2007). Other sugar transporters, i.e., *sugars will eventually be exported transporters* (*SWEET*s), are expressed in phloem cells, including *AtSWEET11* and *AtSWEET12* in the rosette leaves of Arabidopsis (Chen et al., 2012) and the *OsSWEET11* promoter, which specifically induces the expression of *β-glucuronidase* (*GUS*) in the phloem cells of rice (Ma et al., 2017).

A promoter from *glutamine synthetase 3A* (*GS3A*) in *Pisum sativum*, encoding a protein that functions in ammonia assimilation, shows phloem specificity in transgenic *Nicotiana tabacum* and *Medicago sativa* (Brears et al., 1991; Pageau et al., 2006). A gene encoding *Arabidopsis* H$^+$-ATPase isoform 3 (AHA3), a major target for 14-3-3 proteins, is expressed exclusively in the phloem cells of the leaf, root, stem, and flower at all developmental stages (DeWitt et al., 1991; Alsterfjord et al., 2004). Finally, two genes encoding rice phloem proteins RPP16 and RPP17, which are homologous to viral movement proteins, are specifically expressed in the phloem parenchyma cells of the leaf blade and root (Asano et al., 2002).

In this study, we identified a 200-bp region of the *Hd3a* promoter that directs the expression of the GUS marker gene within the parenchyma cells of vascular tissues.

MATERIALS AND METHODS

Plant materials and growth conditions
Plants of *Oryza sativa* 'Longjing27' and 'Taichung65' were used. Seeds of those cultivars were germinated either on a 1/2 Murashige and Skoog (MS) medium containing 3% sucrose or directly in soil as previously reported (Cho et al., 2016). The seedlings were then cultured in controlled growth rooms under SD conditions (12 h light at 28℃/12 h darkness at 22℃; 50% humidity).

Generation of transgenic plants carrying *pHd3a-GUS* fusions
The 1.75-kb fragment of the *Hd3a* promoter region was amplified with the pair of primers as previously reported (Ishikawa et al., 2005; Tamaki et al., 2007). Genomic DNA from 'Dongjin' rice was used as template. Three deletions from that fragment were generated using the primers listed in Supplementary Table S1 (Fig. 1A). The promoter fragments were inserted in the *Kpn*I and *Bam*HI restriction sites of the pGA3519 binary vector that carries the *GUS* coding region followed by the *nopaline synthase* terminator (Yoon et al., 2014). In the constructs, the *Hd3a* coding region located 153 bp from the ATG start codon was fused to the *GUS* coding region within the same reading frame. All constructs were confirmed by sequencing the insertion. The binary vectors were transformed into Agrobacterium tumefaciens LBA4404 (An et al., 1989). Transgenic rice plants

were generated via the *Agrobacterium*-mediated co-cultivation method as previously reported (Lee et al., 1999), using embryonic calli derived from mature seeds of 'Longjing27' and 'Taichung65' rice. Plants carrying this *Hd3a* promoter–GUS chimeric gene were selected by PCR using genomic DNA isolated from hygromycin-resistant seedlings as templates and primers located in the *Hd3a* promoter and *GUS* coding region (Supplementary Table S1). These transgenics were cultured in the growth room until maturity. After their seeds were harvested, we selected two independent transgenic lines with high GUS expression.

Histochemical GUS staining

Histochemical GUS staining was performed as previously described (Yoon et al., 2017). Seeds of the transgenic plants were germinated on an MS medium containing 50 µg L^{-1} of hygromycin and the seedlings were cultured in the controlled growth room under SD conditions. Leaf blades were collected and placed in a GUS-staining solution containing 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide, 50 mM sodium phosphate (pH 7.0), 0.1% Triton X-100, 10 mM EDTA, 1% DMSO, and 1.0 mg mL^{-1} of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid. After vacuum-infiltration for 30 min, the samples were incubated at 37℃ for 10 h. Chlorophylls were removed by incubating in 70% ethanol at 65℃. The tissues were dehydrated in an ethanol series (50, 70, 90, and 100%) and then treated with tert-butyl alcohol. They were either observed immediately under a BX61 optical microscope (Olympus, http://www.olympus-global.com/en/)

or fixed in paraffin and sectioned (10 µm thickness) with a microtome (Model 2165; Leica Microsystems, http://www.leica-microsystems.com/) before being observed under that microscope.

RNA isolation and quantitative RT-PCR analyses

Leaf blades and root tissues were harvested at 7, 14, 21, 28, and 35 DAG from SD-grown 'Longjing27' plants. Total RNA was isolated from the samples using RNAiso Plus (TaKaRa, Shiga, Japan; http://www.takarabio.com) and qualified by a Nano Nanodrop ND-2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA; http://www.nanodrop.com) as described previously (Cho et al., 2016). Complementary DNA (cDNA) was made with 10 ng of the oligo (dT)$_{18}$ primer, 2.5 mM deoxy ribonucleotide triphosphates, and Moloney murine leukemia virus reverse transcriptase. Quantitative real-time RT-PCR (qRT-PCR) was performed with the synthesized cDNAs as templates and primers listed in Supplementary Table S1, using SYBR Premix Ex Taq™ II (TaKaRa) and the Rotor-Gene 6000 instrument system (Corbett Research, Sydney, Australia; http://www.corbettlifescience.com). Rice Ubi1 served as an internal control. At least three biological replicates were analyzed. We used the data only when the melting curve showed a single sharp peak.

RESULTS

Expression patterns of the *Hd3a* promoter–*GUS* fusion

Transgenic plants containing the 1.75-kb *Hd3a* promoter
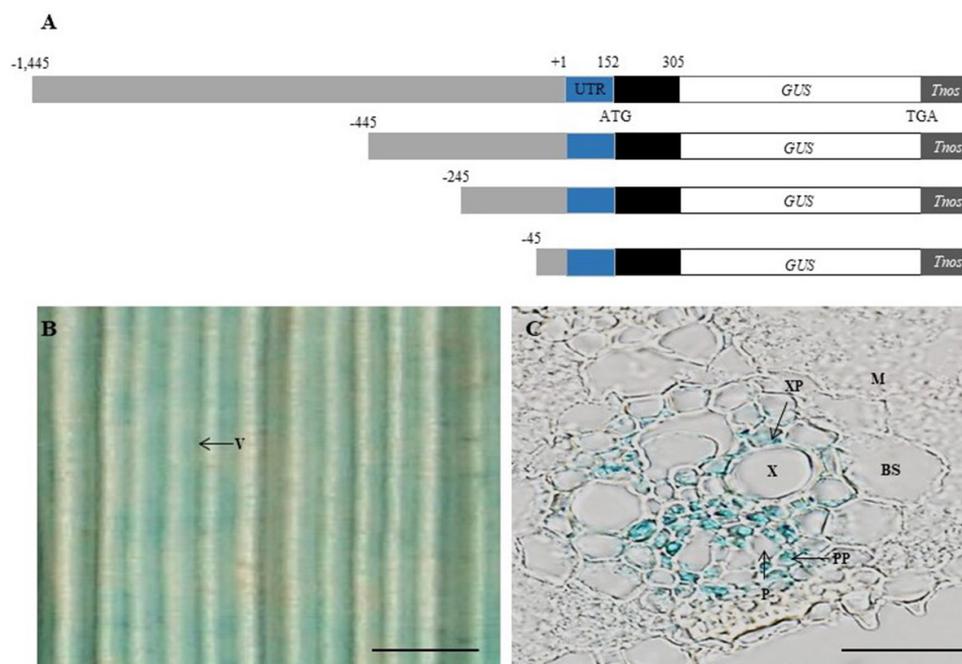
Fig. 1. Schematic representation of *Hd3a* promoter-GUS reporter constructs and histochemical localization of GUS. (A) *Hd3a* genomic fragments comprising -1,445, -445, -245, and -45 bp promoter regions (grey box), the 152-bp 5' UTR (blue box) and 153-bp coding region of *Hd3a* (black box) were connected to *GUS* coding region and *nopaline synthase* terminator (*Tnos*). (B) Leaf blade of -1,445 *Hd3a* promoter–*GUS* transgenic at ZT 1 on 35 DAG. (C) Transverse section of leaf blade in Panel B. BS, bundle sheath cells; M, mesophyll cells; P, phloem; PP, phloem parenchyma; V, vascular bundle; X, xylem; XP, xylem parenchyma. Scale bars =1 mm (B) and 20 µm (C).

showed reporter gene expression in the phloem and xylem parenchyma cells of the leaf blades (Hayama et al., 2003; Tamaki et al., 2007). However, the organ-preferential expression patterns of the construct were not examined. An identical promoter fragment was isolated that consisted of the 1,445-bp promoter region, the 152-bp 5' untranslated region (UTR), and the 153-bp coding region of *Hd3a* (Fig. 1A). The promoter fragment was placed upstream of *GUS* in the same reading frame. Several transgenic rice plants expressing the fusion molecules were generated and two independent lines of the primary transgenics were grown under SD conditions. Because expression of *Hd3a* peaks at approximately 35 DAG under short days (Komiya et al., 2008; Ryu et al., 2009; Lee et al., 2010), we conducted a histochemical GUS assay of leaf blades from the transgenic plants at that time point and found that the *GUS* reporter gene was expressed (Fig. 1B). Cross-sectioning revealed *GUS* staining in the parenchyma cells within the vascular bundles (Fig. 1C). This pattern of tissue expression is similar to that previously reported for *Hd3a* in rice (Tamaki et al., 2007).

We also examined the developmental expression patterns of *Hd3a* and *GUS* in transgenic plants carrying the *Hd3a* promoter–*GUS* construct at 7, 14, 21, 28, and 35 DAG (Fig. 2). Transcript levels of *Hd3a* were low during the early developmental stages but began to increase at 21 DAG before reaching a high level at 35 DAG, as previously reported (Fig. 2A). However, the *GUS* reporter was constitutively expressed in the leaves throughout all five stages (Fig. 2B). Histochemical analyses also indicated that the transgenics expressed the reporter protein constitutively during their development (Figs. 3A). This demonstrated that the 1.75-kb fragment did not contain the regulatory element needed to determine a proper pattern of developmental expression for Hd3a. To examine whether *Hd3a* is preferentially expressed in a certain part of the leaf, we measured expression levels of the gene by qRT-PCR from three different positions. Transcript levels of this gene were similar among samples from the top, middle, and bottom portions of the leaf blade (Fig. 3I). Similar results were obtained for expression levels of the *GUS* reporter (Fig. 3J). Histochemical GUS analyses also revealed that the reporter was expressed at a similar level regardless of position (Figs. 3K-3M).
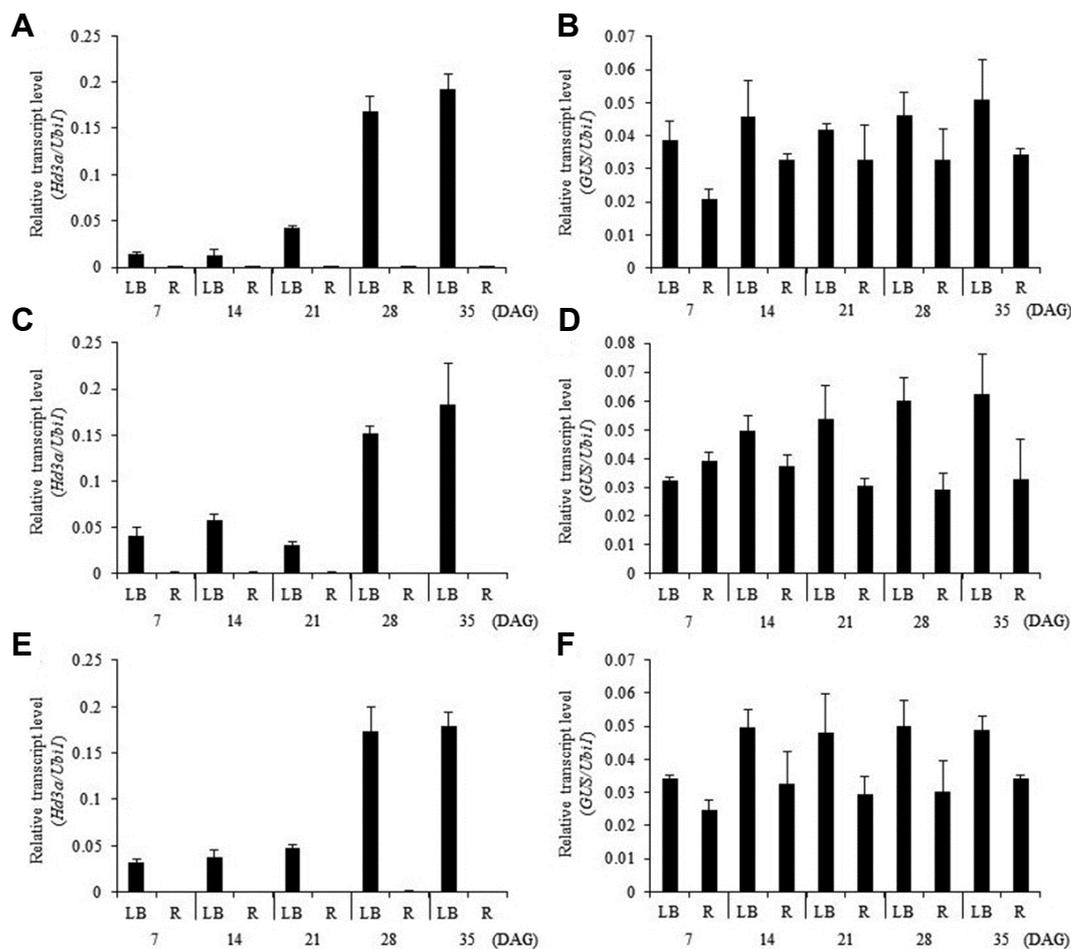
Fig. 2. Developmental expression patterns of *Hd3a* and *GUS* in leaf blade (LB) and root (R). Quantitative real-time RT-PCR analyses of transcript abundance of *Hd3a* (A, C, and E) and *GUS* (B, D, and F) from -1,445 *Hd3a* promoter–*GUS* (A, B), -445 *Hd3a* promoter–*GUS* (C, D), and -245 *Hd3a* promoter–*GUS* (E, F). Error bars represent standard deviations. n = 6. DAG, days after germination.

Transcript of *Hd3a* was not found in the roots at any time during the vegetative growth period (Fig. 2A). The same has been reported previously (Tamaki et al., 2007). However, *GUS* transcript was detectable in roots from transgenic plants expressing the reporter gene under the 1.75-kb *Hd3a* promoter (Fig. 2B), and a considerable amount of transcript accumulated in the roots during all five developmental stages. GUS staining also showed that the reporter was expressed in roots at all stages (Figs. 4A-4E). These results suggested that the promoter fragment did not contain the regulatory elements needed to suppress *Hd3a* expression in the roots.

Identification of the region responsible for vascular cell-specific expression

Although the 1.75-kb fragment did not contain elements for organ and developmental specificities, it did carry those for vascular-tissue specificity. To find the responsible region, we made three deletion constructs (Fig. 1A). The first was a deletion of 1,000-bp upstream region from the 1.75-kb fragment, leaving the 445-bp promoter region. The second was a further 200-bp deletion that resulted in the 245-bp promoter region. The third construct was made by an additional 200-bp deletion from the second construct, leaving only the 45-bp promoter region. All three constructs retained the 152-bp UTR and 153-bp coding region of *Hd3a*.

From several transformed plants, we selected two independent plants from each deletion and stained for GUS activity. Their progeny were grown under SD conditions and their GUS expression patterns were studied at 7, 14, 21, 28, and 35 DAG. The first- and second-deletion constructs showed reporter gene expression that was specific to the vascular tissue in the leaves at all developmental stages (Figs. 3B and 3C). However, plants carrying the third-deletion construct did not have any detectable level of GUS activity (Figs. 3D). Cross-sections of the leaf blades revealed staining in both phloem parenchyma cells and xylem parenchyma cells within the vascular bundles of the three longer constructs (Figs. 3E, 3F, and 3G), but not from the smaller fragment (Fig. 3H). Mesophyll cells and bundle sheath cells were not stained. This indicated that elements responsible for vascular cell-specific expression in the leaves were located in the 200-bp region between -45 and -245 from the transcription initiation site.
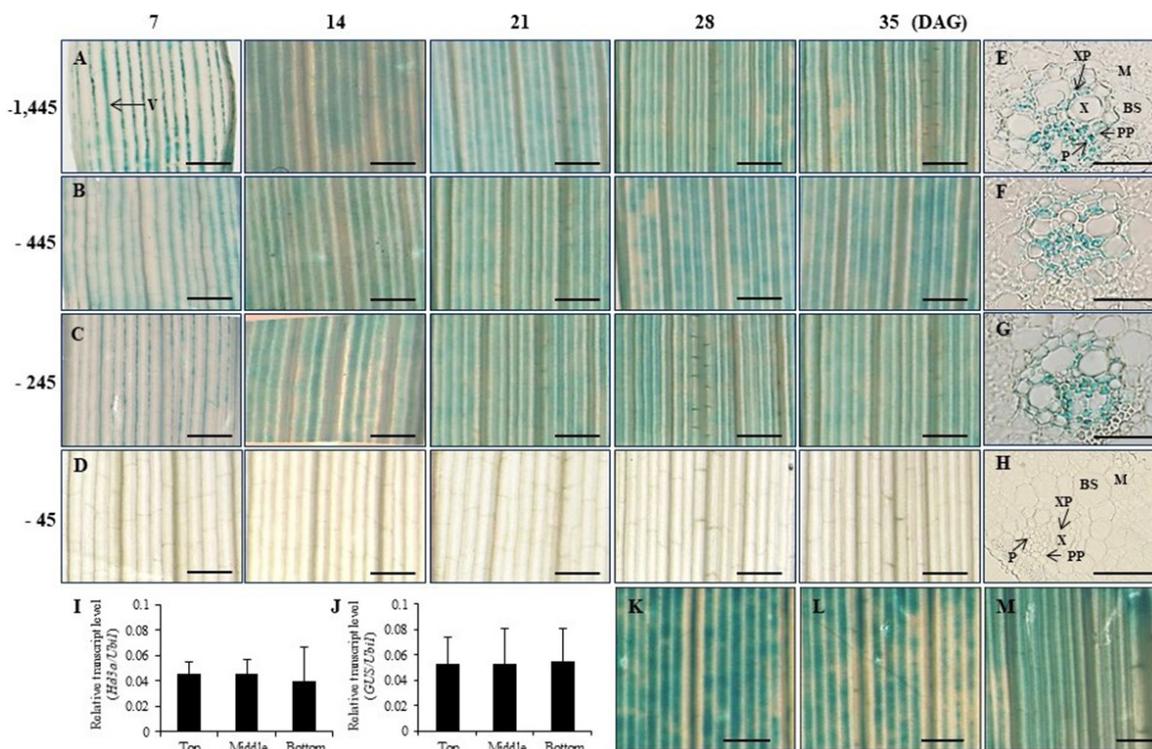
Fig. 3. Histochemical localization of GUS activity in leaf blades at five developmental stages and expression pattern of *Hd3a* at 3 positions along leaf blade top, middle and bottom. Leaf blades of transgenic rice plants carrying -1,445 *Hd3a* promoter–*GUS* (A), -445 *Hd3a* promoter–*GUS* (B), -245 *Hd3a* promoter–*GUS* (C), and -45 *Hd3a* promoter–*GUS* (D). Cross-sections of leaf blades at 35 DAG of -1,445 (E), -445 (F), -245 (G), and -45 (H) construct. Quantitative real-time RT-PCR analyses of *Hd3a* transcript (I) and -1,445 *Hd3a* promoter-driven *GUS* transcript (J) at top, middle, and bottom at ZT 1 on 35 DAG. Histochemical localization of GUS activity in leaf blades in Panel A at top (K), middle (L), and bottom (M). Mean values from two biological replicates are shown. Error bars represent standard deviation in (I and J). n = 6. Scale bars = 20 μm in Panels E, F, G and H; 1 mm in others. BS, bundle sheath cells; M, mesophyll cells; P, phloem; PP, phloem parenchyma; V, vascular bundle; X, xylem; XP, xylem parenchyma.
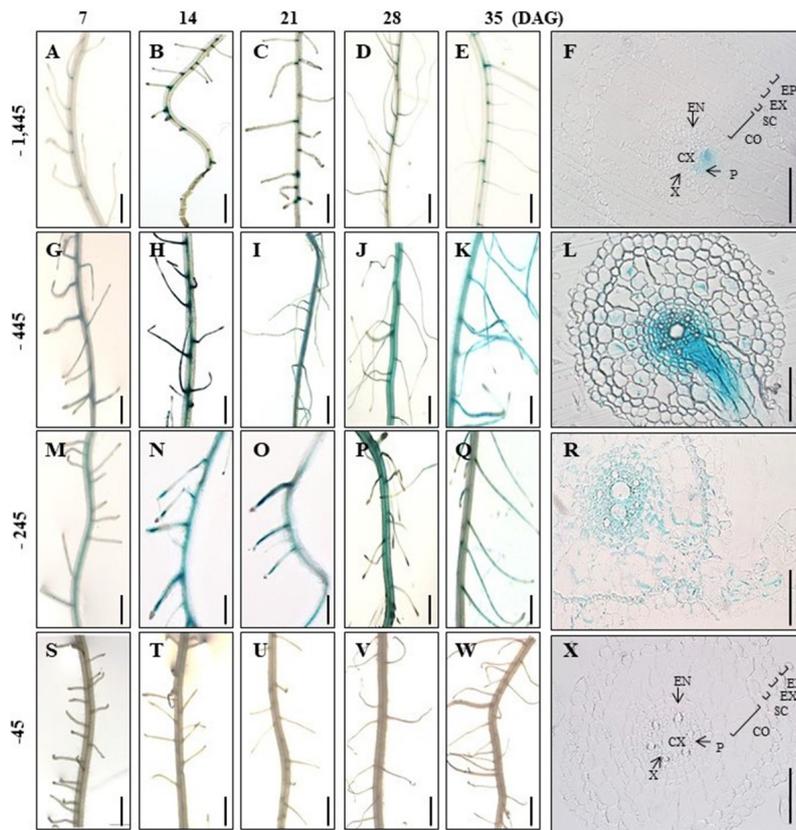
Fig. 4. Histochemical localization of GUS activity in roots at five developmental stages. (A-E) Roots of transgenic plants carrying -1,445 *Hd3a* promoter–*GUS* at 7 (A), 14 (B), 21 (C), 28 (D), and 35 (E) DAG. (G-K) Roots of transgenic plants carrying -445 *Hd3a* promoter–*GUS* at 7 (G), 14 (H), 21 (I), 28 (J), and 35 (K) DAG. (M-Q) Roots of transgenic plants carrying -245 *Hd3a* promoter–*GUS* at 7 (M), 14 (N), 21 (O), 28 (P), and 35 (Q) DAG. (S-W) Roots of transgenic plants carrying -45 *Hd3a* promoter–*GUS* at 7 (S), 14 (T), 21 (U), 28 (V), and 35 (W) DAG. Cross-sections of roots at 35 DAG from -1,445 *Hd3a* promoter–*GUS* (F), -445 *Hd3a* promoter–*GUS* (L), -245 *Hd3a* promoter–*GUS* (R), and -45 *Hd3a* promoter–*GUS* (X). Co, cortex; CX, central xylem; En, endodermis, Ep, epidermis; Ex, exodermis; P, phloem; Sc, sclerenchyma; V, vascular bundle; X, xylem. Scale bars = 50 μm in Panels F, L, R, and X; 5 mm in others.
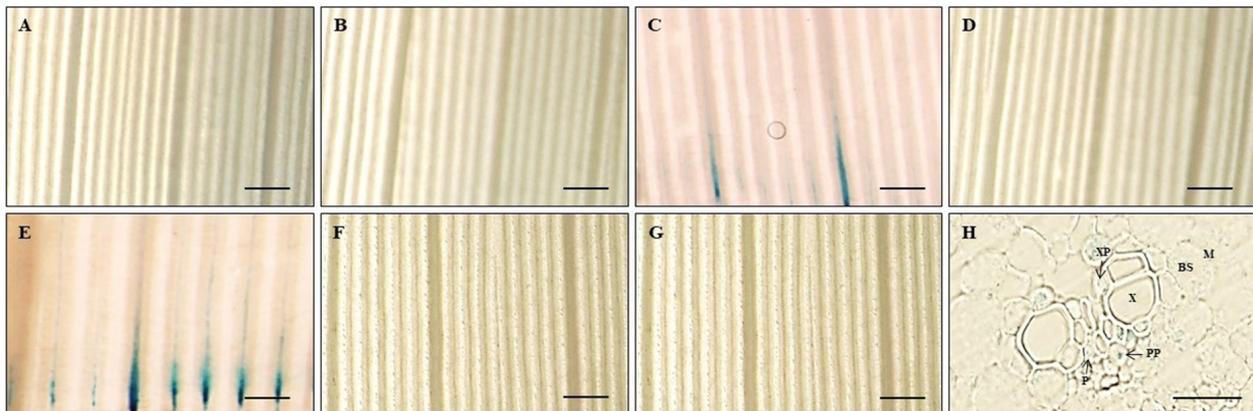
Fig. 5. Histochemical analyses of GUS in 'Taichung65' rice carrying -1,445 *Hd3a* promoter-*GUS*. (A-G) Leaf blades of 7 independent plants were analyzed at 45 DAG. (H) Cross-section of leaf blade in (E). Scale bars: 1 mm (A-G) or 20 μm (H). DAG, days after germination. BS, bundle sheath cells; M, mesophyll cells; P, phloem; PP, phloem parenchyma; V, vascular bundle; X, xylem; XP, xylem parenchyma.

Analyses of roots from the transgenic plants also showed that plants expressing the first- and second-deletion constructs presented vascular tissue-preferential expression at all stages (Figs. 4G-4K, 4M-4Q). However, GUS expression was not detected from plants carrying the third construct (Figs. 4S-4W). This demonstrated that *cis*-elements for vascular-preferential expression in the roots also resided within the 200-bp region identified from the leaf analyses.

Role of Ehd1 in expression of *Hd3a*–*GUS*

Hd3a functions immediately downstream of *Ehd1* (Doi et al., 2004). To examine whether the expression of the 1.75-kb promoter fragment was dependent upon Ehd1, we transformed the 1.75-kb *Hd3a* promoter–*GUS* construct into 'Taichung65' rice, which carried a defective *Ehd1*. Analyses of seven independent transgenic plants showed that five displayed no GUS activity regardless of developmental stage

(Figs. 5A, 5B, 5D, 5F and 5G). However, a low level of GUS staining was observed in vascular tissues from two plants (Figs. 5C and 5E). These findings indicated that Ehd1 is a major regulatory factor for phloem-specific expression of *Hd3a*.

DISCUSSION

We studied the 1.75-kb promoter fragment of *Hd3a* to investigate *cis*-regulatory elements present in the region. Our objective in using this fragment was to demonstrate that the promoter-driven GFP reporter protein is produced in leaf phloem and moves to the SAM. Our findings support earlier conclusions that Hd3a is a mobile signal that transmits the flowering signal from leaves to the shoot apex (Tamaki et al., 2007; Komiya et al., 2009). In this study, the -1.75-kb promoter-driven reporter gene was expressed in the vascular bundles of leaves and in the roots at all developmental stages, but no GUS activity was observed in the SAM (Supplementary Fig. S1). These results are quite comparable to the tissue specificity of *FT* expression in Arabidopsis (Takada and Goto, 2003). In *Arabidopsis,* the 5.7-kb *FT* promoter sequence upstream of the translation start site is required for mediating spatial and temporal expression of the gene (Adrian et al., 2010). Shortening the promoter to 4.0 kb obliterates that ability, indicating that a promoter region of that size is not large enough to determine promoter specificity. Therefore, all of these results suggest that the necessary regulatory elements occur in the region between 4 kb and 5.7 kb for *FT* promoter specificity.

Although the 1.75-kb *Hd3a* promoter fragment lacks elements controlling leaf specificity and the developmental expression pattern of the gene, it confers vascular cell-specific expression within the leaf blades. To identify the cis-regulatory elements involved in the latter, we generated truncated promoters and found that the removal from -1445 to -245 did not affect vasculature specificity. However further deletion of 200 bp abolished such specificity. This indicated that the 200-bp region between -245 and -45 carries the *cis*-elements needed for cell specificity.

Using the rice RTBV promoter, Yin et al. (1997) have identified the GCA repeat element GCATC (N)9 GCATC that is required for phloem-specific expression. Two similar elements, GCATA(N)$_{10}$GCATA and GCACC(N)$_8$GCAGC, have been identified from the phloem-specific promoters of Arabidopsis *H+-ATPase isoform 3* (*AHA3*) and maize *sucrose synthase-1* (*Shl*), respectively (Werr et al., 1985; DeWitt et al., 1991). The GCA repeat is located within the phloem-specific region at $^{-193}$ GCAGA(N)$_9$GCATG^{-175} of the *Hd3a* promoter region. Another phloem-specific element, the GATA motif, has been identified from the CaMV 35S promoter that is strongly expressed in vascular tissues (Lam and Chua, 1989). That element is also present in the *AHA3* promoter as the GAGATGATA motif (Yin et al., 1997) and in the phloem-specific *glutamine synthetase 3A* (*GS3A*) promoter as the TAGTAGATA motif (Brears et al., 1991). The GATA motif exists within the phloem-specific region at $^{-144}$ GAGATGATA^{-136} of the *Hd3a* promoter. Further study is needed to examine whether these elements indeed determine the vascular specificity of that promoter.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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