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d14, a Strigolactone-Insensitive Mutant of Rice, Shows an Accelerated Outgrowth of Tillers

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Recent studies using highly branched mutants of pea, Arabidopsis and rice have demonstrated that strigolactones, a group of terpenoid lactones, act as a new hormone class, or its biosynthetic precursors, in inhibiting shoot branching. Here, we provide evidence that *DWARF14* (*D14*) inhibits rice tillering and may act as a new component of the strigolactone-dependent branching inhibition pathway. The *d14* mutant exhibits increased shoot branching with reduced plant height like the previously characterized strigolactone-deficient and -insensitive mutants *d10* and *d3*, respectively. The *d10-1 d14-1* double mutant is phenotypically indistinguishable from the *d10-1* and *d14-1* single mutants, consistent with the idea that *D10* and *D14* function in the same pathway. However, unlike with *d10*, the *d14* branching phenotype could not be rescued by exogenous strigolactones. In addition, the *d14* mutant contained a higher level of 2′-*epi*-5-deoxystrigol than the wild type. Positional cloning revealed that *D14* encodes a protein of the α/β-fold hydrolase superfamily, some members of which play a role in metabolism or signaling of plant hormones. We propose that D14 functions downstream of strigolactone synthesis, as a component of hormone signaling or as an enzyme that participates in the conversion of strigolactones to the bioactive form.

Keywords: *DWARF 14* • Hormone signaling • Rice • Shoot branching • Strigolactone.

Abbreviations: BAC, bacterial artificial chromosome; CCD, carotenoid cleavage dioxygenase; dCAPS, derived cleaved amplified polymorphic sequence; *epi*-5DS, 2′-*epi*-5-deoxystrigol; GUS, β-glucuronidase; HLS, hormone-sensitive lipase; LC/MS-MS, liquid chromatography-quadruple/time-of-flight tandem mass spectrometry; RT–PCR, reverse transcription–PCR; SA, salicylic acid; SABP2, salicylic acid-binding protein 2; SNP, single nucleotide polymorphism; SSR, simple sequence repeat; X-Gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide.

Introduction

The pattern of shoot branching is one of the critical determinants of the aerial plant architecture (Steeves and Sussex 1989). Shoot branching starts from the generation of axillary buds in the axil of leaves. Axillary buds often become dormant after they are formed, and resume their outgrowth later in development. The fate of axillary buds, to grow or to remain dormant, is governed by the complex interplay of environmental and endogenous cues (McSteen and Leyser 2005).

In the last decade, remarkable progress has been made in understanding the molecular basis of the control of shoot branching through studies of a set of highly branched mutants isolated from pea, Arabidopsis and petunia (for reviews, see Beveridge 2006, Ongaro and Leyser 2008). Four mutants, *max1–max4*, that work in a single genetic pathway were identified in Arabidopsis and five mutants, *ramosous1* (*rms1*) to *rms5* were reported in pea. Extensive physiological analysis and grafting experiments of these mutants revealed the involvement of a graft-transmissible branch-inhibiting hormone in the control of shoot branching. Although the postulated hormone had not been identified, analysis of the mutants inferred that they were deficient in either

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the synthesis or signaling of the hormone. Among the four Arabidopsis genes, *MAX1*, *MAX3* and *MAX4* were predicted to be involved in the production of the hormone, while *MAX2* was predicted to play a role in its perception or signaling. Similarly, in pea, *RMS1* and *RMS5* appear to be involved in the biosynthesis of the hormone while *RMS3* and *RMS4* would be involved in the signaling pathway of the hormone.

Molecular identification of *MAX* genes has demonstrated the validity of the prediction (Stirnberg et al. 2002, Sorefan et al. 2003, Booker et al. 2004, Booker et al. 20005). Consistent with the prediction that *MAX1*, *MAX3* and *MAX4* were involved in the synthesis of the hormone, they encode proteins that function as enzymes. *MAX1* encodes a class III cytochrome P450, CYP711A1 (Booker et al. 2005). *MAX3* and *MAX4* encode carotenoid cleavage dioxygenases (CCDs), leading to the possibility that the branch-inhibiting hormone is synthesized from carotenoids (Sorefan et al. 2003, Booker et al. 2004). On the other hand, *MAX2*, a putative signaling component, encodes a member of the F box protein family that is often involved in the signal transduction of plant hormones (Stirnberg et al. 2002). *RMS1*, *RMS4* and *RMS5* were shown to be orthologous to *MAX4*, *MAX2* and *MAX3*, respectively (Sorefan et al. 2003, Foo et al. 2005, Jhonson et al. 2006). Petunia *Decreased apical dominance1* (*DAD1*) was also shown to be an ortholog of *MAX4/RMS1* (Snowden et al. 2005), indicating that the regulatory pathway for the inhibition of branch shoot outgrowth is well conserved across different species.

Rice is another useful system to study the control of axillary bud outgrowth (Ishikawa et al. 2005). One advantage of using rice is that the phenotype of the branching mutants can be easily distinguished at early stages of plant development. Previously, we reported five tillering dwarf mutants, *dwarf3* (*d3*), *d10*, *d14*, *d17* and *d27* (Ishikawa et al. 2005). *D3* and *D10* have been identified as orthologs of *MAX2/RMS4* and *MAX4/RMS1/DAD1*, respectively, extending the conservation of the mechanism controlling shoot branching to monocots (Ishikawa et al. 2005, Arite et al. 2007). In addition, it was shown that *high tillering dwarf1* (*htd1*) and *d17* are mutant alleles of the rice *MAX3/RMS5* ortholog (Zou et al. 2006, Umehara et al. 2008). Like *RMS1* and *DAD1*, the *D10* mRNA level is up-regulated in the mutants in which the branch-inhibiting hormone pathway is disrupted (Arite et al. 2007). *D14* and *D27* genes remain to be cloned and characterized; however, significant up-regulation of *D10* expression was found in *d14* and *d27* as in *d3*, *d10* and *d17* mutants, implying that *D14* and *D27* most probably are involved in the same branch-inhibiting pathway.

The chemical nature of the predicted branch-inhibiting hormone was revealed recently from studies of pea and rice (Gomez-Roldan et al. 2008, Umehara et al. 2008). Application of strigolactones rescued mutant phenotypes of *d10/rms1* and *d17/rms5*, hormone-deficient mutants, but not that of *d3/rms4*, putative signaling mutants. It was confirmed that endogenous strigolactones were indeed reduced to an undetectable level in roots of *d10* and *d17*, whereas the level was elevated in *d3*, most probably as a result of a feedback regulation. Based on these results, it was concluded that strigolactones or their downstream metabolites constitute the mobile branch-inhibiting hormone. Strigolactones are a group of terpenoid lactones and are structurally related to strigol, the first member of this class of terpenes (**Fig. 1**). Prior to this discovery, strigolactones have been identified twice, first as a trigger of parasitic seed germination (Cook et al. 1972) and then as an inducer of mycorrhizal hypha branching (Akiyama et al. 2005). This discovery, combined with previous findings, suggested that strigolactones may act not only as inhibitors of shoot branching but also as mediators of nutrition uptake and the control of plant development.

Regardless of the remarkable progress which has been made, still little is known about the molecular mechanisms by which strigolactones act to control shoot branching. Also, it has yet to be determined whether strigolactones are the active form of the hormone or if they are precursors that need to be converted to the bioactive form in order to function in the plant. Here, we report that rice *d14-1* shows reduced sensitivity to strigolactones and accumulates elevated levels of endogenous strigolactones. Positional cloning revealed that *D14* encodes a protein of the α/β-hydrolase superfamily. We propose that *D14* is a novel player in the strigolactone pathway and functions at a late step of active hormone synthesis or in its signaling pathway.

Results

d14 is a new strigolactone-insensitive mutant

d14 is a recessive tillering dwarf mutant (Ishikawa et al. 2005). As we reported previously, *D10* expression, which is subjected to a feedback regulation, is elevated in *d14-1*, suggesting that *D14* probably functions in the strigolactone pathway along with *D3*, *D10* and *HTD1/D17* (Arite et al. 2007). To confirm this notion, we produced *d10-1* and *d14-1* double mutants and analyzed their phenotype. Both *d10-1* and *d14-1* show increased branching and dwarf phenotypes, and they are indistinguishable from each other in appearance

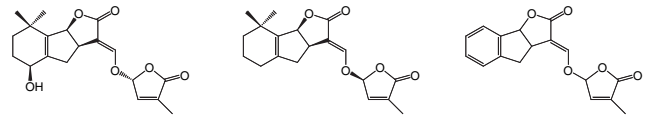

Fig. 1 Chemical structures of strigolactones.

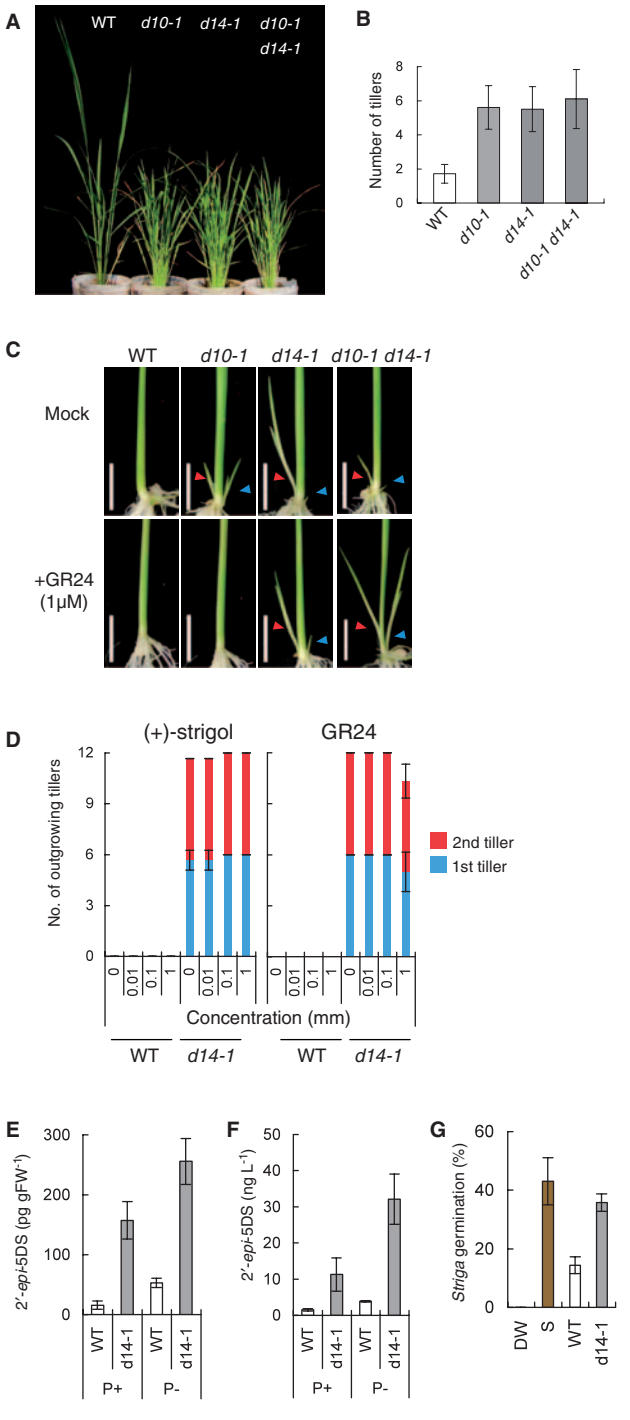

Fig. 2 Analysis of strigolactones in the *d14-1* mutant. (A) Phenotype of *d10-1 d14-1* double mutants at 6 weeks after germination. (B) Numbers of tillers in wild-type (WT), *d10-1*, *d14-1* and *10-1 d14-1* double mutant plants at the stage of emergence of the eighth leaf. Error bars represent the SD ($n = 8$). (C) Response of *d10-1*, *d14-1* and *d10-1 d14-1* to the application of GR24. Two-week-old WT and mutant plants were treated with 1 μM GR24. Tillers in the axil of the first (blue arrowhead) and second (red arrowhead) leaves do not grow in WT plants but grow in mutants. The outgrowth of tillers in *d10-1*, but not in *d14-1* or *d10-1 d14-1*, was suppressed by application of GR24. Scale bars = 1 cm. (D) Response of *d14-1* to (+)-strigol (left) and GR24 (right) applications. Total numbers of tillers showing outgrowth (>2 mm) in six seedlings are shown. Blue and red bars indicate the first and second leaf buds, respectively. Error bars represent the SD ($n = 3$). (E) LC/MS-MS analysis of endogenous levels of *epi*-5DS in the WT and *d14-1* mutant in the presence (P+) or absence (P−) of inorganic phosphate. Error bars represent the SD ($n = 3$). (F) LC/MS-MS analysis of *epi*-5DS levels in root exudates of the WT and *d14-1* mutant in the presence (P+) or absence (P−) of inorganic phosphate. Error bars represent the SD ($n = 3$). (G) Germination rate of Striga (*Striga hermonthica*) seeds in the presence of root exudates of the WT and *d14-1* mutant. Approximately 50 seeds were used in each experiment. DW, distilled water; S, 0.1 μM (+)-strigol. Error bars represent the SD ($n = 3$).

(Ishikawa et al. 2005). The double mutant plants also exhibited the same phenotype as *d10-1* and *d14-1* (**Fig. 2A, B**), suggesting that *D14* works in the same strigolactone pathway.

To understand the role of *D14*, we examined whether the *d14-1* mutant phenotype could be rescued by the application of GR24, a synthetic strigolactone analog (**Fig. 2C**). As reported previously, application of 1 μM GR24 to the hydroponic medium completely inhibited the outgrowth of the first and second axillary buds of *d10-1*, a strigolactone-deficient mutant (Umehara et al. 2008). In contrast, growth of the first and second leaf buds of *d14-1* was not suppressed by the same treatment with GR24. Application of GR24 to *d10-1 d14-1* double mutant plants did not rescue the branching phenotype (**Fig. 2C**). The sensitivity of the *d14-1* mutant to strigolactones was examined in more detail (**Fig. 2D**). The responses of *d14-1* plants to (+)-strigol, a natural strigolactone, and GR24 were measured in parallel with other *d* mutants, and the results for *d3-1*, *d10-1* and *d17-1* mutants have been published previously (Umehara et al. 2008). Outgrowth of tillers in *d14-1* was not suppressed by (+)-strigol or GR24, mimicking the responses of *d3-1*, a strigolactone-insensitive mutant (Umehara et al. 2008). This indicates that *d14-1* has reduced strigolactone sensitivity. However, our current experiments do not distinguish whether *d14-1* mutant plants are totally insensitive to strigolactones or whether their tiller bud outgrowth is still inhibited in response to higher doses of strigolactones.

Endogenous levels of 2′-*epi*-5-deoxystrigol (*epi*-5DS, **Fig. 1**), a native strigolactone of rice, were determined in roots by liquid chromatography-quadruple/time-of-flight tandem mass spectrometry (LC/MS-MS). We have previously reported that *d3-1* roots accumulated much higher levels of *epi*-5DS than do wild-type roots when phosphate ion (P) is depleted in the medium, whereas *epi*-5DS is undetectable in

d10-1 irrespective of the nutrient conditions (Umehara et al. 2008). We show here that the levels of *epi*-5DS in *d14-1* roots and root exudates were significantly higher than those in wild-type roots under both P-rich and P-deficient conditions (**Fig. 2E, F**).

Next, we carried out a highly sensitive parasitic seed germination assay using *Striga hermonthica* seeds whose germination is induced by strigolactones (**Fig. 2G**). *d14-1* root exudates in P-deficient medium showed a stronger germination-stimulating activity than did those of the wild type, in accordance with the increase in *epi*-5DS levels determined by LC-MS/MS.

Isolation of the D14 gene by positional cloning

We have isolated the *D14* gene by map-based cloning. Rough mapping delimited the *D14* locus within a 3 cM region on chromosome 3 (**Fig. 3A**). Subsequently, the locus was further narrowed to a 3.6 kb interval on bacterial artificial chromosome (BAC) clone AC146702 by fine mapping. We compared sequences of the eight putative genes predicted in this region between the wild-type and *d14-1*. In the *d14-1* mutant, a 615 bp insertion was found in an intron of a gene encoding a putative α/β-hydrolase superfamily protein (Os03g0203200/AK070827) (**Fig. 3B**). The inserted sequence was shown to be an endogenous active transposon *nDart1* (Tsugane et al. 2006) (**Supplementary Fig. S1**). Reverse transcription–PCR (RT–PCR) analysis showed that the mRNA of Os03g0203200 was below the level of detection in *d14-1* (**Fig. 3C**), suggesting strongly that the insertion of *nDart1* into Os03g0203200 is the cause of the *d14-1* phenotype. To confirm this, we introduced a 4.8 kb genome fragment of Os03g0203200, containing the 2.7 kb upstream region, the open reading frame and the 1.0 kb downstream sequences, into *d14-1*. The transformed plants showed similar phenotypes to the wild type in both plant height and tiller development (**Fig. 3D**). The dominant co-segregation between the introduced genome fragment and complementation of the mutant phenotype was further confirmed in subsequent generations (data not shown). These results led to the conclusion that *D14* corresponds to Os03g0203200.

D14 is a member of the α/β-hydrolase superfamily

D14 encodes a protein of 318 amino acids that is classified as a member of the α/β-hydrolase superfamily (**Supplementary Fig. S2**). There is a closely related homolog of *D14* (Os03g0437600) in the rice genome which we refer to as *D14-like*. Two *D14* homologs (At3g03990 and At4g37470) were found in Arabidopsis, and phylogenetic analysis showed that they are orthologs of *D14* and *D14-like*, respectively (**Fig. 4**). *D14* homologs are found in a wide range of plant species including ferns (*Selaginella moellendorfir*) and bryophytes (*Physcomitrella patens* and *Marchantia polymorpha*). Interestingly, all *D14* homologs in gymnosperm, fern and

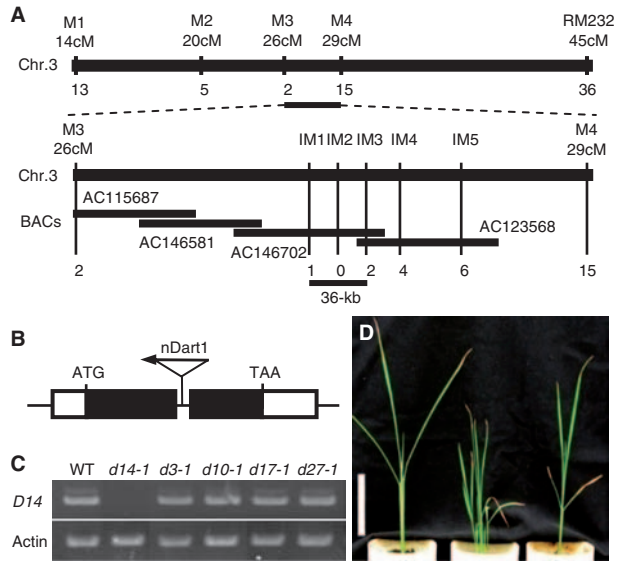

Fig. 3 Positional cloning of *D14*. (A) Fine mapping of the *D14* locus. Location of the *D14* locus was narrowed down to a 36 kb region between IM1 and IM3 on chromosome 3. The number indicates recombinants identified from 65 F_2 plants. (B) A schema of the *D14* candidate gene (Os03g0203200). An endogenous transposon *nDart1* (black arrow) is inserted in the first intron of the putative *D14* gene. The black and white boxes indicate the coding region and the untranslated region, respectively. (C) RT–PCR analysis of the *D14* candidate gene (Os03g0203200) in *d* mutants. Total RNAs isolated from the shoot apex were used for the analysis. The number of cycles was 29. (D) Complementation of *d14-1* by the genome region of Os03g0203200. Left, wild type (Shiokari); center, *d14-1*; right, a complemented plant. Scale bars = 10 m.

bryophyte species are classified into the *D14-like* subfamily, while the *D14* subfamily consists of only genes from angiosperm species.

The proteins of the α/β-hydrolase superfamily do not show significant sequence similarities but exhibit structural similarities, with three conserved amino acids, a nucleophilic residue, an acidic residue and a histidine residue in the catalytic center (Nardini and Dijkstra 1999, Ollis et al. 1992). Among the α/β-hydrolase superfamily members, *D14* and *D14* homologs show a significant sequence similarity to *Regulator of Sigma B* (*RsbQ*) of *Bacillus subtillis* (**Fig. 4**). The three amino acids, called the catalytic triad, Ser147, Asp268 and His297, are also conserved in the *D14* and *D14* homologs (**Supplementary Fig. S2**).

Tissue specificity of D14 mRNA expression

Tissue specificity of *D14* expression was examined by RT–PCR analysis. *D14* mRNA was detected in all tissues examined except for root tips (**Fig. 5A**). Relatively high levels of *D14* mRNA accumulation were detected in leaves and the first leaf buds. The spatial distribution of *D14* gene expression was further examined by using the *GUS* (β-glucuronidase)

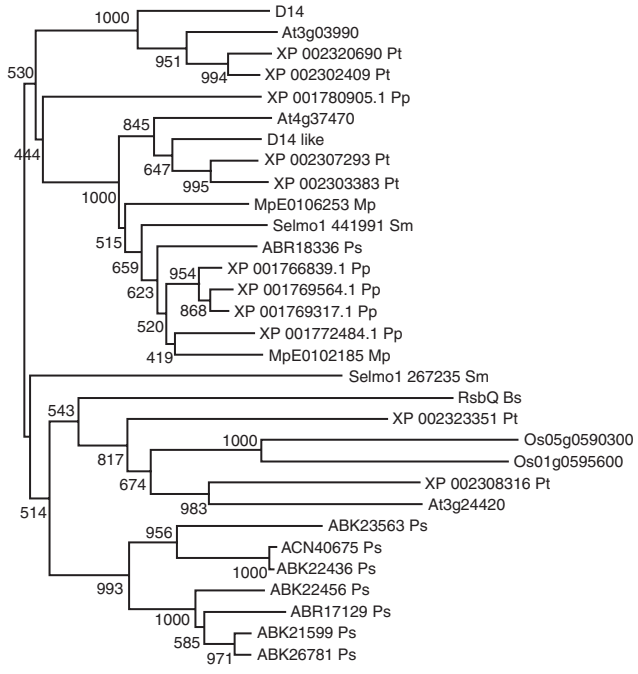

Fig. 4 Phylogenetic analysis of *D14* homologs. Phylogenetic analysis was performed on protein sequences predicted from expressed sequence tag (EST) sequences from various plant species and RsbQ protein from *Bacillus*. EST sequences were collected from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and Selaginella Genomics (http://xselaginella.genomics.purdue.edu/). The unrooted tree was generated using the ClustalX (2.09) program by the neighbor-joining method (http://www.clustal.org/). Bootstrap values from 1,000 replicates are indicated at each node. At, *Arabidopsis thaliana*; Bs, *Bacillus subtilis*; Mp, *Marchantia polymorpha*; Os, *Oryza sativa*; Pp, *Physcomitrella patens*; Ps, *Picea sitchensis*; Pt, *Populus trichocarpa*; Sm, *Selaginella moellendorfii*.

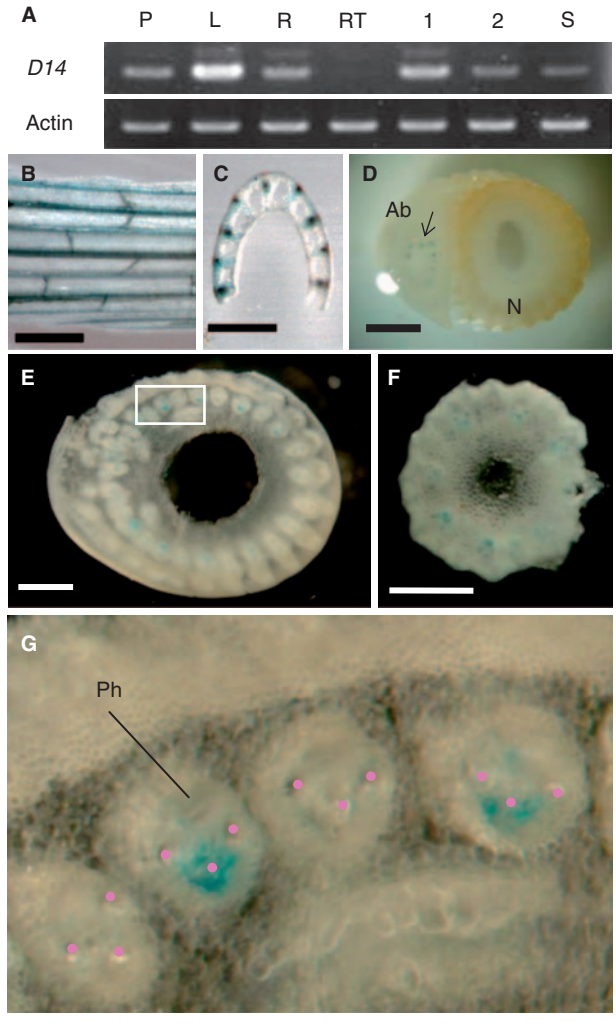

Fig. 5 Expression pattern of *D14* mRNA. (A) RT–PCR analysis of *D14* expression in various organs. P, panicle; L, leaf; R, root; RT, root tip; 1, first leaf bud; 2, second leaf bud; S, shoot apices. The number of PCR cycle was 26. (B–G) Expression pattern of *D14* mRNA. The *D14* expression pattern was examined by using GUS as a reporter. Longitudinal section of the leaf sheath (B), transverse section of the leaf sheath (C), transverse section of the stem at the lower node (D, E) and the neck node. GUS activity is visible in cells surrounding the xylem in the axillary bud (arrow) (F). A close-up view of the region enclosed within the white square in F (G). Ab, axillary bud; N, node; Ph, phloem. Meta xylems are indicated with pink dots. Scale bars = 100 μm (B); 500 μm (C, F); 1 mm (D, E).

gene as a reporter. An approximately 2.7 kb upstream region of D14, which was used in the complementation test, was used as a promoter to drive *GUS*. Three independent transgenic lines were used for analysis. GUS activity was low in all transgenic lines examined and variable in strength among different lines, but *D14:GUS* expression was consistent in that it is only detectable in the vasculature. Examination of transverse sections revealed that *D14:GUS* is expressed in parenchyma cells surrounding the xylem in leaves, stems and axillary buds (**Fig. 5B–G**).

Discussion

The *d14-1* mutant exhibits a dramatic increase in tiller numbers and a reduction in height, resembling the *d3*, *htd1/d17* and *d10* mutants (Ishikawa et al. 2005, Zou et al. 2006). Here, we showed that the *d10-1 d14-1* double mutants are indistinguishable from either single mutant, confirming that *D14* indeed works in the strigolactone pathway. Furthermore, unlike the case with *d10-1*, phenotypic defects of the *d14-1* mutant were not rescued by the application of (+)-strigol or GR24, indicating that *d14-1* has reduced sensitivity to strigolactone. Consistent with this, *d14-1* mutant plants accumulate an elevated level of *epi*-5DS, a major endogenous strigolactone, as often seen for hormone levels in hormone-insensitive mutants. Positional cloning of the *D14* gene revealed that it encodes a protein of the α/β-hydrolase superfamily. Based on these results, we propose that *D14* is a new component in the strigolactone pathway and acts in a

step downstream of the synthesis of strigolactones. D14 could function as the enzyme that catalyzes the metabolic conversion(s) of strigolactones to the as yet unknown bioactive form of the hormone or a component for the perception or transduction of the hormonal signal (**Fig. 6**). To determine whether *D14* works in the biosynthesis or signaling of the hormone, grafting experiments could provide information. If a wild-type rootstock could rescue the phenotypic defects in a *d14* scion this would suggest that *D14* functions in the synthesis of the active hormone. However, unlike in Arabidopsis and pea, grafting of rice plants is technically very difficult. Therefore, analysis of Arabidopsis and pea *D14* orthologs is important in order to examine whether D14 is a common component in the branch-inhibiting hormone pathway. Besides the grafting experiments, further biochemical studies of D14 will be necessary to elucidate its exact role in the strigolactone pathway.

Members of the α/β-hydrolase superfamily exhibit similar overall 3D structures and possess the invariant catalytic triad: a nucleophilic residue, an acidic residue and histidine (Ollis et al. 1992, Nardini and Dijkstra 1999). Among the α/β-hydrolase superfamily proteins, D14 resembles RsbQ of *B. subtillis* at the level of the amino acid sequence (Brody et al. 2001). *RsbQ* participates in the stress signaling cascade that leads to the activation of Sigma Factor B (SigB) through the activation of RsbP. Although the enzymatic activity of RsbQ is still under debate, crystallographic analysis of RsbQ demonstrated that it has a hydrophobic cavity that can potentially accommodate a small compound (Kaneko et al. 2005). The binding with this unknown small molecule is thought to be essential for its function. The catalytic triad and some residues that constitute the hydrophobic cavity in RsbQ (Phe27, Val97 and Phe196) are conserved in D14 and its homologs (**Supplementary Fig. S2**). The sequence and structural similarities between D14 and RsbQ suggest an intriguing idea that D14 might directly interact with strigolactones or downstream metabolites.

There is evidence that some members of the α/β-hydrolase superfamily act as a high-affinity binding protein of small molecules (receptors) without performing a hydrolytic reaction. GID1, a soluble receptor of gibberellin, belongs to the α/β-hydrolase superfamily with a similarity to hormone-sensitive lipases (HSLs) (Ueguchi-Tanaka et al. 2005). GID1 lacks the conserved histidine residue, which increases the nucleophilicity of the hydroxyl group of the serine (nucleophile) residue, in the catalytic triad of HSLs. Thus, GID1 does not possess hydrolase activity. Recent analysis of GID1 crystal structures revealed that, instead of acting as a nucleophile that attacks a carbonyl carbon to initiate hydrolysis, the conserved serine residue in the catalytic triad of GID1 is responsible for forming a hydrogen bond with the carboxyl group of gibberellins (Murase et al. 2008, Shimada et al. 2008). SABP2, a salicylic acid (SA)-binding protein,

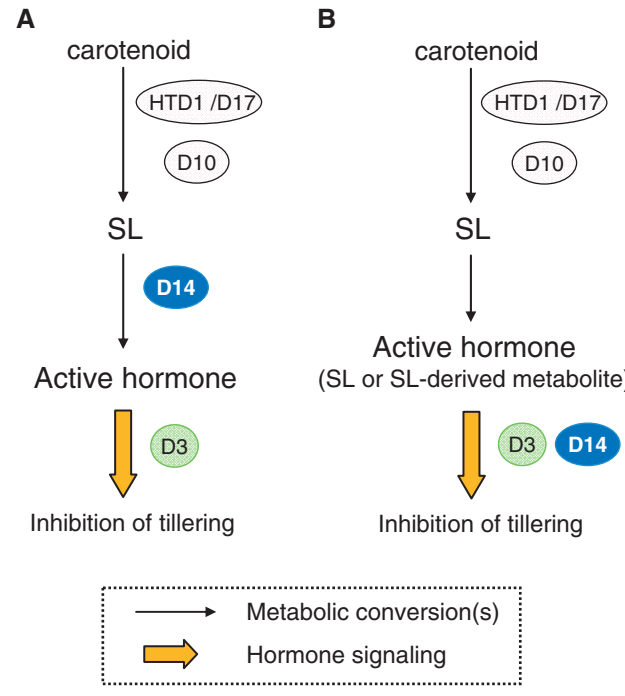

Fig. 6 Models of D14 function in the strigolactone pathway. (A) D14 works in the metabolic conversion(s) of strigolactones to the bioactive hormone. D3 functions in hormone signaling. (B) D14 functions as a signaling component of the strigolactone pathway. SL, strigolactones.

is another member of the α/β-hydrolase superfamily (Kumar et al. 2003, Forouhar et al. 2005). Although SABP2 was initially purified as an SA-binding protein, it has the conserved catalytic triad and does function as an esterase that converts methyl salicylate to SA. SA is bound in the active site and acts as a potent inhibitor of the catalytic activity. It still has to be investigated further whether SA binding to SABP2 plays any role in signaling besides inhibiting the esterase activity. These recent findings indicate the substantial role of α/β-hydrolase superfamily proteins in metabolism and signaling of small molecules such as plant hormones.

The site of action of the branch-inhibiting hormone to suppress outgrowth of axillary buds is yet to be determined. Analysis using a genetic mosaic technique demonstrated that *MAX2*, a putative strigolactone signaling component, works in either the bud or surrounding tissues to suppress bud outgrowth (Stirnberg et al. 2007). Consistently, *MAX2:GUS* expression was observed in the whole region of axillary buds. In this study, we showed that *D14* mRNA is relatively highly expressed in axillary buds. A higher level of *D14* mRNA accumulation in the first leaf bud, which is to be dormant, than in the second leaf bud, which will grow, suggests that *D14* may be a limiting factor for the repression of bud outgrowth.

On further investigation of cell specificity of *D14* expression by using *GUS* as a reporter, the *D14* promoter was active

in xylem parenchyma cells in leaves, stems and axillary buds. As we previously reported, *D10:GUS* was also expressed in xylem parenchyma cells (Arite et al. 2007). However, in contrast to the *D10* promoter which is active in both roots and stems, we were unable to detect *D14:GUS* activity in roots. Because D10 catalyzes an early step of strigolactone synthesis, expression of *D10* in xylem parenchyma cells in roots and stems implies the possibility that strigolactones are synthesized in cells surrounding the xylem and loaded into the xylem to be transported upward. Consistent with this idea, *MAX1:GUS* is also expressed in the cambial region and xylem-associated parenchyma cells (Booker et al. 2005). We showed that the promoter of *D14*, which works after the synthesis of strigolactones, is active in xylem parenchyma cells in axillary buds. This suggests that strigolactones transported through the xylem are unloaded and further processed or perceived by D14.

Our phylogenetic analysis shows that *D14* homologs are classified into two groups, namely *D14* and *D14-like* subfamilies. It is interesting to note that both *D14* and *D14-like* subfamilies exist in angiosperm species, while only members of the *D14-like* subfamily have so far been found in non-angiosperm plants. Although we need further analysis by using a larger number of D14 homologs from a variety of plant species to provide more conclusive interpretations, these observations may suggest that the *D14* subfamily is confined to angiosperms and that it may have evolved to perform a specialized function that is unique to angiosperms. The origin of symbiosis and shoot branching is ancient (Bonfante and Genre 2008). Arbuscular mycorrhizal fungi form symbiosis with gymnosperms, ferns and bryophytes, suggesting that these non-angiosperm plants also produce strigolactones. In fact, the presence of genes encoding putative CCD7 and CCD8 in *P. patens* suggests that strigolactone synthesis occurs widely in plants, including non-vascular plants (Gomez-Roldan et al. 2008). Elucidation of the function of *D14* homologs in both angiosperm and non-angiosperm plants may reveal how the roles of the two *D14* subfamilies evolutionarily diverged and whether the *D14-like* subfamily also plays a role in strigolactone metabolism or signaling.

Materials and Methods

Plant materials

d3-1, *d10-1* and *d14-1* mutants (cv. Shiokari) were described previously (Ishikawa et al. 2005). Plants were grown in a growth chamber (14 h of darkness at 28°C, 10 h of light at 24°C) or in a glasshouse under natural conditions.

Strigolactone analysis

Strigolactone treatment, strigolactone analysis by LC-MS/MS and *S. hermonthica* germination assays were carried out according to Umehara et al. (2008).

Strigolactone treatment and analysis

Strigolactone treatment and LC/MS-MS analysis of *epi*-5DS were performed as described previously (Umehara et al. 2008). In brief, surface-sterilized rice seeds were incubated in sterile water in the dark for 2 d. The germinated seeds were transferred into hydroponic culture medium (Kamachi et al. 1991) solidified with 0.6% agar and cultured for 5 d. Each seedling was then transferred to a glass vial containing a sterilized hydroponic culture solution and grown for an additional 7 d (total 14 d). For the phosphate depletion experiment, phosphate ion was omitted from the medium during the hydroponic culture for 7 d. *epi*-5DS levels in roots and hydroponic culture media (for root exudates) were determined by LC/MS-MS using deuterium-labeled *epi*-5DS as an internal standard. Strigolactone treatment was carried out by including (+)-strigol or GR24 in hydroponic solution containing phosphate ion for 7 d. *Striga hermonthica* germination assays were performed as described previously (Sugimoto and Ueyama 2008). Rice seedlings were grown hydroponically in phosphate-depleted medium as described above and the collected medium was extracted with ethyl acetate. *Striga hermonthica* seeds were pre-conditioned on moist glass fiber filter paper at 30°C for 12 d, and then incubated with the ethyl acetate fraction for 24 h at 30°C before scoring for germination. De-ionized water and (+)-strigol solution were used as negative and positive controls, respectively.

Mapping of D14

To map the *D14* locus, the *d14-1* mutant was crossed with a wild-type plant (cv. Kasalath). Rough mapping was performed with simple sequence repeat (SSR) markers (M1–M4 and RM232) using 17 mutant plants obtained in the F_2 population. Sixty-five F_2 mutant plants were used for fine mapping. An SSR marker (IM1) and four derived cleaved amplified polymorphic sequence (dCAPS) markers (IM–IM5) generated based on single nucleotide polymorphisms (SNPs) were used for the fine mapping. Primer sets used for mapping are 5′-AATCCGGTTGAGGTTGACAC-3′ and 5′-ATTTCAGTTCGGCGAGAGG-3′ (M1), 5′-GAATGCACTTAGGGTCAAAAG-3′ and 5′-AACTTGGCGTGGCACATATT-3′ (M2), 5′-TGAGTTAAACCCCTGAAAACAG-3′ and 5′-CCCATTGATTGTTCTCGAAAG-3′ (M3), 5′-AGAGCGAAACCCTAGGCAAC-3′ and 5′-CAATCCTAGCTATAACCTGGACA-3′ (M4), 5′-CCGGTATCCTTCGATATTGC-3′ and 5′-CCGACTTTTCCTCCTGACG-3′ (RM232), 5′-GATGTCGCGAAACAAAATCC-3′ and 5′-CGTCCGTAGCTAAAAGACTG-3′ (IM1), 5′-CCCAATATTTCAGTTAGTGGTGA-3′ and 5′-GCTAGGAGAATTGGGGGTTT-3′ (IM2), 5′-CAGCCGGACTAAAATCTTTCTT-3′ and 5′-CTGCTGCTCGGGCTACTG-3′ (IM3), 5′-TTCAAACTCGACGTGTGGAC-3′ and 5′-CGTTCGGTCTTTTTGGTCTC-3′ (IM4) and 5′-TCGCTAGCTACTTTTCTG

ATGCGGATGCATG-3′ and 5′-TGTGTTTTGTCCGATTGA CC-3′ (IM5).

Complementation of the d14-1 phenotype

BAC DNA (OSJNBa0009J13) was digested with *Bam*HI and *Xba*I, and a 4.9 kb DNA fragment containing the entire putative *D14* gene was cloned into *pPZP* (Hajdukiewicz et al. 1994). The resultant binary vector was introduced into *d14-1* via *Agrobacterium*-mediated transformation.

RT–PCR

RT–PCR was performed according to Arite et al. (2007). PCR was performed using the following primer sets: *D14*, 5′-GTGCTGTCGCATGGCTTC-3′ and 5′- GCAGGTCGTCG ACGTAGG-3′; and *Actin*, 5′-CAATCGTGAGAAGATGAC CC-3′ and 5′-GTCCATCAGGAAGCTCGTAGC-3′.

Analysis of D14 expression pattern

To construct pD14:*GUS*, approximately 2.7 kb of the *D14* promoter region was amplified by PCR with a set of primers (5′-GGATCCCCTTGTCTAAGACC-3′ and 5′-CACACCAGC GCGGCGGATTG-3′) using BAC DNA (OSJNBa0009J13) as a template. The amplified product was cloned into pENTR1A (Invitrogen, Calsbad, CA, USA) and subsequently cloned into pGWB3 (Nakagawa et al. 2007) with LR clonase (Invitrogen). The resultant binary vector was introduced into Nipponbare via *Agrobacterium*-mediated transformation. Regenerated T_0 transgenic plants were used for histochemical staining of GUS activity. Leaves and stems were cut by hand, treated with 90% acetone for 15 min on ice and stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) solution containing 0.5 mg ml^{-1} X-gluc, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.1% Triton X-100, 10 mM EDTA and 100 mM $NaPO_4$ (pH 7.0). Samples were deaerated at the initiation of staining with X-gluc solution.

Supplementary data

Supplementary data are available at PCP online.

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