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ORIGINAL PAPER

Isolation of rice dwarf mutants with ectopic deposition of phenolic components including lignin in parenchyma cell walls of internodes

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Abstract Rice internodes must have the proper shape to support high-yielding panicles. The shape of internodes is controlled by various factors involved in their formation, such as developmental patterns, cell division, cell elongation, and cell wall biosynthesis. To understand the regulation of internode development, we screened dwarf mutants to identify those with a phenotype of ectopic deposits of phenolic components in parenchyma cell walls of internodes. We named these mutants *ectopic deposition of phenolic components1* (*edp1*). Two alleles were identified, *edp1-1* and *edp1-2*. Furthermore, these mutants showed disordered cell files in internode parenchyma. These abnormal phenotypes were very similar to that of a previously reported *dwarf50* (*d50*) mutant. Genetic analyses of *edp1* mutants revealed that the *edp1* loci are distinct from *d50*. Our results indicate that analyses of *edp1* mutants as well as the *d50* mutant will be useful for understanding the molecular mechanisms behind ectopic deposition of cell wall phenolic components in internode parenchyma cells and the regulation of internode development.

Keywords Abnormal internode parenchyma cells · Dwarf mutant · Ectopic deposition of phenolic components · Cell wall synthesis

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Introduction

Dwarfism is a frequently observed phenotype induced by mutation in plants. This is possibly because plant height is one of the most easily observable phenotypes and is controlled by various pathways, factors and genes. Plant height is an important trait in plant breeding and has been the focus of recent intensive studies. More than 60 dwarf mutants have been reported in rice (http://www.gramene.org/rice_mutant/). Many genes associated with dwarfism are related to biosynthesis or signaling of phytohormones, such as brassinosteroids (BRs) and gibberellins (GAs) (Mandava 1988; Clouse and Sasse 1998; Wu et al. 1999; Yamamuro et al. 2000; Ashikari et al. 2002; Hong et al. 2002, 2003, 2005; Mori et al 2002; Sasaki et al. 2002; Fujioka and Yokota 2003; Itoh et al. 2004; Sakamoto et al. 2004; Tanabe et al. 2005; Nakamura et al. 2006). These mutants show characteristic phenotypes. BR mutants produce deep green and erect leaves, a unique internode elongation pattern, small seeds, and the absence of mesocotyl and internode elongation in the dark (Yamamuro et al. 2000; Hong et al. 2002, 2003, 2005; Mori et al. 2002;

Tanabe et al. 2005). GA mutants show deep green rough leaves, but no other abnormal morphology (Sakamoto et al. 2004).

Several reports have described dwarf mutants with phenotypes that differ from those of typical BR- or GA-related mutants. These dwarf mutants are classified into several groups according to their abnormal patterns of internode elongation and other morphological characteristics. For example, several mutants exhibit short elongation or no elongation of specific internodes, such as the second internode from the top, without gross abnormality of plant shape. This type of dwarf mutant is caused by mutations *Short second internode 1* (*Ssi1*), *ssi2*, *ssi3* and *ssi4* (Wu et al. 2000; Sunohara et al. 2006). The shortening of lower internodes observed in *d6* mutants is caused by the loss of function of a KNOTTED1-like homeobox transcription factor, OSH15, which is involved in the maintenance of meristem function and cellular differentiation (Sato et al. 1999). Several other mutants, like *Twisted dwarf 1* (*Tid1*), exhibit dwarfism with twisted leaves and a dominantly inherited mutation. *TID1* encodes an α-tubulin protein comprising microtubules, indicating that the organization of microtubules is involved in rice internode elongation (Sunohara et al. 2009).

Rice internodes differentiate by the development of the intercalary meristem, and analysis of dwarf mutants is used to investigate the mechanisms of biological processes, including determination of developmental patterns, cell differentiation, cell elongation and cell division. Thus, the investigation of internode development using dwarf mutants with phenotypes unrelated to characteristic BR or GA signaling or biosynthesis should shed light on the mechanisms controlling these processes.

A rice dwarf mutant, Fukei 71 (F71), has been shown to be due to a single recessive gene, *dwarf 50* (*d50*) (Futsuhara 1968), and dwarfed internodes of F71 exhibit irregularly shaped and collapsed internode parenchyma cells, resulting in disordered parenchyma cell files (Kitano and Futsuhara 1981, 1982). Our previous study revealed that, in the parenchyma cells of elongated internodes, ectopic deposition of the polysaccharide-linked hydroxycinnamoyl esters, which specifically exist in cell walls of commelinoid monocotyledons, is induced by *d50* (Nishikubo et al. 2000).

In order to reveal the mechanisms behind ectopic deposition of cell wall phenolic components in internode parenchyma cells of F71, we screened dwarf rice mutants and isolated two lines with ectopically deposited phenolic components in parenchyma cells. We designated the mutants *ectopic deposition of phenolic components* (*edp*) mutants. Furthermore, we performed genetic analyses of *edp* mutants, and characterized their internode parenchyma cells by quantitative and qualitative analyses.

Materials and methods

Plant materials and growth conditions

Wild-type (WT) rice plants (*Oryza sativa* spp. *japonica* cv. Kinmaze) and *N*-methyl-*N*-nitrosourea (NMU) induced mutant lines were used in this study. These lines were grown under natural field conditions in a research field of Tokyo University of Agriculture and Technology, Fuchu, Tokyo, Japan. Seeds of NMU-induced mutant lines were kindly provided by Prof. Hikaru Sato (Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka, Japan).

Histochemical observation of phenolic components in cell wall

For histochemical localization of phenolic components including lignin in the cell wall, the Mäule and Wiesner reactions were performed according to a standard protocol (Srivastaba 1996). Samples of third internodes fixed in formalin:acetic acid:70% ethanol (1:1:18) were washed with water, and hand-cut sections were used for each reaction. For the Mäule reaction, sections were treated for 5 min with 1% potassium permanganate at room temperature, rinsed in distilled water, decolorized with 1 N hydrochloric acid for 20 min, washed in distilled water, treated with a few drops of concentrated ammonia solution, and then observed with a DMLB light microscope (Leica, Wetzler, Germany). For the Wiesner reaction, sections were incubated for 2 min in phloroglucinol solution (2% in ethanol), treated with a few drops of 12 N hydrochloric acid, and then observed with the DMLB light microscope.

Anatomical observation of rice internodes

Samples of third internodes were harvested after the heading stage from WT and *edp* mutants. Samples were fixed in formalin:acetic acid:70% ethanol (1:1:18) overnight at room temperature. Each fixed segment was dehydrated through a graded ethanol series (50, 60, 70, 80, 95 and finally 100% twice, for at least 1 h in each case) and then embedded in Paraplast Plus paraffin wax (McCormick Scientific, Richmond, IL, USA). For anatomical analysis, 16-μm sections were cut with a carbon knife on a microtome and stained with 0.25% Toluidine Blue O (Sigma-Aldrich Japan, Tokyo, Japan), and then observed with the light microscope.

Genetic analysis

To test for allelism between the two *edp* mutants, the *edp* mutants were crossed with each other. The morphology of

F_1 progeny of the cross between the two *edp* mutants was analyzed. Furthermore, the recessive mutant *d50*, which exhibits a similar phenotype in internode parenchyma cell walls with that of *edp* mutants, was crossed with *edp1-1* and *edp1-2* and the morphology of F_1 progeny was analyzed.

Analysis of total phenolic components

The cell wall residue of internode parenchyma and whole internode was collected from *edp* mutants and WT plants at the internode elongation stage. The pith parenchyma regions were excised with a razor blade. The collected samples were ground into a fine powder, extracted with 80% ethanol three times at 100°C for 1 h, and then the residue was completely dried in a vacuum oven. The cell wall phenolic components including lignin and cell wall-linked phenolics were measured by the acetyl bromide method (Iiyama and Wallis 1990) using 10 mg of parenchyma cell wall residue.

Analysis of alkali-labile phenolics in cell wall

The level of hydroxycinnamic acids linked to the cell walls via alkali-labile linkages, such as ester bonds, was measured following alkaline hydrolysis of the extract-free residues and subsequent analysis by gas chromatography (GC). Parenchyma cell wall residue (20 mg) was treated with 5 ml of 4 M sodium hydroxide at 60°C for 1 h under nitrogen, and subsequent experiments proceeded according to the method described by Nishikubo et al. (2000).

To determine molecular weight distribution of the alkali-labile phenolics, we used gel permeation chromatography (GPC) analysis after neutralization with 4 M hydrochloric acid and freeze-drying. The freeze-dried samples were dissolved in 10 ml boron trifluoride methanol complex methanol solution (Wako, Osaka, Japan), methylated at 80°C for 2 h, filtered through a 0.45-μm filter (Millex-LH; Millipore, Tokyo, Japan) and added into 100 ml of distilled water on ice. The solution obtained was acidified with 12 M hydrochloric acid to pH 1 and extracted three times with ethyl acetate. The ethyl acetate-soluble fraction was recovered, and the solution was evaporated to dryness. The residue obtained was dissolved in 1 ml of *N,N*-dimethylformamide (Wako), and GPC analysis was performed using 50 μl of solution. Phenolic components were separated on paired Shodex Asahi pak GF-510HQ and GF-310HQ columns (Showa Denko, Tokyo, Japan) at 40°C, and the molecular weight of phenolic components was identified based on retention time using an L-4000 UV detector (Hitachi High-Tech, Tokyo, Japan).

Analysis by pyrolysis-gas chromatography (Py-GC)

The Py-GC system consisted of a JHP-5 Curie-point pyrolyzer (Japan Analytical Industry Co. Ltd., Tokyo, Japan) and a GC-17A gas chromatograph (Shimadzu, Kyoto, Japan). Each 200-μg sample of cell wall residue derived from parenchyma or whole internodes was tightly wrapped in 50-μm-thick ferromagnetic pyrofoil. The sample was inserted into a sample tube, and then a sample holder that was centered in the pyrolyzer and heated to 270°C. After the pyrolysis system was flushed with helium gas for 15 s, pyrolysis was allowed to proceed at 500°C for 4 s. The products were transferred to the GC via a transfer needle heated to 270°C. Volatile products were separated on a DB-1 column (Agilent Technologies, Tokyo, Japan). The column temperature was kept at 50°C for 1 min and programmed to increase to 300°C at 5°C min^{-1}. Products were detected by a flame ionization detector operated at 270°C with helium used as a carrier gas. Identification of the products was based on retention times of standard compounds, interpretation of the results of Py-GC, and comparison with published data (Ralph and Hatfield 1991) as described previously (Kuroda et al. 1990, 1995).

Results

Isolation and characterization of *edp* mutants

In order to elucidate the mechanisms regulating ectopic deposition of cell wall phenolic components in parenchyma cell walls of internodes, we screened an NMU-mutagenized mutant collection prepared from the rice cultivar Kinmaze for isolation of mutants with an altered pattern of phenolic deposition. To this end, hand-cut sections of 3rd internodes were stained by the Mäule and Wiesner reactions. Among 50 dwarf mutant plants screened, we found two mutants with a dramatic alteration in phenolic staining pattern. In the internodes of WT, both Mäule and Wiesner staining were clearly observed in cortical fibers and in large and small vascular bundles, but no staining was observed in parenchyma cells (Fig. 1). In contrast, in most parenchyma cells of mutants, ectopic Mäule staining was also observed in cortical fibers and vascular bundles (Fig. 1a). Wiesner staining was also observed in the parenchyma of mutant internodes, although the ectopic staining area was smaller than that of Mäule staining (Fig. 1b). Although the parenchyma regions of each mutant had expanded, the thickness of internodes was almost same as WT. Magnified images of parenchyma cells of mutants showed that the ectopic staining was observed in cell wall and intercellular spaces (Fig. 1). Therefore, we defined these mutants as *ectopic deposition of phenolic components* (*edp*) mutants.

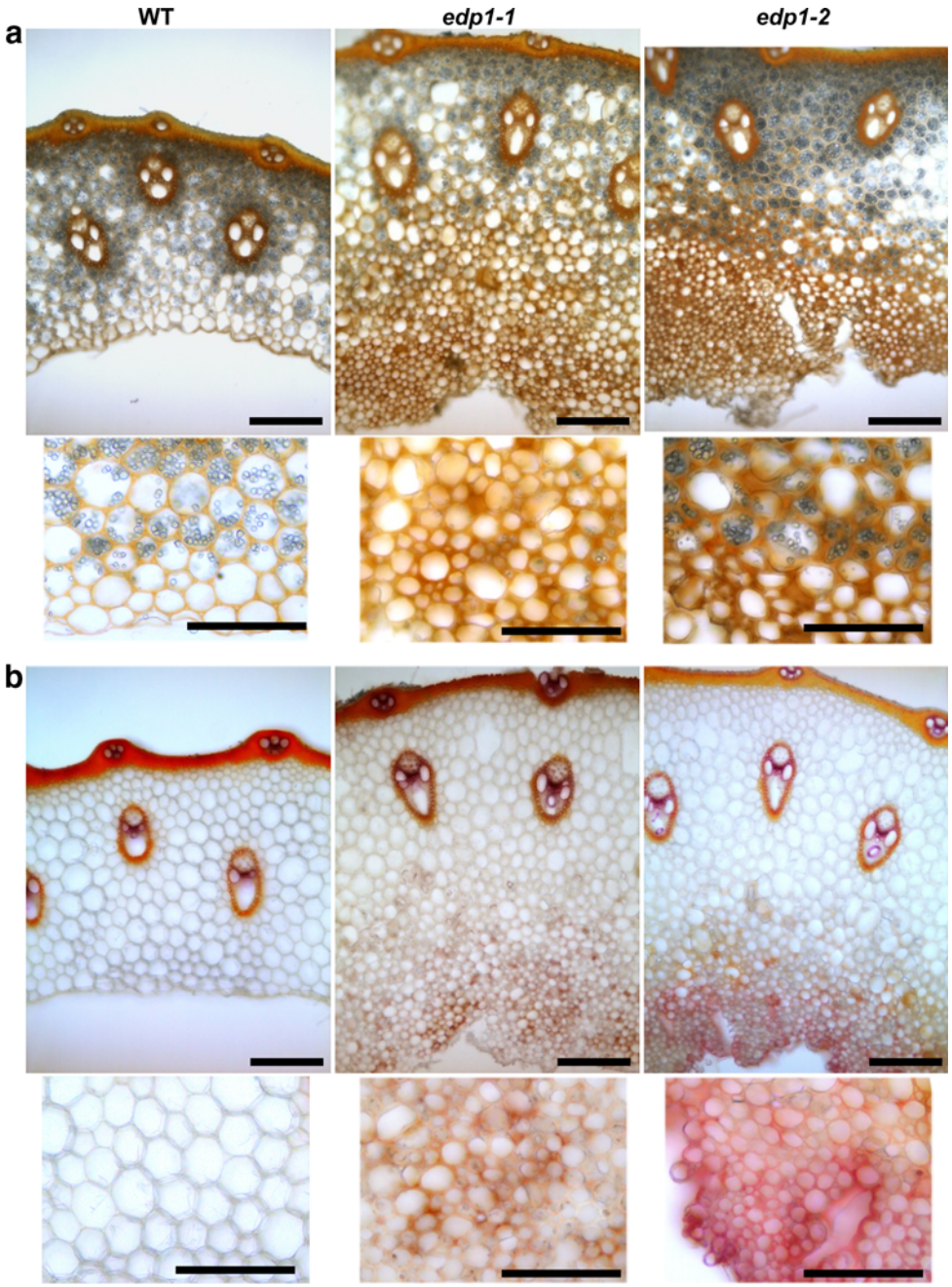

Fig. 1 Histochemical analysis of phenolic components in cell walls of 3rd elongated internodes. **a** Mäule reaction of WT and *edp1* mutants showing ectopically stained parenchyma cells in *edp1* mutants. Parenchyma cells in the *edp1* mutants are shown at high magnification at the bottom. **b** Wiesner reaction of WT and *edp1* mutants showing ectopically stained parenchyma cells in *edp1* mutants. Parenchyma cells in the *edp1* mutants are shown at high magnification at the bottom. *Bars* 200 μm

Internode elongation patterns of *edp* mutants

A dwarf phenotype in rice is generally caused by a reduction in internode length. Based on the elongation pattern of internodes, rice dwarf mutants are classified into several types. The WT internode length increases gradually with higher internode position (Fig. 2). In the *edp* mutants, the lengths of the panicles and the first internodes were almost the same as those of WT; however, the lengths of the second and subsequent internodes were dramatically reduced compared to WT (Fig. 2). This type of internode elongation pattern was similar to that of the Fukei 71 (*d50*) mutant that we previously described in detail (Fig. 2b; Nishikubo et al. 2000). Furthermore, the morphology of *edp* mutants was similar to that of *d50* (Fig. 2a).

In order to understand the cell morphology of the dwarf-phenotype *edp* mutants, we observed longitudinal sections of dwarfed 3rd internodes. All WT parenchyma cells were fully elongated and arranged in fine cell files aligned with the internode elongation axis (Fig. 3a). In contrast, most parenchyma cells in *edp* mutants exhibited irregular shapes and sizes, resulting in an unorganized cell file in parenchyma tissue (Fig. 3b, c). Because parenchyma cell files were formed in the cell division region, we observed the

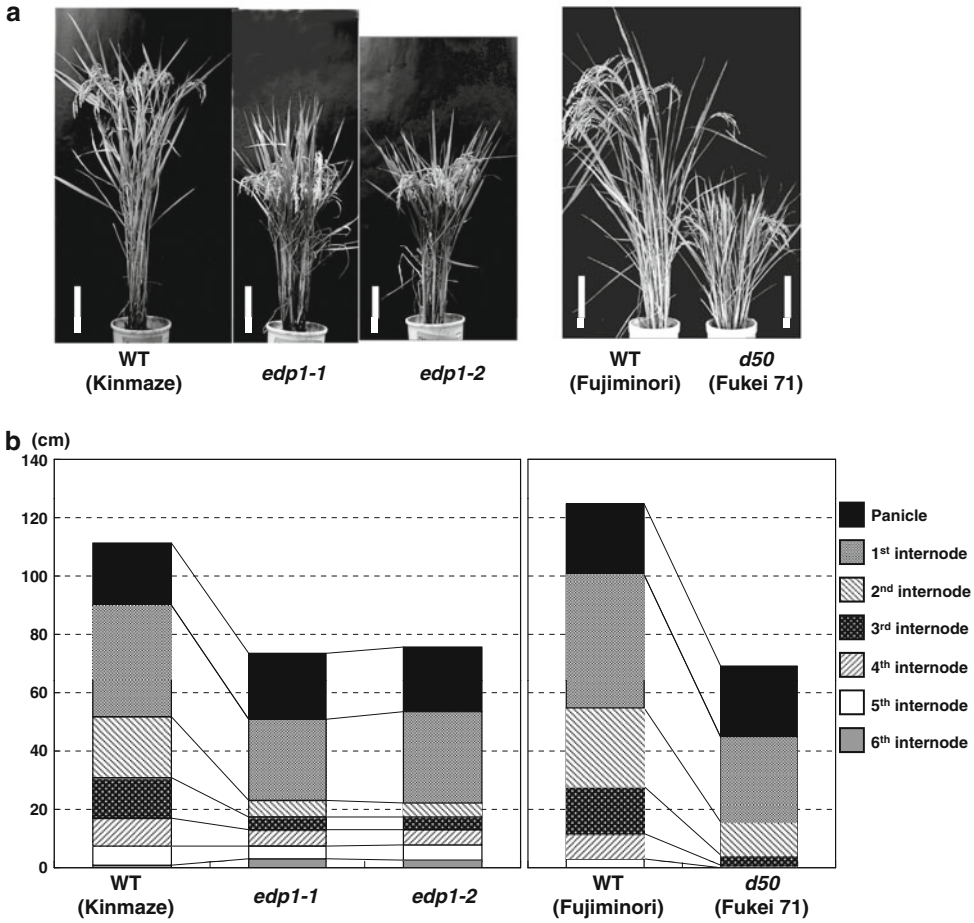

Fig. 2 Characterization of internode elongation pattern in *edp1* mutants. **a** WT, *edp1* mutants and *d50* mutant plants showing dwarf phenotypes in *edp1* and *d50* mutants. **b** Internode elongation pattern in WT, *edp1* mutants and *d50* mutant showing a nearly identical internode elongation pattern in *edp1* and *d50* mutants. *Bars* 20 cm

basal part of elongating internodes, which contain young dividing parenchyma cells. WT parenchyma cells of the cell division region exhibited fine cell files along the internode elongation axis and consisted of nearly equally sized parenchyma cells (Fig. 3d, g). On the other hand, parenchyma cell files in *edp* mutants were disturbed, especially in the inner region of parenchyma tissues (Fig. 3e, f). Magnification of parenchyma cells of *edp* mutants showed an irregular direction of cell division in many cells (Fig. 3h, i arrowheads), which has also been observed in the *d50* mutant (Kitano and Futsuhara 1982).

Genetic analysis of *edp* mutants

To determine whether the two *edp* mutants are allelic, they were crossed with each other. Of the 20 F_1 plants examined, all showed a dwarf phenotype (Fig. 4, left) and ectopic Mäule staining in the parenchyma cells (data not shown), indicating that these two *edp* mutants could not complement each other and therefore are allelic. The mutant locus was defined as *edp1* and the two alleles named *edp1-1* and *edp1-2*.

Because we observed that the ectopic deposition of cell wall phenolic components in the parenchyma, the internode elongation patterns and the irregularly oriented cell division of parenchyma cells in *edp1* mutants were all very similar to the *d50* mutant, we crossed the *d50* mutant, Fukei 71, and *edp1* mutants. The resulting F_1 progeny plants exhibited almost the same plant height as WT (Fig. 4, center and right), indicating that the *d50* and *edp1* loci complement each other. These results revealed that the *d50* and *edp1* loci are different and that the *edp1* gene is recessive.

Levels of cell wall phenolic components in parenchyma of *edp1* mutants

To determine whether the ectopic Mäule and Wiesner staining of internode parenchyma in *edp1* mutants is due to altered cell wall composition, we compared the levels of total phenolic components of parenchyma cell walls in mutants and WT plants. Acetyl bromide analysis showed that the WT parenchyma cell wall contained 31.1 ± 5.1 mg/g phenolic components including lignin in total cell wall residues (Fig. 5a, left). In the *edp1-1* and *1-2* mutants, the total phenolic contents were 64.1 ± 0.7 mg/g and 92.9 ± 1.0 mg/g, respectively (Fig. 5a, left), indicating that the phenolic content was increased about 2 ∼ 3

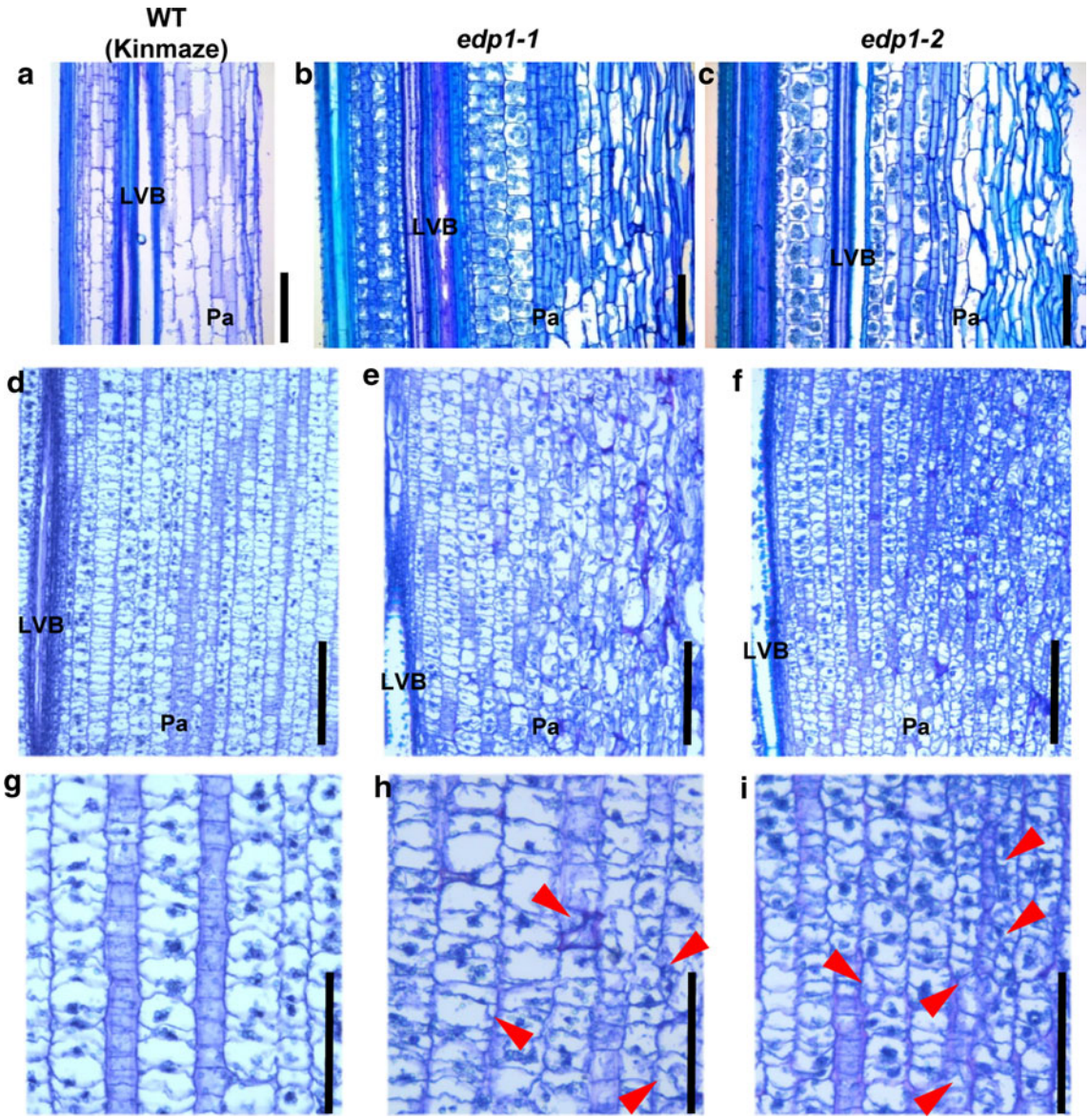

Fig. 3 Anatomical observation of internodes from WT and *edp1* mutants. **a** Longitudinal section of elongated internodes from WT. **b, c** Longitudinal sections of elongated internodes from *edp1* mutants, showing unorganized cell files in parenchyma (Pa) in *edp1* internodes. **d** Longitudinal sections of bottom region of elongating internodes containing dividing parenchyma cells of WT. **e, f** Longitudinal sections of bottom region of elongating internodes of *edp1* mutants. Inner region of parenchyma cell files of *edp1* mutants were disrupted in this region. **g–i** Magnification of **d–f** showing abnormally oriented cell division in parenchyma cells of *edp1* mutants (*arrowheads*). LVB, large vascular bundle. *Bars* 200 μm in **a–f**, 100 μm in **g–i**

times over WT in the parenchyma of *edp1* mutants (Fig. 5a, left).

Type II cell walls, found only in commelinoid monocotyledonous plants including rice, are specifically rich in low molecular weight phenolic acids (*p*-coumaric and ferulic acid) ester-linked to arabinoxylan (Iiyama et al. 1994; Yokoyama and Nishitani 2004; Vogel 2008). Cell wall-linked hydroxycinnamoyl esters were examined by mild alkaline hydrolysis with subsequent analysis by GC. The parenchyma cell wall of WT contained 1.0 ± 0.4 mg/g *p*-coumaric acid and 3.5 ± 0.4 mg/g ferulic acid in total cell wall residues (Fig. 5a, right). On the other hand, the *edp1-1* mutant contained 3.6 ± 0.3 mg/g *p*-coumaric acid and 6.2 ± 0.7 mg/g ferulic acid, and the *edp1-2* mutant contained 6.5 ± 0.3 mg/g *p*-coumaric acid and 7.2 ± 0.4 mg/g ferulic acid (Fig. 5a, right), indicating that the parenchyma cell walls of *edp1* mutants had higher levels of hydroxycinnamoyl esters than those of WT.

The *edp1* mutants contained higher levels of hydroxycinnamoyl esters than WT; however, the increased total phenolic content of *edp1* mutants could not account for the increase in hydroxycinnamoyl esters. These results differed

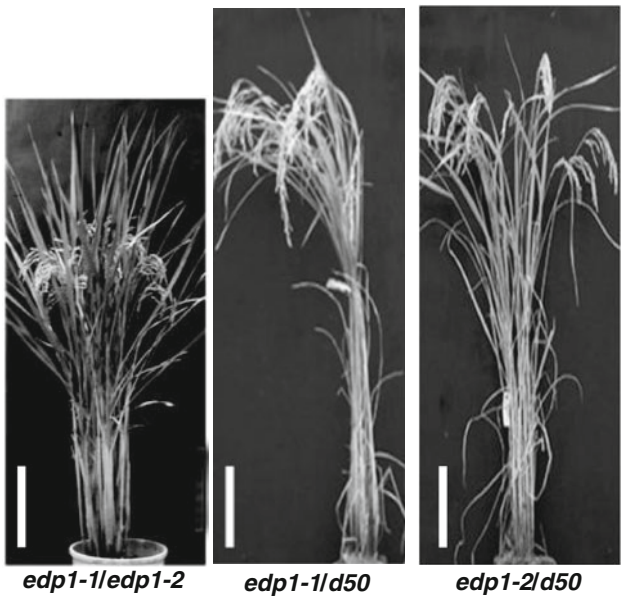

Fig. 4 Allelism tests between the two *edp* mutant lines and the *d50* mutant. F_1 progeny obtained from crosses between the two *edp* mutants showed a dwarf phenotype, indicating that these mutations are allelic. On the other hand, F_1 progeny obtained from crosses between each *edp1* mutant and the *d50* mutant exhibited WT plant height, indicating that the *edp1* and *d50* loci are different and that the *edp1* gene is recessive. *Bars* 20 cm

from those for *d50* mutants, in which the level of hydroxycinnamoyl esters increased to almost the same level as the increased total phenolic components (Fig. 5b), which is consistent with the results of Nishikubo et al. (2000).

Qualitative analysis of phenolic components in *edp1* parenchyma cell wall

In order to identify the main phenolic component in parenchyma cell wall of the *edp1* mutants, we analyzed the cell walls qualitatively using GPC and Py-GC. GPC analysis of cell wall phenolic components in internode parenchyma extracts obtained by alkaline hydrolysis of WT and *edp1* showed that, even though peak 1 and 3 of *edp1-2* differed compared to those of WT and *edp1-1*, the molecular weight distribution of the cell wall phenolic components was almost the same in WT and *edp1* mutants (Fig. 6). These results imply that the increased phenolic components in parenchyma cell walls of *edp1* mutants are the same components that exist in WT parenchyma cell walls, such as lignin, as suggested by Wiesner staining (Fig. 1).

To analyze lignin characteristics in parenchyma cell walls of *edp1* mutants, we performed Py-GC analysis on parenchyma cell wall residues of *edp1* mutants and whole internodes of WT, which contain a large amount of lignin. As shown in Fig. 7, cell wall residues of whole internodes of WT and parenchyma of *edp1* mutants exhibited almost the same peak pattern. Peaks 1–7 in Fig. 7 are expected to correspond to phenol, guaiacol, 4-ethylphenol, 4-vinylphenol, 4-vinylguaiacol, syringol and acetosyringone, respectively, which are characteristic peaks of lignin, as reported by Kuroda et al. (1995). Peaks corresponding to 4-vinylphenol and 4-vinylguaiacol, which are derived from *p*-coumaric and ferulic acids, were relatively high in all samples. All characteristic peaks of lignin were detected in parenchyma cell walls of *edp1* mutants as well as whole internodes of WT (Fig. 7). These results suggest that the parenchyma cell walls of *edp1* mutants deposit lignin and hydroxycinnamoyl esters ectopically.

Discussion

The *edp1* mutation affects the elongation pattern of lower internodes and formation of longitudinal cell files

During internode elongation of rice, many biological processes, including developmental patterns, cell differentiation, cell elongation and cell division, are regulated. However, regulatory mechanisms other than those related to GA or BR are poorly understood. To understand these mechanisms, isolation and analysis of dwarf mutants distinct from GA- and BR-related mutants are required.

In this study, we isolated rice mutants *edp1-1* and *1-2*, which exhibited a dwarf phenotype without any other abnormal morphology. Analysis of their internode elongation pattern showed that the panicle and uppermost 1st internode were almost the same length as in WT (Fig. 2), suggesting that the *edp1* mutation should not affect flower initiation, which is important for reproduction of rice plants. Instead, the *edp1* mutation decreased the length of the second and lower internodes, and induced formation of irregular cell files in dwarfed internode parenchyma (Figs. 2, 3). This type of internode elongation pattern was reported in the rice *d6* mutant, which has a defective homeobox gene, *OSH15*; however, irregular cell files in internode parenchyma were not observed in *d6* (Sato et al. 1999). These observations suggest that internode development of the uppermost (1st) internode and lower (2nd and lower) internodes is regulated differently and that several mechanisms could be involved in the internode elongation pattern.

Dwarfed internodes of *edp1* mutants ectopically deposit hydroxycinnamoyl esters and lignin in parenchyma cell wall

Our histochemical observations demonstrated that the *edp1* mutants ectopically deposited cell wall phenolic components, including lignin, specifically in the parenchyma cell

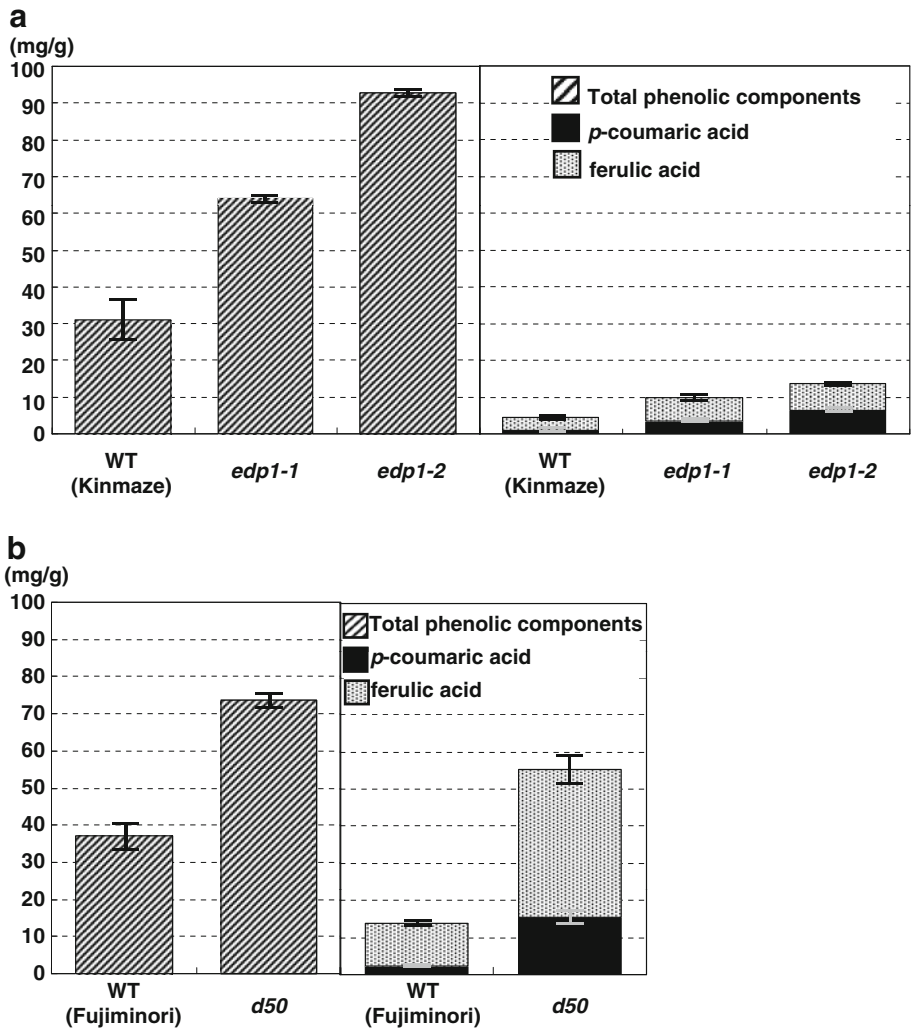

Fig. 5 Phenolic component levels in parenchyma cell wall residues. **a** Total phenolic components and alkaline-soluble ester-linked *p*-coumaric and ferulic acids levels in WT (Kinmaze) and *edp1* mutants, showing an increased level in total phenolic components but not as great an increase in alkaline-soluble ester-linked hydroxycinnamic acids. **b** Total phenolic components and alkaline-soluble ester-linked *p*-coumaric and ferulic acids levels in WT (Fujiminori) and the *d50* mutant, showing increased levels of total phenolic components and alkaline-soluble ester-linked hydroxycinnamic acids ($n = 3$)

wall (Figs. 1, 5, 6, 7). The loci corresponding to three ectopic lignification mutants, *eli1*, *elp* and *det3*, have been reported in *Arabidopsis*. The *eli1* mutant has a defect in a cellulose synthase subunit, CesA3 (Caño-Delgado et al. 2000, 2003). Another ectopic lignification mutant, *elp1*, has a mutation in a gene for a chitinase-like protein, *At*CTL1 (Zhong et al. 2000, 2002). The *det3* mutant was originally identified in a screen for dark photomorphogenic mutants (Cabreray Poch et al. 1993), and was later shown to have ectopic lignification (Caño-Delgado et al. 2000; Newman et al. 2004). The *DET3* locus encodes the C-subunit of the vacuolar-type ATPase (V-ATPase) (Schumacher et al. 1999). These three mutants accumulate lignin ectopically in pith tissue of most organs such as roots, leaves, hypocotyls and stems (Caño-Delgado et al. 2000, 2003; Zhong et al. 2000, 2002; Newman et al. 2004; Rogers et al. 2005). However, the relationship between these three genes and ectopic deposition of lignin is poorly understood.

In rice, only one mutant, F71 (*d50*), that exhibits ectopic deposition of cell wall phenolic components has been isolated. In general, rice cell wall is classified as a Type II cell wall (Yokoyama and Nishitani 2004), and Type II cell walls have mainly two types of phenolic components, lignin and low molecular weight phenolic acids ester-linked to arabinoxylan (Iiyama et al. 1994; Vogel et al. 2008). In the *d50* mutant, low molecular weight phenolic acids (*p*-coumaric and ferulic acids) ester-linked to the cell wall accumulate ectopically in parenchyma cell walls of internodes (Nishikubo et al. 2000).

In this study, we identified *edp1* mutants that ectopically deposit phenolic components in parenchyma cell walls of internodes, and revealed that the causal gene is different from *d50* (Fig. 4). Quantitative analyses of phenolic and ester-linked low molecular phenolic acids in the parenchyma cell walls of *edp1* mutants and WT showed that *edp1* contains a higher level of cell wall phenolic components (Fig. 5). Furthermore, total phenolic and low molecular weight phenolic acid contents in *edp1-2* were higher than in *edp1-1* (Fig. 5). Because genetic analysis showed that *edp1-1* and *1-2* are allelic (Fig. 4), *edp1-2* is a

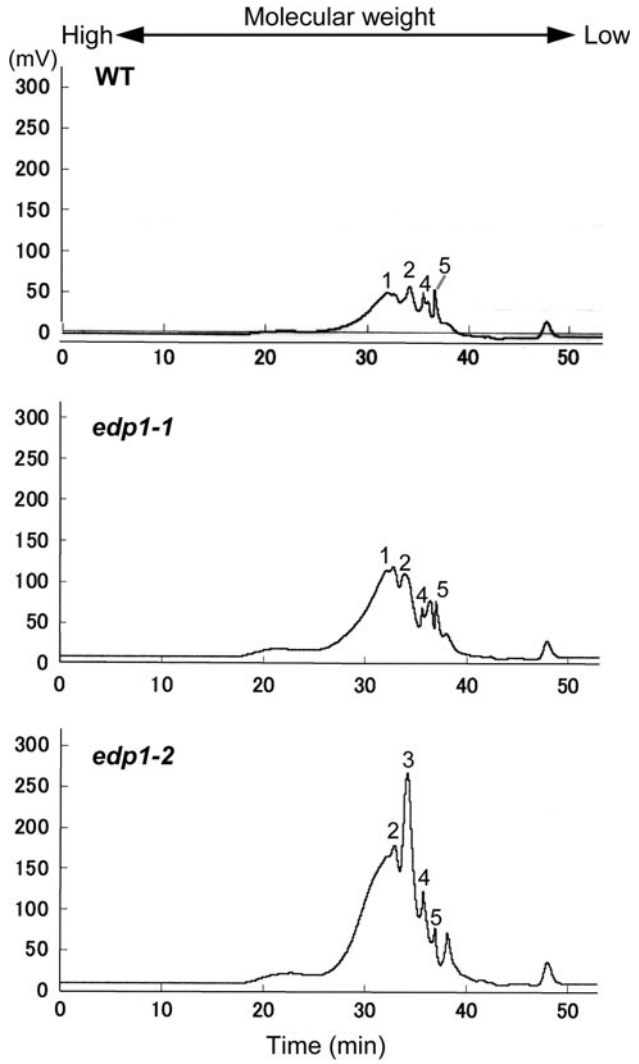

Fig. 6 Molecular weight distribution of alkaline-soluble phenolic components in parenchyma cell wall of *edp1* mutants analyzed by GPC. Almost the same molecular distribution was observed for *edp1* mutants and WT. *Peaks 1–5* are predicted as having molecular weight 605, 552, 376, 255 and 218, respectively based on authentic samples

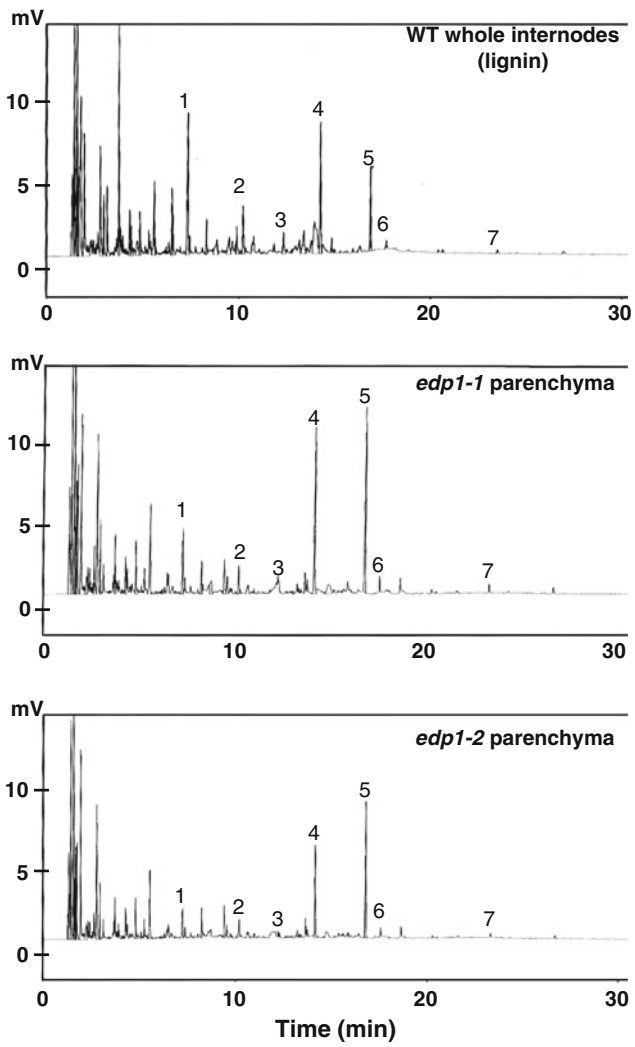

Fig. 7 Py-GC chromatograms of cell wall from whole WT internodes containing large amounts of lignin and parenchyma cell wall from *edp1* mutants. Chromatograms of *edp1* parenchyma cell walls (*middle* and *bottom* panels) showed almost the same peak patterns as that of whole WT internode (*top* panel)

more severe mutant, which is consistent with the more intense Wiesner staining of parenchyma cells in *edp1-2* than *edp1-1* (Fig. 1). Therefore, the *edp1-1* and *edp1-2* mutants are useful for studying the mechanisms of regulation of spatial, temporal and quantitative deposition of cell wall phenolic components. To understand these control mechanisms, identification and characterization of the causal gene of *edp1-1* and *1-2* will be indispensable.

Ectopic deposition of cell wall phenolic components accompanies abnormal cell division in *edp1* mutants

Anatomical analyses revealed that the disordered cell files in *edp1* mutants were caused by irregularly oriented cell division in parenchyma cells (Fig. 3). This phenotype was almost the same as that of the *d50* mutant (Kitano and Futsuhara 1982). However, irregularly oriented cell division is not always induced by ectopic deposition of cell wall phenolic components. For example, rice *d61* mutants show altered cell division (Nakamura et al. 2006), but ectopic accumulation of phenolic components has not been analyzed. Therefore, irregular cell division and ectopic deposition of phenolic components may be controlled by specific pathways. Our preliminary genetic analysis revealed that *d50* encodes a putative inositol polyphosphate 5-phosphatase, which may be involved in phosphoinositide signaling pathways required for many essential cellular functions such as cytoskeleton organization, endocytosis and vesicular trafficking in eukaryotes. Identification and function of the *edp1* gene and further analysis of *edp1* and

d50 mutants should shed light on the regulatory mechanisms coordinating cell division, deposition of cell wall phenolic components and phosphoinositide signaling during internode development.

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