Inactivation of the CTD phosphatase-like gene OsCPL1 enhances the development of the abscission layer and seed shattering in rice

Hyeonso Ji1,†, Sung-Ryul Kim2,†, Yul-Ho Kim3, Hakbum Kim1, Moo-Young Eun4, Il-Doo Jin5, Young-Soon Cha6, Doh-Won Yun1, Byung-Ogh Ahn3, Myung Chul Lee3, Gang-Seob Lee3, Ung-Han Yoon3, Jung-Sook Lee3, Yeon-Hee Lee3, Seok-Cheol Suh1, Wenzhu Jiang6, Jung-II Yang2, Ping Jin2, Susan R. McCouch8, Gynheung An2,* and Hee-Jong Koh6,*

1Department of Agricultural Bio-resources, National Academy of Agricultural Science (NAAS), Suwon 441-707, Korea,
2National Research Laboratory of Plant Functional Genomics, Division of Molecular and Life Science, Pohang University of Science and Technology (POSTECH), Pohang 790-784, Korea,
3National Institute of Crop Science, Suwon 441-857, Korea,
4Department of Agronomy, Kyungpook National University, Daegu 702-701, Korea,
5School of Environment and Agriculture, Sunchon National University, Sunchon 540-742, Korea,
6Department of Plant Science, Research Institute of Agriculture and Life Sciences, and Plant Genomics and Breeding Institute, Seoul National University, Seoul 151-921, Korea,
7National Agrobiodiversity Center, National Academy of Agricultural Science (NAAS), Suwon 441-707, Korea, and
8Department of Plant Breeding, Cornell University, Ithaca, NY 14853-1901, USA

Received 31 July 2009; revised 12 September 2009; accepted 22 September 2009; published online 5 November 2009.

*For correspondence (fax +82 2 873 2056; e-mail heejkoh@snu.ac.kr; fax +82 54 279 0659; e-mail genean@postech.ac.kr).
†These two authors contributed equally to this work.

SUMMARY

Although susceptibility to seed shattering causes severe yield loss during cereal crop harvest, it is an adaptive trait for seed dispersal in wild plants. We previously identified a recessive shattering locus, sh-h, from the rice shattering mutant line Hsh that carries an enhanced abscission layer. Here, we further mapped sh-h to a 34-kb region on chromosome 7 by analyzing 240 F2 plants and five F3 lines from the cross between Hsh and Blue&Gundil. Hsh had a point mutation at the 3¢ splice site of the seventh intron within LOC_Os07g10690, causing a 15-bp deletion of its mRNA as a result of altered splicing. Two transferred DNA (T-DNA) insertion mutants and one point mutant exhibited the enhanced shattering phenotype, confirming that LOC_Os07g10690 is indeed the sh-h gene. RNA interference (RNAi) transgenic lines with suppressed expression of this gene exhibited greater shattering. This gene, which encodes a protein containing a conserved carboxy-terminal domain (CTD) phosphatase domain, was named Oryza sativa CTD phosphatase-like 1 (OsCPL1). Subcellular localization and biochemical analysis revealed that the OsCPL1 protein is a nuclear phosphatase, a common characteristic of metazoan CTD phosphatases involved in cell differentiation. These results demonstrate that OsCPL1 represses differentiation of the abscission layer during panicle development.

Keywords: rice, shattering, abscission layer, carboxy-terminal domain phosphatase, Oryza sativa CTD phosphatase-like 1, sh-h.

INTRODUCTION

Wild rice species have been domesticated via artificial selection of important traits, such as flowering time, fertility, seed number and size, grain quality, and seed shattering. Although shattering diminishes yields during crop harvests, it is an adaptive trait for seed dispersal by wild plants. Susceptibility to shattering results from the formation of an abscission layer, and the subsequent degradation of its middle lamellae and cell walls (Osborne, 1989; Patterson, 2001; Roberts et al., 2002). This abscission layer forms just below the sterile lemma in the rice grain pedicel, approximately 16–20 days before heading, when the young panicle is 5–30-mm long (Jin, 1986). As the seeds mature, the cells of
the abscission layer degrade, increasing the chances that the grain will detach from the mother plant (Jin and Inouye, 1982). The pedicel breaking tensile strength (BTS), which is inversely proportional to shattering degree, decreases over the 10–20 days after heading (DAH) in shatter-prone varieties (Ji et al., 2006).

Linkage studies revealed the presence of rice shattering loci on chromosome 11 (Nagao and Takahashi, 1963), chromosome 1 (Oba et al., 1990), chromosome 4 (Eiguchi and Sano, 1990; Nagai et al., 2002) and chromosome 3 (Fukuta and Yagi, 1998). Also, rice shattering QTLs have been reported on chromosomes 1, 3, 4, 7, 8 and 11 (Xiong et al., 1999; Cai and Morishima, 2000; Bres-Patry et al., 2001; Thomson et al., 2003). However, only two rice shattering genes have been identified through map-based quantitative trait loci (QTL) cloning. The qSH1 gene, a major QTL on chromosome 1, encodes a BEL1-type homeobox gene, and a single nucleotide polymorphism (SNP) in the 5′ regulatory region of qSH1 causes loss of expression only at the abscission layer, and has led to a decline in seed shattering over the history of rice domestication (Konishi et al., 2006). Another major QTL on chromosome 4, named sh4, encodes a predicted transcription factor with a DNA binding domain. A single amino acid substitution resulting from an SNP in the putative DNA binding domain also causes a reduction in seed shattering (Li et al., 2006).

In Arabidopsis, several genes have been identified that underline the development of a dehiscence zone, which is comparable with the rice abscission layer. Two redundant MADS-box genes, SHATTERPROOF1 (SHP1) and SHP2, specify valve margin identity (Liljegren et al., 2000). The myc/bHLH gene, ALCATRAZ (ALC), promotes the differentiation of a strip of labile, non-lignified separation-layer cells sandwiched between layers of lignified cells at the valve margin (Rajani and Sundaresan, 2001). INDEHISCENT (IND), encoding an atypical bHLH protein, also plays an important role in the lignification and development of this separation layer at the valve margin (Liljegren et al., 2004). The expression of the valve margin identity genes is limited to the valve margin through negative regulation by FRUITFUL (FUL) in the valves, and by REPLUMLESS (RPL) in the replum (Roeder et al., 2003; Liljegren et al., 2004; Dinneny et al., 2005).

Previously, we identified a rice shattering mutant line, Hsh, that was derived from a non-shattering japonica rice variety, Hwacheong, through treatment with N-methyl-N-nitrosourea (MNU). We located the recessive shattering locus, sh-h, between markers RM7161 and RM8262 (598 kb) on chromosome 7 (Ji et al., 2006). Here, we report that sh-h encodes a carboxy-terminal domain (CTD) phosphatase-like 1 (OsCPL1) protein that is similar to the metazoan CTD phosphatases, which are involved in cell differentiation.

RESULTS

Fine mapping of sh-h

Compared with the wild-type Hwacheong line, the shattering mutant line, Hsh, has a well-developed abscission layer and a drastically reduced grain pedicel BTS (Figure 1a,b). To implement fine mapping of the shattering locus sh-h, we genotyped 240 F2 plants derived from a cross between Hsh and Blue&Gundil, using six SSR markers (RM8262, RM21160, RM21171, RM180, RM21182 and RM7161). These markers were developed from the sequences of six BAC clones (AP005259, AP005171, AP004273, AP003943, AP003837 and AP003748) spanning the RM8262–RM7161 interval on rice chromosome 7. We found that sh-h is located between RM180 and RM7161 (Figure 2a).

Among the 16 genes in this interval, the genomic region of the six most plausible candidate genes was sequenced from Hwacheong, Hsh and Blue&Gundil lines. Those genes are annotated as encoding a leucine-rich repeat protein (LOC_Os07g10630), putative polygalacturonases (LOC_Os07g10680, LOC_Os07g10700, LOC_Os07g10730 and LOC_Os07g10740) and a putative NLI interacting factor (LOC_Os07g10690). Three SNP markers were developed based on the newly found SNPs (Tables S1 and S2). Further, mapping was based on the five F2 lines derived from F2 plants that have recombination between RM180 and RM7161, and sh-h was found to be located in the 34-kb interval between SN1 and SN3 (Figure 2a).

Identification of sh-h

Among the eight genes located between SN1 and SN3, LOC_Os07g10690 was considered to be a strong candidate for the sh-h locus because of the presence of a point mutation at the 3′ end splice site of its seventh intron. Sequencing of the RT-PCR products of Hwacheong and Hsh amplified with the RTL1 primer pair (Table S3) revealed that, compared with Hwacheong, a 15-bp deletion was induced by altered splicing in the mRNA isolated from Hsh (Figure 2b). Because the consensus sequence for splicing at this ‘AG’ site was changed to ‘TG’, the next ‘AG’ sequence, 15-bp downstream, was used as a new splice site. This was confirmed by length polymorphism of RT-PCR products amplified with the RTL4 primer pair (Figure 2c; Table S3).

The open reading frames (ORFs) of LOC_Os07g10690 cDNA of Hwacheong and Hsh were translated in silico. Alignment of these two protein sequences showed that the 15-bp deletion in the Hsh mRNA resulted in the deletion of five amino acids in the C-terminal region of the CTD-like phosphatase domain (CPDc) (Figure 3a). A BLAST search with the LOC_Os07g10690 protein of Hwacheong revealed that it had high levels of identity with several proteins from various organisms. The 10 closest proteins were selected for analysis and the conserved regions were aligned with the published structure of the human SCP1 protein (Kaminski...
et al., 2004), which contains the CPDc domain (Figure 3b). The five deleted amino acids occurred two amino acids downstream of the aspartic acid and asparagine residues, which are situated within the phosphatase active site (Figure 3b). Therefore, this deletion might have led to structural deformation of the active site, which would inhibit protein function.

To confirm that the mutation in LOC_Os07g10690 causes the enhanced shattering phenotype, we searched for the mutant lines with insertions in LOC_Os07g10690 from the transferred DNA (T-DNA) end sequence database (An et al., 2003). Two independent T-DNA lines (1B09125 and 3A13234) had a T-DNA insertion in the third intron of the gene, and LOC_Os07g10690 transcripts were not detected in these lines (Figure 2d,e), indicating that both mutants are null alleles (Jeong et al., 2002). Furthermore, by screening for enhanced shattering mutants from the T-DNA tagging population, we identified one more mutant line (1B03129), which had a point mutation (G→T) in the eighth exon of LOC_Os07g10690 that changed a conserved serine residue to isoleucine in the CPDc domain (Figures 2d and 3a,b). Pedicel BTS values for these three mutated lines were greatly reduced compared with those of the wild-type lines and their original variety, Dongjin, and the abscission layer of the three mutants was prominent (Figure 1a,b). These data demonstrate that LOC_Os07g10690 underlies the shattering locus sh-h. We named this gene OsCPL1. We used double-stranded RNA (dsRNA)-mediated interference to silence OsCPL1 (Miki et al., 2005). Nineteen independent OsCPL1-RNAi transgenic plants had markedly reduced BTS values and levels of OsCPL1 expression, thereby supporting the notion that this gene is correct for the shattering locus sh-h (Figure 4).

Expression of OsCPL1

We conducted an RT-PCR analysis of OsCPL1 using three primer pairs (RTF, RTP5 and RTL1). Expression was detected in the leaves and roots at all growth stages, and in young panicles of various lengths from both Hwacheong and Hsh lines (Figure 5a).

For a detailed evaluation of the OsCPL1 expression pattern, we generated transgenic plants expressing the GUS reporter gene under control of the OsCPL1 promoter. Histochemical analysis of young panicles (1 cm in length) showed that GUS was strongly expressed in all panicle tissues, and in the stem immediately below the panicle (Figure 5b). Expression was also detected in the region surrounding the abscission layer in young spikelets, but became weaker as spikelets matured (Figure 5c). Longitudinal sectioning revealed that GUS was strongly expressed in the abscission layer of young spikelets (Figure 5h–i). However, in older spikelets no expression was observed in the abscission layer, and expression was detected in the floral organs and vascular tissues of the pedicel (Figure 5k). At the heading stage, when the panicle is about 22-cm long, weak GUS expression was detected in the parenchyma tissues and vasculature, but not in the sclerenchymatous pedicel tissue (Figure 5c,l). In the roots, GUS was expressed in the

Figure 1. Shattering degree and anatomy of the abscission layer region.
(a) Comparison of the breaking tensile strength (BTS) of grain pedicels from shattering mutants and their respective wild types. The unit of BTS, gf, is the abbreviation of grams force. One gf is the force exerted by 1 g of mass under the influence of standard gravity on Earth. (b) Anatomy of the abscission layer region in the grain pedicel. Photographs in each panel were taken at a 200× magnification; AL, abscission layer; P, pedicel; SL, sterile lemma; VB, vascular bundle.
Figure 2. Identification of sh-h.
(a) Fine mapping placed sh-h in a 34-kb region between SNP markers SN1 and SN3 in BAC clone AP003943. Among the eight genes in this region, LOC_Os07g10690 corresponds to sh-h. Open bars represent the shattering Hsh genotype region; black and gray bars represent homozygous non-shattering and heterozygous genotypes, respectively. Hatched bars correspond to recombination intervals. S and NS indicate shattering and non-shattering phenotypes, respectively.
(b) Sequence alignment of RTL1 RT-PCR products of LOC_Os07g10690 in Hwacheong and Hsh lines reveals a 15-bp deletion in the mRNA of Hsh, caused by a point mutation at the 3' end splice site of its seventh intron. Rectangles represent exons, solid lines represent introns and filled rectangles indicate protein coding regions.
(c) The 15-bp deletion was confirmed by length polymorphisms of RT-PCR products amplified with the RTL4 primer pair.
(d) Schematic diagram of independent T-DNA mutant lines. RB and LB denote the right border and left border of T-DNA, respectively. Lines 1B09125 and 3A13234 have T-DNA insertions at 360 and 455 bp, respectively, from the translation initiation site. Mutant line 1B03129 has a point mutation (G → T) in the eighth exon, changing a conserved serine residue to isoleucine at the CPDc domain.
(e) RT-PCR of LOC_Os07g10690 in two T-DNA insertional mutants; TT, homozygous mutant; WW, homozygous wild type; TW, heterozygous line.
tips, lateral primordia and vasculature (Figure 5d). GUS was also weakly detected in mature leaves (Figure 5e). These observations indicate that OsCPL1 functions in the development of the abscission layer in young panicles.

Cellular localization and phosphatase activity

To identify the subcellular location of OsCPL1, we fused the full-length ORF of OsCPL1 to the GFP gene. We expressed this OsCPL1:GFP fusion construct in protoplasts isolated from seedling leaves and from a rice Oso cell line that originated from the roots. The majority of GFP signal was detected in the nucleus in both types of protoplast (Figure 6), suggesting that OsCPL1 localizes to the nucleus. The protein encoded by OsCPL1 was 462 amino acids long. To determine whether this protein has phosphatase activity, we produced an N-terminal maltose binding protein (MBP)-tagged truncated protein (tOsCPL1, amino acids 158–462) containing the functional CPDc domain (Figure 7a). The recombinant fusion protein was purified from soluble bacterial lysates on a maltose affinity column. We used this truncated form because the MBP:OsCPL1 protein was not detectable in Escherichia coli, whereas MBP:tOsCPL1 was produced as a soluble form. The purified protein had obvious phosphatase activity against p-nitrophenyl phosphate (pNPP), and its optimal pH was 7.0 (Figure 7b). The Mg$^{2+}$ ion was necessary for normal activity of the MBP:tOsCPL1 protein, whereas Mn$^{2+}$ and Ca$^{2+}$ ions were less effective as co-factors (Figure 7c). MBP fusions of truncated OsCPL1 proteins derived from the Hsh and 1B03129 mutants were also produced, and their phosphatase activities were measured. The MBP:tOsCPL1 protein from Hsh had almost no activity, and that from 1B03129 had very little activity (Figure 7d). We concluded that OsCPL1 functions in the development of the abscission layer in young panicles.
that the five-amino-acid deletion and the one-amino-acid substitution, from serine to isoleucine, in the OsCPL1 proteins abolished phosphatase activity, which in turn enhanced the development of the abscission layer and seed shattering.

DISCUSSION

Inactivation of OsCPL1 enhances the development of the abscission layer and seed shattering in rice. Therefore, this gene represses the formation of the abscission layer. The OsCPL1 protein harbors the CTD phosphatase domain, and we have provided evidence that this protein is a nuclear phosphatase, as are the metazoan CTD phosphatases, which are involved in cell differentiation.

This CTD phosphatase domain is involved in the dephosphorylation of the CTD of the largest subunit of RNA polymerase II (RNAP II) in eukaryotes. The CTD of RNAP II contains multiple repeats of an evolutionarily conserved heptapeptide with the consensus sequence Tyr\(^1\)-Ser\(^2\)-Pro\(^3\)-Thr\(^4\)-Ser\(^5\)-Pro\(^6\)-Ser\(^7\). The number of repeats varies among different organisms, ranging from 26 or 27 in yeast to 52 in mammals. It serves as a scaffold for a variety of accessory factors involved in the capping, splicing, polyadenylation and cleavage of transcripts (Orphanides and Reinberg, 2002; Hartzog, 2003; Palancade and Bensaude, 2003; Meinhart et al., 2005). Progression of RNAP II through the transcription cycle is regulated by both the state of CTD phosphorylation and the specific site of phosphorylation within the consensus repeat. Thus, CTD phosphatases may regulate the transcription of target genes by dephosphorylating the CTD of RNAP II.

CTD phosphatases belong to the third major group of phosphatases. This most recently discovered group is characterized by the catalytic signature DXDXT/V (Kerk et al., 2008). The essential yeast protein, FCP1, which is involved in the recycling of RNAP II after the termination of transcription, was the first CTD phosphatase to be identified (Kobor et al., 1999). Since its identification, additional members of the CTD phosphatase family with diverse functions have been reported. The human small CTD phosphatase 1 (SCP1) preferentially catalyzes the dephosphorylation of Ser\(^5\) within the consensus repeats of CTD, and causes neuronal gene silencing in non-neuronal genes (Yeo et al., 2003, 2005). *Xenopus* Os4 (XOs4; also called XSCP2) induces a secondary axis in young embryos, suggesting that it has a role in early development (Zohn and Brivanlou, 2001). Both XOs4 and human SCP2 dephosphorylate the Smad1 protein and attenuate signaling of the bone morphogenetic protein (BMP) (Knockaert et al., 2006). SCP2 also interacts with the androgen receptor (AR), and attenuates androgen-dependent transcription, probably by regulating promoter clearance of RNAP II during steroid-activated transcription (Thompson et al., 2006). Human RBSP3 (HYA22; also called SCP3) is a tumor suppressor: its role is probably to dephosphorylate the RB1 protein, and thereby block the cell cycle at the G1/S transition (Kashuba et al., 2004). The Dullard protein, which is required for neural development in *Xenopus*, promotes the degradation and dephosphorylation of BMP receptors (Satow et al., 2002, 2006; Kim et al., 2007). It is also reportedly involved in nuclear membrane biogenesis (Kim et al., 2007). Although HSPC129 (Qian et al., 2007) and UBLCP1 (ubiquitin-like domain containing CTD phosphatase 1; Zheng et al., 2005) are RNAP-II CTD phosphatases, their specific functions are unknown. In plants, four Arabidopsis CTD phosphatase-like family members, AtCPL1–AtCPL4, are RNAP-II CTD phosphatases (Koïwa et al., 2002, 2004; Bang et al., 2006; Ueda et al., 2008). In addition to the CTD phosphatase domain, AtCPL1 and AtCPL2 possess a double-stranded RNA-binding motif (DRM), whereas AtCPL3 and AtCPL4 contain the breast cancer 1 C terminus (BRCT) domain (Koïwa et al., 2002). AtCPL1 and AtCPL3 are negative regulators of stress-responsive genes, and are modulators of plant growth and development.
development (Koiwa et al., 2002; Xiong et al., 2002). AtCPL2 acts as a multifunctional regulator of plant growth and responses to stress and auxin (Ueda et al., 2008). AtCPL3 and AtCPL4 are paralogs that resemble the prototypic CTD phosphatase, FCP1: whereas the former specifically facilitates ABA signaling, the latter is necessary for normal plant growth (Bang et al., 2006).

In summary, the CTD phosphatases have roles in cell differentiation, growth and development, cell cycling, and hormonal and stress responses. They dephosphorylate RNAP-II CTD or other phosphoproteins, and generally act as repressors of mRNA transcription or signaling pathways. They are often simultaneously expressed in several tissues and involved in multiple functions. CTD phosphatases in the genomes of Arabidopsis, green algae (Chlamydomonas reinhardtii and Ostreococcus tauri), rice (Oryza sativa), and Populus trichocarpa have been cataloged and divided into 11 subclusters by phylogenetic analysis (Kerk et al., 2008).

Figure 5. Expression of OsCPL1. (a) RT-PCR analysis of OsCPL1, using three primer pairs (RTL1, RTP5 and RTF). VL and YR, young leaves and roots, respectively, from 17-day-old seedlings; ML and MR, leaves and roots, respectively, of plants in the middle growth stage (2 months old and 1 month before heading); Pa, young panicles 2–20 mm in length; Pb, young panicles 20–40 mm in length; Pc, young panicles 40–100 mm in length; PL and PR, leaves and roots, respectively, of plants in the young panicle stage; OL and OR, old leaves and roots, respectively, from plants 2 weeks after heading. H and S denote the Hwacheong and Hsh lines, respectively. (b–l) OsCPL1 promoter-driven GUS expression. (b) Young panicle, 1 cm in length. (c) Flowers from panicles 3.0-, 6.5-, 14.0- or 22.0-cm long. (d) Root. (e) Mature leaf. (f) Dark-field image of (j). (g) Dark-field image of (k). (h–l) Longitudinal sections of flowers in panicles 1.0-, 3.0-, 6.5-, 14.0- or 22.0-cm long. Dark-field images show GUS activities as purple signals.

Figure 6. Subcellular localization of the OsCPL1:sGFP fusion protein. Protoplasts derived from rice mesophyll cells (a–d) and rice Oc cells (e–h) were co-transformed with fusion constructs OsCPL1:sGFP, driven by the maize ubiquitin promoter, and NLS:mRFP, driven by the CaMV 35S promoter. The empty vector (pUbi-sGFP) was introduced into rice mesophyll protoplasts (i, j) and used as a control plasmid. Fluorescence signals were visualized using confocal laser scanning microscopy. (a, e, i) Fluorescence images showing localization of GFP protein. (b, f) Fluorescence images showing the localization of RFP fused with NLS (nuclear localization signal peptide: ‘PKKKRKV’). (c, g, j) Bright-field images (c, g, j). (d, h) Merged images showing the co-localization of GFP and RFP.
Among the 19 rice gene members annotated as having the CTD phosphatase domain, OsCPL1 (designated O07g10690) belongs to the subcluster that consists of animal/algal/plant sequences of unknown function, and is located between the SCP and Dullard subclusters. OsCPL1 shares a common feature with both subclusters: they are all nuclear phosphatases involved in cell differentiation. Therefore, the members of the OsCPL1-containing subcluster may also regulate cell differentiation. The other rice CTD phosphatases might be involved in diverse processes, as suggested by their close phylogenetic relationship with proteins such as the AtCPLs. Our study has now established the groundwork for elucidating the functions of CTD phosphatases in crop plants.

Considering that several QTLs for rice shattering have been detected, numerous genes appear to be involved in the differentiation of abscission layer cells. Two rice shattering genes identified to date, qSH1 and sh4, are dominant for abscission layer development and seed shattering (Konishi et al., 2006; Li et al., 2006). OsCPL1 is the third known player in the shattering process in rice, and was the first recessive shattering gene to be identified. Both qSH1 and sh4 promote the formation of the abscission layer (Konishi et al., 2006; Li et al., 2006), and qSH1 is genetically epistatic to sh4 (Onishi et al., 2007). Because of its recessive shattering trait, OsCPL1 acts as a repressor of abscission layer differentiation. This is comparable with human SCP1, which blocks the expression of neuronal genes in non-neuronal cells (Yeo et al., 2005). The negative influence of SCPs on the transcription of neuronal genes may be mediated by dephosphorylation of the CTD of RNAP II, as the phosphatase-inactive forms of SCPs promote neuronal differentiation (Yeo et al., 2005). OsCPL1 may repress the expression of genes involved in abscission layer cell differentiation by dephosphorylating the CTD of RNAP II or other phosphatase substrates. Here, we identified two mutant lines, Hsh and 1B03129, that produce forms of OsCPL1 that markedly reduce phosphatase activity (Figure 7d), and that show enhanced development of the abscission layer and seed shattering. Therefore, phosphatase activity seems to be crucial for the normal functioning of OsCPL1. The abscission layer forms approximately 16–20 days before heading, when the young panicle is 5–30-mm long (Jin, 1986). In grain pedicels at this stage, OsCPL1 expression is greater in the vascular tissue, and is stronger in the perivascular tissue than in the region farther away from the vascular tissue (Figure 5h). Therefore, the expression of genes involved in abscission layer cell differentiation may be particularly suppressed in the vascular and perivascular tissues.

The most likely candidate gene for the jointless-2 locus, which controls the development of the pedicel abscission zone in tomato, has been proposed to be ToCPL1, a gene harboring the CTD phosphatase domain (Budiman et al., 2004; Lewis et al., 2006). If this is confirmed, we may infer that CTD phosphatases in various crop plants have a role in abscission layer formation. Whether this protein dephosphorylates the CTD of RNAP II remains to be tested. Likewise, the interactions among this gene and other identified rice shattering-related genes (i.e. qSh1 and sh4) should be further analyzed to decipher the genetic network underlying abscission layer development and seed shattering in rice.

**EXPERIMENTAL PROCEDURES**

**Plant materials and fine mapping**

We analyzed 240 F2 plants from the Hsh × Blue&Gundil cross that had been used in a previous mapping study (Ji et al., 2006). Based on our earlier finding that sh-h is located in the RM8262–RM7161 interval on rice chromosome 7, we downloaded the DNA sequences of BAC clones in this interval from the TIGR database (http://www.tigr.org/tdb/e2k1/osa1/). SSR sites in these BAC clones were identified with the SSRIT program (Temnykh et al., 2001) on the GRAMENE website (http://www.gramene.org/db/searches/ssrtool), and SSR markers were designed. Polymorphic markers were used to genotype those F2 plants. After narrowing the sh-h region down to RM180–RM7161, we planted five F3 lines derived from F2 plants.

---

**Figure 7.** Phosphatase activity of OsCPL1 protein.
(a) Maltose binding protein (MBP)-tagged truncated OsCPL1 proteins (MBP:tOsCPL1) derived from Hwacheong (Hwa), Hsh and 1B03129 (1B) were produced in Escherichia coli and purified on a maltose affinity column. Purified protein (3 μg of each) was loaded onto a 10% SDS–PAGE gel.
(b) Phosphatase activity in a pH gradient.
(c) Phosphatase activity in the presence and absence of various cation co-factors.
(d) Comparison of phosphatase activities among the MBP:tOsCPL1 proteins derived from Hwacheong (Hwa), Hsh and 1B03129 (1B) lines.
that had recombination in that interval. After harvesting two panicles per plant at 50 days after heading, we used a digital force gauge (FGC-1B; Shimpo, http://www.shimpo.com) to measure the BTS of 10 grain pedicels from the uppermost part of each panicle. CAPS and dCAPS markers were designed using the dCAPS Finder 2.0 program (http://helix.wustl.edu/dcaps/dcaps.html).

Analysis of the candidate gene

RNA was extracted, using an RNeasy Plant Mini Kit (Qiagen, http://www.qiagen.com), from the young leaves and roots of 17-day-old seedlings, from the leaves and roots of plants in the middle growth stage (2 months old and 1 month before heading), from the young panicles (2–20, 20–40 and 40–100 mm long), leaves, and roots of plants in the young panicle development stage, and from the leaves and roots of plants 2 weeks after heading. cDNA was synthesized from 2 μg of total RNA by reverse transcription, using Super-Script III (Invitrogen, http://www.invitrogen.com), according to the manufacturer’s instructions. The details of the three primer pairs (RTF, RTPS and RTL1) used for RT-PCR analysis are summarized in Table S3. Primer pair RTL1 was designed to include the point mutation site at the 3′ end splice site of the seventh intron. Actin cDNA was amplified as a standard, using primers 5′-GGAGGACATCCACTACATC-3′ and 5′-CATACAGACCCCTTCTGC-ATT-3′. RT-PCR products obtained with the RTL1 primer pair were sequenced. The ORF sequences were in silico-translated and their domain structure was analyzed using the CD Search program (Marchler-Bauer and Bryant, 2004) on the NCBI website (http://www.ncbi.nlm.nih.gov/). Conserved regions were aligned with the published structure for the human SCP1 protein (Kamenski et al., 2004), using the ClustalW2 program on the EMBL-EBI website (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Three mutant T-DNA lines were selected from T-DNA mutant pools based on their possession of the enhanced shattering phenotype and on our flanking sequence analysis (An et al., 2003). Their F1 progenies were genotyped with primers designed based on T-DNA flanking sequences, and F2 progeny lines of mutants, hetero and wild genotypes were planted. The OsCPL1 transcript level was measured by RT-PCR, using primers 5′-CTGGGACACTGAGGAATA-3′ and 5′-CCTACAAGAGGACTTATCCA-3′. BTS values for grains from homozygous wild-type and homozygous mutant plants were measured with a digital force gauge. To observe the anatomy of the abscission layer, young panicles were harvested just before flowering, and about 10 flower pedicel samples per panicle were fixed, embedded in paraffin, sectioned with a microtome and stained, as described by Ji et al. (2006).

RNAi experiment

To generate the OsCPL1-RNAi construct for OsCPL1 gene suppression, we amplified a 441-bp fragment of OsCPL1 cDNA, first with primers OsCPL1-RNAI-F (5′-AAAAAGCAGGCTACATGACCCATCTC-ATTG-3′) and OsCPL1-RNAI-R (5′-AGAAAGGTGGTTTTATTCCTGC-ATTGCTG-3′), and then with primers attB1 (5′-GAGGGACAAAGT GTGTCATCCAAAAGCAGGCT-3′) and attB2 (5′-GGAGACCAGCTTTG-TACAGAAAGCTGGGT-3′). The resulting attB-PCR products were cloned, by a BP clonase reaction (Invitrogen), into the Gateway™ pDONR 201 cloning vector (Invitrogen), which carries two recombination sites (attL1 and attL2). Subsequently, we used an LR clonase reaction (Invitrogen) to insert the OsCPL1 cDNA fragment in opposite directions into two regions, each flanked by recombination sites (attR1 and attR2) in the destination vector pANDA1 (Miki et al., 2005). The resulting RNAi construct was introduced into Agrobacterium strain LBA 4404, which was used to transform rice calli induced from mature embryos of the Dongjin variety, according to the method described by Hiei et al. (1994). RNAi plants were regenerated from transformed calli after selection for hygromycin resistance. The regenerated plants were confirmed by PCR analysis with specific primers for the Bar gene, namely Bar-F (5′-TCTGACCATGTCGAAATCCTACATC-3′) and Bar-R (5′-CTGAAGTCCAGTGCCAGAAACCCA-3′), and for the gus linker, namely GUS-F (5′-ATTACCTTTCAGCTGGAAGAGAT-3′) and GUS-R (5′-ATGCACTGATACCTTCTACTC-3′). BTS values for mature grains from transformed plants were measured with a digital force gauge. We selected 19 plants that showed good seed set and significantly reduced BTS values compared with Dongjin. T1 lines from these transgenic plants were grown, and RNA was extracted, using an RNeasy Plant Mini Kit, from three transgenic seedlings per line. A 1-μg aliquot of total RNA was reverse-transcribed using Super-Script III (Invitrogen). One percent of the synthesized first-strand cDNAs was used for real-time quantitative RT-PCR analysis, using the following sets of gene-specific primers: for OsCPL1, QRT-F (5′-GAGGGAAACTCTTTAAGAGTCTAAGGCTCTTG-3′) and QRT-R (5′-CCAGCTTTCATCAGGATGCGATC-3′), and for ubiquitin (the control), RUB2-F (5′-AATCAGCGCTTTGGTGAGCTG-3′) and RUB2-R1 (5′-ATGCAATAAGGACAAATTGAGGACA-3′) (Chen et al., 2007). Real-time quantitative RT-PCR analysis of RNAi plants was conducted with SYBR® GREEN PCR Master Mix (Applied Biosystems, http://www.appliedbiosystems.com) and an Mx3005P real-time PCR system (Stratagene, http://www.stratagene.com), according to the manufacturer’s instructions. For semi-quantitative RT-PCR of OsCPL1, the primer pair for RTL1 (Table S3) was used.

Subcellular localization of the OsCPL1:GFP fusion protein in rice mesophyll cells

The full-length OsCPL1 coding region without the stop codon was PCR-amplified with primers 5′-ccccggATCGAGTTGGGAGAT-3′ and 5′-tctagaCTTTCGAGGTCCATTGAAAG-3′, which harbor restriction sites (set in lower case) for cloning. This OsCPL1 PCR product was cloned into the pG3A452 vector containing an sGFP coding sequence under the control of the maize ubiquitin promoter. A vector that generates an NLS:mRFP fusion protein was used as a marker of the nucleus. The protoplast preparation and transformation procedures were as described previously (Han et al., 2006; Woo et al., 2007). Briefly, leaves from wild-type seedlings (10 days after germination) were dissected with a razor blade and incubated with gentle agitation in an enzyme solution (1.5% cellulase, RS, 0.5% macerozyme, 0.1% pectolyase, 0.6 M mannitol, 10 mM 2-(N-morpholine)-ethanesulfonic acid (MES), 1 mM CaCl2, and 0.1% bovine serum albumin) for 4 h at room temperature. Approximately 0.5 ml of protoplasts (1 × 10^6 ml^-1) was mixed with about 20 μg each of OsCPL1:sGFP and NLS:mRFP plasmid DNA, and the mixture was electroporated in a 4-mm-wide cuvette in the Gene Pulser Xcell (Bio-Rad, http://www.bio-rad.com), set at 300 V and 450 μF. After 15–20 h of incubation at 28°C in darkness, the transformed protoplasts were examined with a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, http://www.zeiss.com) equipped with filter sets for GFP and RFP.

Phosphatase assay of OsCPL1 protein

The ORF of OsCPL1 cDNA was PCR-amplified with primer pair 5′-CACTGTCTTCCGAGTACAGCAGACCGGAAAAAG-3′ and 5′-CAGCTCT- AACGTTCACAATTGCGAGCT-3′, and then inserted into pRSET E. coli expression vectors (Invitrogen) containing N-terminal 6x His or MBP tags. A partial gene that encodes a truncated form of the OsCPL1 protein (tOsCPL1) containing only the C-terminal functional
domain (158–462 amino acids) was also amplified by PCR with primer pair 5′-GAAGGGATCCTCGTTGAGAAC-3′ and 5′-CAGTCTAAGCTTCAATGTTGATA-3′, and was inserted into the pRSET expression vector. These constructs were introduced into the Top 10 E. coli strain, and protein synthesis was induced. The bacterial lysates were divided into soluble and insoluble forms and electrophoresed on SDS–PAGE gels. Only the MBP:tOsCPL1 fusion protein was produced in a soluble form, and was purified on a maltose affinity column.

Phosphatase activity was tested with pNPP as the substrate. Buffers of various pH values were prepared: acetate–sodium acetate buffers of pH 4.4, 5.0 and 5.8, potassium phosphate–sodium phosphate buffers of pH 5.2, 6.3, 7.0 and 7.7, and Tris–HCl buffers of pH 7.4, 8.0 and 8.9. Reaction buffers containing 5 mM DTT and 10 mM MgCl₂ were made from these pH buffers. The enzyme was assayed in a 50-μl reaction volume that included 10 μg of purified MBP:tOsCPL1 protein, 5 mM pNPP and 35 μl of reaction buffer. After incubation at 30°C for 4 h, the OD₄₀₀ was measured on a NanoDrop Spectrophotometer (http://www.nanodrop.com). The effect of cationic co-factors was tested by the addition of MgCl₂, MnCl₂, CaCl₂ or no co-factor, in Tris–HCl buffer (pH 7.4), a buffer that prevents precipitation while sustaining high phosphatase activity. Activities were compared among MBP:tOsCPL1 fusion proteins from Hwacheong, Hsh and 1B-03129 lines using potassium phosphate–sodium phosphate buffer (pH 7.0) with 5 mM DTT and 10 mM MgCl₂.

Construction of the OsCPL1 promoter–GUS fusion and GUS assay

A genomic DNA fragment (from 2647 to 18 bp from the translation start site) containing the promoter region of OsCPL1 was amplified by PCR, using primers 5′-ggtaccTCTGCTTGGTGCTT-3′ and 5′-actagtCTCCAAACCATCTGGATCA-3′, and was inserted into the pB340/OS vector. These constructs were introduced into the Agrobacterium tumefaciens strain EHA105 harboring the Ti plasmid pBI121. The Agrobacterium:pOsCPL1 promoter–GUS fusion was used to transform rice protoplasts. The GUS activity was measured based on the quantification of a colorimetric assay (Jefferson, 1987). The activity of the pOsCPL1 promoter–GUS fusion was assayed in transgenic rice plants (OsCPL1::GUS). The transgenic lines were identified from GUS staining in young leaf tissues. The GUS activity was assayed by histochemical GUS staining method (Jefferson, 1987).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Single nucleotide polymorphism (SNP) sites found by sequencing candidate genes for sh-h, and development of SNP markers.

Table S2. Detailed information on single nucleotide polymorphism (SNP) markers developed based on sequencing of candidate genes.

Table S3. Primers used in the RT-PCR of the LOC_Os07g10880 gene. Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References


© 2009 The Authors


Mekhedov, L. (2007) Constitutively wilted 1, a member of the rice YUCCA gene family, is required for maintaining water homeostasis and an appropriate root to shoot ratio. Plant Mol. Biol. 65, 125–136.

Mekhedov, L. (2007) Constitutively wilted 1, a member of the rice YUCCA gene family, is required for maintaining water homeostasis and an appropriate root to shoot ratio. Plant Mol. Biol. 65, 125–136.

Mekhedov, L. (2007) Constitutively wilted 1, a member of the rice YUCCA gene family, is required for maintaining water homeostasis and an appropriate root to shoot ratio. Plant Mol. Biol. 65, 125–136.

