ABNORMAL SHOOT IN YOUTH, a Homolog of Molybdate Transporter Gene, Regulates Early Shoot Development in Rice

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ABSTRACT

We analyzed the abnormal shoot in youth (asy) mutant to understand the phase-specific regulation of shoot development. asy showed various shoot abnormalities, including small leaves due to the precocious termination of cell division, defects in leaf blade-sheath boundary formation, and abnormal shoot apical meristem maintenance at the early vegetative stage. These defects recovered with advanced development. ASY encodes a DUF791 domain protein, which is part of the major facilitator superfamily. Despite stage-specific phenotypes, the ASY expression level was roughly constant throughout development. A paralog of ASY, ASL, exists in the rice genome and is supposed to have redundant functions. ASL expression was relatively low in early-stage embryos but increased at later stages. Thus, asy phenotypes were limited to the stage when ASL expression was suppressed. A homology search revealed that ASY is a homolog of the Chlamydomonas CrMoT2 gene, which encodes a molybdate transporter. ASY was suggested to encode a molybdate transporter based on its sequence similarity with CrMoT2 and predicted transmembrane topology. This is the first report of a CrMOT2-type molybdate transporter in higher plants.

Keywords: ABNORMAL SHOOT IN YOUTH; Early Vegetative Stage; Shoot Meristem; Rice; Molybdenum; Transporter

1. Introduction

The genetic regulatory mechanism of shoot development has been studied in many higher plants such as Arabidopsis, rice, and maize. CUP-SHAPED COTYLEDON 1 (CUC1), CUC2, and SHOOT MERISTEMLESS play important roles in differentiation of the shoot apical meristem (SAM) in Arabidopsis [1-3]. However, distinct genes related to small RNA metabolism in rice such as SHOOTLESS 1 (SHL1), SHL2, SHOOT ORGANIZATION 1 (SHO1), SHL4/SHO2, and WAVY LEAF have decisive roles in SAM formation [4,5]. The mechanism of SAM maintenance is another important aspect of shoot development. A large number and various classes of genes are involved in SAM maintenance, including CLAVATA, WUSCHEL, FAS, and FSM [6-9]. These genes function throughout the plant life cycle; however, plants show phases of development that include embryogenesis, and vegetative and reproductive phases. The vegetative phase is further divided into juvenile and adult phases. These phases indicate that plant development is regulated by phase-specific genes as well as by genes acting throughout the life cycle.

Recent advances in plant developmental biology have shown that the juvenile-adult phase change during vegetative development is an important event. Because a large number of traits differ between the juvenile and adult phases [10-12] and several genes associated with phase changes have been cloned [13-16], it is expected that juvenile or adult phase-specific regulatory mechanisms of shoot development are operating. Accordingly, another interesting aspect of plant vegetative development is the phase-specific regulation of gene expression.

In this study, we identified the abnormal shoot in youth (asy) mutant, which showed abnormal phenotypes during the early vegetative phase but near-normal later development. The asy causal gene encodes a molybdate transporter. The asy phenotype is similar to the shootless (shl) phenotype in rice, which is associated with small leaves and defects in leaf blade-sheath boundary formation. However, the asy phenotype is unique in that it shows precocious termination of cell division and abnormal shoot apical meristem maintenance at the early vegetative stage. These defects recover with advanced development. The asy phenotype is similar to the CrMoT2 phenotype in Chlamydomonas, which encodes a molybdate transporter. ASY encodes a DUF791 domain protein, which is part of the major facilitator superfamily. Despite stage-specific phenotypes, ASY expression level is roughly constant throughout development. A paralog of ASY, ASL, exists in the rice genome and is supposed to have redundant functions. ASL expression is relatively low in early-stage embryos but increases at later stages. Thus, asy phenotypes are limited to the stage when ASL expression is suppressed. A homology search revealed that ASY is a homolog of the Chlamydomonas CrMoT2 gene, which encodes a molybdate transporter. ASY was suggested to encode a molybdate transporter based on its sequence similarity with CrMoT2 and predicted transmembrane topology. This is the first report of a CrMOT2-type molybdate transporter in higher plants.
transporter. Our findings indicate that a specific trace element plays a distinct role in plant development, including leaf morphogenesis, cell division, and shoot meristem maintenance.

2. Materials and Methods

2.1. Plant Materials

We identified a single recessive mutant from M2 population of rice (Oryza sativa L., ssp. japonica cv. Taichung 65) mutagenized with N-methyl-N-nitrosourea. Since the mutant developed weak plants at the early stage and recovered almost normal stature at the late vegetative stage, we named this mutant abnormal shoot in youth (asy). Taichung 65 was used as wild type. In the experiments on early vegetative seedlings, seeds were surface sterilized and grown on MS plates that contained 4.6 g/L Murashige and Skoog plant salts mixture [17], 30 g/L sucrose, 5 mg/L myo-inositol, 5 ng/L thiamine hydrochloride, 25 ng/L nicotinic acid, 25 ng/L pyridoxine hydrochloride, 0.1 mg glycine and 10 g/L agar (adjusted to pH 5.8 with KOH) under constant white light at 28˚C. Adult plants were grown in pots or paddy field. Transgenic plants were grown in biohazard phytotron.

2.2. Histological Analysis

For paraffin sectioning, samples were fixed in FAA (formaldehyde: glacial acetic acid: ethanol [1:1:18]) for 24 h at 4°C, dehydrated in a graded ethanol series, and embedded in Paraplast plus (McCormick Scientific). Microtome sections (8 μm thick) were stained with Delafield’s hematoxylin, and then observed under a light microscope.

2.3. In Situ Hybridization

Shoot apices 7-day after germination (DAG) of wild type and asy were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 48 h at 4°C, and then dehydrated in a graded ethanol series. The dehydrated samples in 100% ethanol were replaced with butanol and embedded in Paraplast plus (McCormick Scientific). Paraffin sections (8 μm thick) were applied to microscope slides coated with MAS (Matsunami Glass). Digoxigenin-labeled antisense RNA probes of OSH1 and Histone H4 were prepared as described [18]. In situ hybridization and immunological detection with alkaline phosphatase were performed according to the methods of Kouchi and Hata (1993) [19].

2.4. Map-Based Cloning of the ABNORMAL SHOOT IN YOUTH Gene

To map the ASY1 locus, asy homozygous plants (O. sativa L. ssp. japonica) were crossed with cv. Kasalath (ssp. indica), and the F2 and F3 populations were examined for recombination between the mutation and PCR-based polymorphic markers. The ASY locus was mapped in the 58.9 to 61.4 cM region on chromosome 10 using STS and CAPS markers obtained from the rice genome database (http://rgp.dna.affrc.go.jp/E/publicdata/caps/index.html). By finding recombination break points among 420 asy plants, the ASY locus was limited within a 100-kb region, including 20 candidate genes annotated in the RAP-DB database, and covered by three overlapping BAC clones.

2.5. Complementation Test

The 6.8-kb ASY genomic DNA including 3-kb upstream and 1-kb downstream was used for complementation test. This fragment was introduced into Agrobacterium tumefaciens strain EHA101 and transformed into asy homozygous plants by the Agrobacterium mediated transformation method [20].

2.6. Transient Expression Assay

For construction of fluorescence fusion protein, the coding region of ASY was amplified with primers that included appropriate restriction sites. It was translationally fused to 5’ terminus of the sGFP gene driven by 35S promoter of the cauliflower mosaic virus (p35S::ASY-sGFP). Then it was introduced into onion epidermal cells using a particle bombardment with gold particles according to the manufacturer’s instructions (PDS-1000/He; Bio-Rad, Hercules, CA, USA). The cells were observed using a fluorescence microscope (BZ-8000; Keyence Co.).

2.7. Expression Analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. For RT-PCR, 1 μg of RNA after DNase I digestion was reverse transcribed with the SuperScript III First Strand Synthesis System and oligo (dT) primer according to the manufacturer’s instructions (Invitrogen). Resulting cDNA was used for RT-PCR with gene specific primers; ASY-ReTiF: TTCAGGTTTGCAAGCCAAAAAGC and ASY-ReTiR: AACAGGAGTCCACAGGAAA (for amplification of ASY cDNA), ASL-ReTiF: GGAGCACAAACAGAGGT and ASL-ReTiR:
cells were enlarged and increased in number in the leaf blade (Figures 1(d)). Transverse sections of the leaf blade revealed that air spaces had formed in the second leaf blades and infrequently in the third leaf blades of asy but not in subsequent leaf blades (Figure 1(g)). Because air spaces are characteristic of wild-type leaf sheaths, this result suggests that leaf blade and sheath identities are mixed in asy leaves.

3.2. Defects in the asy Shoot Meristem and Embryo

The leaf abnormalities and seedling lethality we observed suggested abnormalities in the asy SAM. Longitudinal sections of SAMs revealed that although the width of the asy SAM was comparable to that of wild type, the height was smaller than that in wild type (Figures 2(a)-(d)). We also found a SAM in which many cells were vacuolated in an extreme dwarf asy plant (Figure 2(d)). As in the case of leaf phenotypes, SAM defects in asy were also recovered at 21 DAG (Figures 2(e) and (f)). Thus, asy was proposed to have a significant role in the SAM at the early (juvenile) stage of vegetative development.

Because the first three leaves are formed in rice during seed development, we examined asy embryos at 10 days after pollination (DAP). Although all organs were formed in the embryos, morphological abnormalities were observed in the scutellum, coleoptile, and shoot apex (Fig-
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3.3. Characterization of asy by Marker Gene Expression

Phenotypic abnormalities such as small leaves and an aberrant SAM suggest that related gene expression is disturbed in asy. First, we examined cell division activity by in situ hybridization probed with histone H4. At 4 DAG, when the fourth leaf primordium was being formed, a large number of histone H4 signals were uniformly observed in second (P3 stage), third (P2), and fourth (P1) leaf primordia in wild type (Figure 3(a)). In contrast, although the fourth leaf primordium (P1) showed many histone H4 signals comparable to those in wild type, histone H4 transcripts disappeared from the distal region of the asy third leaf primordium (P2) (Figure 3(b)). This tendency became more remarkable in the second leaf primordium; the expression domain was limited to a narrow basal region and the expression level decreased (Figure 3(b)). This result indicates that asy terminated cell division precociously, resulting in small leaves.

To examine SAM activity, we performed in situ hybridization probed with the OSH1 gene, which is expressed in indeterminate cells of the SAM. At 7 DAG, some asy SAMs showed a narrower OSH1 expression domain (Figures 3(c) and (d)), while other asy SAMs failed to express OSH1 (Figure 3(e)). Thus, asy is defective in SAM maintenance. Presumably, vacuolated and/or OSH1-non-expressing SAMs are inactive and the corresponding plants die at an early stage of development.

3.4. Identification of ASY Gene

asy homozygous plants were crossed with ssp. indica cv. Kasalath to identify the ASY gene using a map-based cloning method. The ASY locus was roughly mapped at 58.9 - 61.4 cM on chromosome 10 (Figure 4(a)). By fine mapping using 420 plants, candidates were confined to 20 genes from the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/). Through sequencing, we identified a G-to-A substitution in Os10g0519600. The substitution occurred in the conserved AG dinucleotide of the splice acceptor site in the third intron (Figure 4(a)). We performed a complementation test by introducing a 6.8-kb genomic fragment containing the coding region of Os10g0519600 into the asy mutant. T2 lines carrying the 6.8-kb genomic fragment (PZP-gASY) fully complemented the asy mutant phenotype (Figure 4(b)). Thus, we concluded that Os10g0519600 is the asy causal gene. Reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that ASY transcripts of various sizes with unusual splicing or non-splicing in the third intron were produced in asy seedlings at 5 DAG (Figure 4(c)).

ASY is comprised of eight exons and seven introns,
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Figure 4. Molecular characterization of ASY gene. (a) Physical mapping of the ASY locus. The number shows recombination events. Black and white boxes indicate exons and untranslated regions, respectively. Arrow indicates mutation site in asy; (b) Complementation test. The left-side four plants are asy mutants transformed with 6.8-kb genomic ASY fragment (PZP-gASY). The right-side three plants are asy mutants transformed with empty vector (PZP); (c) Multiple splicing products in asy mutant. The upper panel shows RT-PCR analysis of ASY transcripts in wild-type (Lane 1) and asy (Lane 2, 3) seedlings at 5 DAG. Three different sizes of transcripts were detected. The lower illustration shows predicted mutant forms of ASY proteins corresponding to the transcripts in the upper panel. Arrowheads indicate mutation site in asy. The transcript as shown by (3) contains complete 3rd intron; (d) The phylogenetic tree of DUF791 domain-containing proteins from three monocots Oryza sativa (Os), Zea mays (Zm), Sorghum bicolor (Sb), four eudicots Arabidopsis thaliana (At), Vitis vinifera (Vv), Populus trichocarpa (Pt), Medicago truncatula (Mt), one fern Selaginella moellendorffii (Sm), one moss Physcomitrella patens (Pp), three green algae Chlamydomonas reinhardtii (Cr), Ostroecoccus lucimartius (Ol), Ostroecoccus tauri (Ot) and red alga Cyanidioschyzon merolae (Cm). The alignment was obtained from ClustalW method and tree was build using MEGA5.1 software.

and encodes a protein composed of 456 amino acid residues (Figure 5(a)). The deduced amino acid sequence of ASY showed that it belongs to the DUF791 family, which is part of the major facilitator superfamily, with a domain of unknown function. The ASY protein contained a predicted 11 transmembrane domains and was proposed to act as a transporter [22] (Figure 5(b)).

BLAST search against the rice protein database using the ASY amino acid sequence revealed that a paralogous DUF791 family gene exists in the rice genome, Os03g0114800, which had 93.2% identity with ASY. We named this paralog ASY-like (ASL) (Figures 4(d) and 5(a)).

A phylogenetic tree constructed from the DUF791 domain of ASY showed that DUF791 family proteins were divided into two clades. ASY group proteins, in which ASY and ASL are included, are well conserved not only in plants but also in algae (Figure 4(d)). In con-
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Figure 5. Alignment of amino acid sequences of **ASY** homologs and predicted topology. (a) Multiple alignment among **ASY**, **ASL** in *Oryza sativa*, **ASY** orthologs in *Arabidopsis thaliana* (At1g64650), *Selaginella moellendorffii* (XP_002977775) and **CrMOT2** in *Chlamydomonas reinhardtii*. Amino acids shaded in black are conserved in all 5 proteins examined, and those in grey conserved in more than three proteins. Underlines indicate transmembrane regions predicted by TMpred software. Arrowhead shows the position of **asy** mutation causing splicing defects in **asy**. DUF791 domain is marked by double underline. Dashed overlines shows four conserved motifs reported by [23]; (b) Schematic illustration of predicted **ASY** transmembrane topology using TMpred software.

Contrast, the other group of proteins is found only in plants and has little significant conservation to **ASY** group proteins. For example, rice Os08g0113800, one of the three DUF791 domain-containing proteins in rice, has 23.2 and 22.8% identity with **ASY** and **ASL**, respectively.

The **ASY** group includes **CrMOT2**, which has been recently identified to be a molybdate transporter in *Chlamydomonas* [23]. **ASY** has 53.1% amino acid identity with **CrMOT2**. In addition, the deduced transmembrane topology was shared between **ASY** and **CrMOT2** (Figure 5(b)).

Next, we measured the molybdenum (Mo) content in mature seeds. The Mo contents (ng/mg dry weight) in whole mature seeds, mature embryos, and endosperm were 0.311 ± 0.010, 0.480 ± 0.028, and 0.287 ± 0.014 (mean ± standard deviation), respectively, in the **asy** mutant and 0.324 ± 0.009, 0.452 ± 0.023, and 0.308 ± 0.005, respectively, in wild type. We could not detect any differences in Mo content in mature embryos, endosperm, or whole seeds between wild type and **asy**.

### 3.5. **ASY** Expression Pattern

**ASY** was deduced to localize to the membrane based on its amino acid sequence. Therefore, we examined the subcellular localization of **ASY** using onion epidermal cells transformed with 35S::**ASY**-GFP. GFP signals were detected mainly in the plasma membrane (Figures 6(a) and (b)), suggesting that **ASY** acts as a transporter. **ASY** expression in different organs was examined by RT-PCR. **ASY** was expressed in all organs examined (Figure 6(c)), although the expression level fluctuated to some extent depending on the organ. **ASL** showed a similar expression pattern, but its expression level was relatively low in 5 DAP embryos and young inflorescences (Figure 6(c)). Next, the expression of **ASY** and **ASL** during embryogenesis and at the early vegetative stage was...
Expression level was represented relative to that of ASY and ASL. Thus, No rice mutants have been reported to show early development, precocious termination of cell division in leaves during the juvenile phase seedlings, including abnormal embryo formation, precocious termination of cell division in leaves 7 - 14 DAP seeds and in 4 - 14 DAG seedlings. Expression level was represented relative to that of ACTIN.

examined by real-time PCR. ASY was expressed rather constantly during seed development, whereas it was low until 10 DAP but largely increased at 14 DAP (Figure 6(d)). ASY expression was higher than that of ASL in 7 DAP seeds when the second leaf primordium was forming in the embryo (Figure 6(d)). After germination, ASY and ASL expression was comparable until 7 DAG, but the ASL expression level was much higher than that of ASY at 10 and 14 DAG (Figure 6(d)). These expression patterns suggest that the early stage-specific phenotypes of asy, despite nearly constant expression in all organs and at all stages, resulted from the expression of its paralog ASL.

4. Discussion

No rice mutants have been reported to show early development-specific phenotypes and recovery at later stages. Thus, asy is a unique mutant in that ASY plays important roles only during early development. Phenotypic defects in asy were observed in various traits of embryo and juvenile phase seedlings, including abnormal embryo formation, precocious termination of cell division in leaves associated with small leaves, aberrant leaf morphogenesis, and defective SAM maintenance. These abnormalities eventually caused seedling death. Interestingly, these stage-specific phenotypes were not caused by stage-specific ASY expression. A highly homologous gene, ASL, exists in the rice genome. ASL transcripts showed a similar expression profile to that of ASY. Notably, ASY expression was relatively higher than that of ASL in 7 DAP seeds when the rice embryo developed second and third leaf primordia. Thus, the different expression levels of these genes during early embryogenesis might be responsible for the asy mutant defects. Although functional redundancy between ASY and ASL has not been confirmed, the high level of expression of ASL at the late stage explains the temporal specificity of asy phenotypes. A loss-of-function ASL mutant is not available. To clarify the relationship between ASY and ASL, identification of the asl mutant and its detailed functional analysis are needed in the future.

ASY is a homolog of Chlamydomonas CrMoT2, which encodes a molybdate transporter, and is also found in humans [23]. ASY is the first homolog of CrMoT2 reported in higher plants. Our results suggest a significant role for the trace element Mo in rice development. The sequence similarity and subcellular localization of ASY suggest that it also acts as a molybdate transporter. Two genes, MOT1 and MOT2, encode molybdate transporters in Arabidopsis, but they are different from ASY/CrMoT1 [24-28]. In the presence of Mo, the Arabidopsis mot1 mutant does not show severe phenotypes [24,26,29]. In contrast, asy showed more severe phenotypes than Arabidopsis mot1 under appropriate soil conditions (seemingly in the presence of sufficient Mo), suggesting a functional difference between ASY and MOT1.

No significant differences were detected in the seed and embryo Mo contents between wild type and asy, although severe defects were observed in asy embryos. Because the Km value of Chlamydomonas CrMoT2 for molybdate is higher than that of Arabidopsis MOT1 and CrMOT1 [24,26], ASY may have a different function from the MOT1-type molybdate transporter. It is possible that ASY creates a local concentration gradient of Mo, which is required for organ development. It is also conceivable that a significant difference in Mo content can be detected when early stage seeds at 7 DAP are examined, because ASL is predominant at later stages. Questions about Mo uptake activity, Mo affinity, and the detailed subcellular localization of ASY in planta should be addressed in future studies.

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REFERENCES


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