Gene silencing of barley P23k involved in secondary wall formation causes abnormal tiller formation and intercalary elongation

Ai Oikawa¹, Kazuya Nagaï¹, Kiyoaki Kato² and Shin-ichiro Kidou*³

¹ Cryobiosystem Research Center, Faculty of Agriculture, Iwate University, Morioka, Iwate 020-8550, Japan
² Department of Crop Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan
³ Graduate School of Natural Sciences, Nagoya City University, Mizuho, Nagoya, Aichi 467-8501, Japan

P23k is a monocot-unique protein that is highly expressed in barley. Our previous loss-of-function studies in barley leaves indicated that P23k, localized to tissues where cell wall polysaccharides accumulate, might contribute to secondary wall formation in the leaf. However, the P23k loss-of-function analysis was limited to the leaf, which is a vegetative organ. Considering the involvement of P23k in secondary wall formation, a dramatically altered phenotype is expected in the stem of P23k gene-silenced barley, where marked secondary wall deposition occurs during the reproductive growth stage. To test this hypothesis, barley striped mosaic virus–based virus-induced gene silencing of P23k was performed. Abnormal tiller formation and arrested intercalary elongation were observed in P23k-silenced barley. From these results, we speculated that cell wall architecture was altered by P23k gene silencing. Consistent with this idea, we observed a marked decrease in the amount of cell wall polysaccharides stained with calcofluor and down-regulation of the cellulose synthase-like CslF6 gene involved in (1,3;1,4)-β-D-glucan synthesis. Taken together, these results suggest that P23k is possibly involved in determining secondary wall architecture and contributes to tiller formation and intercalary elongation in barley.

Key Words: Hordeum vulgare, P23k, virus-induced gene silencing, secondary wall formation, (1,3;1,4)-β-D-glucan, tiller, intercalary elongation.

Introduction

Tiller number is an important agronomic trait for grain production in cereals such as rice, wheat and barley. Although tillering (branching) is also found in dicots, it differs significantly from that of monocots: the tiller in monocots is derived from the lower, unelongated portion of the stem, while branching in dicots often occurs from the upper, elongated part of the stem. The formation of an axillary meristem, which enables tiller development at a late growth stage, involves changes in cell proliferation and differentiation at certain positions on the stem, accompanied by drastic and concerted modifications in gene expression (Talbert et al. 1995, Schmitz et al. 2002, Li et al. 2003, Sorefan et al. 2003, Takeda et al. 2003) and action of hormones such as auxins (Reinhardt et al. 2000, 2003, Benkova et al. 2003, Xu et al. 2005, Carraro et al. 2006). Despite progress in identification of genes expressed in the tiller or branch, our knowledge of their molecular basis is still fragmented. The reason for the restriction of the tiller to the lower, unelongated portion of the stem, especially in monocots, remains unclear.

A schematic representation of the stem architecture of the monocot barley (Hordeum vulgare L.) is shown in Fig. 1. In monocots, the internodes of the upper stem elongate following the development of an intercalary meristem just above each node in the reproductive growth phase. Therefore, the stem elongation is termed intercalary elongation. The cells produced by the intercalary meristem exhibit marked expansion in a specific region called an elongation zone during the intercalary elongation (Evans 1965, Fisher 1970, Bleecker et al. 1986). The first (uppermost) internode can elongate to a maximum of 30 cm and is the longest of the internodes, indicating that the stem length is determined by the degree of cell expansion from the intercalary meristem. In contrast, internodes of the lower, unelongated part of the stem do not have the ability to elongate since cell division in the intercalary meristem is repressed even when the shoot apical meristem passes into the reproductive phase. Thus, cell expansion in the elongation zone is considered to be the major determinant of the growth rate of the whole internode (Kende et al. 1998).

Plant cell expansion is regulated by the mechanical properties of the cell wall and cell wall synthesis (Taiz 1984). The plant cell wall is composed of an organized matrix of cellulose, hemicellulosic and pectic polysaccharides protein and lignin, and is conveniently considered to be of two types, a thin primary wall and a thicker secondary wall. The primary wall is the part of the wall laid down by young and...
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undifferentiated cells such as parenchyma cells, while secondary wall deposition occurs after the cell has stopped growing, as in sclerenchyma cells (Talmadge et al. 1973). The cell wall is an intricate structure involved in the determination of cell size and shape, growth and development, and intercellular communication. By analyzing the direct attachment between cellulose and microtubules using live-cell imaging, Paredez et al. (2006) suggested that the cellulose-microtubule network might be important for intercalary elongation. Nemoto et al. (2004) reported that microtubules are involved in the rate and direction of cell expansion in intercalary elongation. Sauter and Kende (1992) also hypothesized that elevated levels of (1,3;1,4)-β-D-glucan (a hemicellulosic component) contribute to elongation growth of internodes by increasing the extensibility of the cell wall. However, evidence for a direct role of cell wall synthesis in intercalary elongation remains to be demonstrated in Gramineae, although it has recently been shown that mutation in the cellulose synthase-like family (Csl) F6, a (1,3;1,4)-β-D-glucan synthase, causes slightly shorter culms in barley (Tonooka et al. 2009).

Barley P23k was originally identified as a protein expressed in barley seedlings (Kidou et al. 2006). P23k is strongly expressed in the scutellum of imbibed barley seeds, and was synchronously down-regulated with consumption of the starchy endosperm. In addition, expression analyses of P23k mRNA in barley leaves showed up-regulation of P23k transcripts with increased photosynthetic activity (Oikawa et al. 2007). These results indicated that P23k might play a role in sugar metabolism. Furthermore, the P23k protein is also expressed abundantly in vascular bundles and sclerenchyma where secondary wall formation is active in vegetative leaves of barley (Oikawa et al. 2007). Virus-induced gene silencing (VIGS) of P23k in the vegetative growth stage led to abnormal leaf development, asymmetric orientation of the main veins, and cracked leaf edges caused by mechanical weakness (Oikawa et al. 2007). From these results, we speculated that P23k is also involved in cell wall synthesis.

In this study, based on P23k expression and the phenotype observed following P23k VIGS, we showed that P23k is involved in modulating secondary wall architecture and regulation of tiller formation in barley.

Materials and Methods

Plant material and growth conditions
Barley (H. vulgare cv. Minorimugi) seeds were surface-sterilized for 30 min with 2% (w/v) NaClO and then germinated inside wet towels in the dark at 25°C. The germinated seeds were transplanted into 1/5 Hoagland No. 2 liquid medium (pH 5.5) and placed in a cold room for six weeks with a 16 h light and 8 h dark cycle for vernalization treatment. The vernalized barley plants were grown in a greenhouse.

Preparation of infectious BSMV RNAs and plant inoculation
The barley striped mosaic virus (BSMV) plasmid vectors used in this study were described previously (Holzberg et al. 2002, Oikawa et al. 2007). Infectious BSMV RNAs were prepared from each plasmid by in vitro transcription using the mMESSAGE mMACHINE High Yield Capped RNA Transcription Kit (Ambion, Austin, TX, USA). Five microliters of each transcript, which included the BSMV RNAs α, β, and genetically modified γ, were combined with 40 μl FES buffer (Pogue et al. 1998) and inoculated into the first and second leaves of barley plants at the five- or six-leaf stage.

Expression analysis by RT-PCR and immunoblot analysis
To generate first-strand cDNA, 1 μg total RNA was annealed with 10 μM random hexamers in a 20 μl reaction.
mixture, and extended using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (TOYOBO) at 42°C for 1 h. One microliter of each reverse transcription (RT) reaction was used as template in 20 μl PCR reactions containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 4 mM dNTPs, 0.2 units Ex Taq polymerase (Takara) and 1 μM primers. The primers used for gene amplification were as follows: P23k (5′-GGTACGGAACGGAATAGC-3′ and 5′-TCGCCACACAAGGCTTTGATGTT-3′); HvEF-1A (5′-TTCAACGTCAAGAAGCTGGGCT-3′ and 5′-ACACAATAAACCAAGCGACTA-3′); BSMVγ (5′-GAATCCAAAACCTGCTCAACC-3′ and 5′-CATTCGGTTGGAACCT-3′); HvCesA1 (5′-ATAACCCAAGCGACTA-3′ and 5′-CAAGCA-3′); HvCslF6 (5′-CATACCTCGGGAGGCAAGCA-3′ and 5′-TTCAACCTTTGCGGCTCT-3′); HvCesA1 (5′-ATGATGAAACTCGCAGCTTC-3′ and 5′-TCATGCCTTAGAACTAGGCC-3′); HvGSL (5′-CATAGTACGTTGTTATGGC-3′ and 5′-CAACCGAGCGCATCTTTCC-3′). Thermal cycling was conducted as follows: denaturing at 94°C for 1 min followed by 30 cycles of denaturing at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min. The resulting products were separated by 2% agarose gel electrophoresis and visualized by EtBr staining. The specificity of the primers was tested using a buffer consisting of 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 1.5 mM MgCl₂, 1 mM PMSF, and 5% glycerol, and then subjected to 12% (v/v) SDS-PAGE following the procedure of Laemmli (1970). After transferal to a polyvinylidene fluoride membrane, immunoblot analysis was performed according to the manufacturer’s (Amersham Pharmacia) instructions. Total protein was detected using Coomassie Brilliant Blue staining as a control.

**Histochemical analysis by in situ hybridization**

Selected BSMV uninfected barley tissues were fixed for 5 h at room temperature in a fixative solution (3.7% formaldehyde, 5% acetic acid and 50% ethanol). Fixed tissues were dehydrated in a series of ethanol and xylene solutions and embedded in paraffin (Paraplast Plus, Sigma). Embedded tissues were then sectioned at a thickness of 7 μm and placed on poly-L-lysine coated microslide glass (Matsunami). Sections were deparaffinized with xylene and rehydrated through a graded ethanol series. They were subsequently pretreated with proteinase K (Boehringer Mannheim) at 37°C for 30 min, dehydrated in a graded ethanol series, and dried under vacuum for 1 h. For the preparation of probes, a partial p23k-1 cDNA clone (Kidou et al. 2006) was used. P23k sense and antisense probes were generated by *in vitro* transcription using a DIG RNA Labeling Kit (Boehringer Mannheim). Hybridization signals were detected using a DIG Nucleic Acid Detection Kit (Boehringer Mannheim).

**Histochemical localization of cell wall polysaccharides**

The deparaffinized sections were treated with 0.005% aqueous calcofluor (fluorescent brightener 28; Sigma) or 0.5% aqueous toluidine blue O, and visualized with a fluorescence microscope (Olympus, Japan).

**Results**

**The construction and molecular assessment in VIGS of P23k**

To address the relationship between P23k function and barley cell wall formation during the reproductive stage, we attempted to silence P23k expression in barley by BSMV-based VIGS. The efficiency of P23k gene silencing, P23k mRNAs were analyzed as a control. To gain better insight into the level of P23k gene expression, we examined the expression of P23k at both the transcriptional and translational levels. RT-PCR analyses revealed the reduced expression of P23k mRNAs and the amplified BSMVγ mRNAs in P23k VIGS barley (Fig. 2A). Detection of the BSMVγ gene suggests that BSMVγ P23k-ir is infected with P23k VIGS barley. Furthermore, immunoblotting using an anti-P23k antibody did not detect any P23k protein in...
Silencing of P23k results in abnormal tiller formation in barley. These results indicated that VIGS of P23k triggered by BSMV-γP23k-ir led to the complete down-regulation of P23k expression at both the transcriptional and translational levels.

Formation of additional tillers in the upper region of the stem and suppression of intercalary elongation by P23k VIGS treatment

In monocots, tillers normally form only at nodes of the lower, unelongated stem region and fail to develop from the upper, elongated portion of the stem (Fig. 1 and Fig. 3A). However, formation of additional tillers was stimulated above the sixth internode by P23k VIGS treatment (Fig. 3B). Another phenotype observed in the heading stage is illustrated in Fig. 3C. Although some shoots produced flag leaves, subsequent heading of the panicle from the additional tiller was arrested. In addition, P23k VIGS resulted in development of an abnormal organ from some of the

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**Fig. 3. Barley striped mosaic virus-based VIGS of P23k and phenotypic changes in barley.** Virus symptoms were recorded at 20 to 40 days post-inoculation in BSMV-γP23k-ir. (A) Normal tillering of BSMV-uninfected barley. (B) Abnormal tillering from the upper, elongated part of the stem. The leaf blades and leaf sheaths developed from below the elongated stem were removed to show the morphological abnormality. (C) Abnormal sequential tillering and arrested heading of the panicle. (D) Abnormal stem with growth-arrested leaves. (E) Higher-magnification image of the abnormal stem. (F) Micrograph of a longitudinal section through the abnormal stem.
growth-arrested leaves (Fig. 3D and 3E). Figure 3F shows a micrograph of a longitudinal section of this organ. The spikelet was observed within the organ. However, subsequent intercalary elongation was inhibited in the spikelet. These phenotypes were observed in 10 out of 30 P23k VIGS barley plants.

Tissue-specific expression of P23k in the stem at the reproductive stage of BSMV uninfected barley

In situ hybridization analyses were performed to examine P23k expression in the upper, elongated portion of the stem. P23k mRNA was detected in developing sclerenchyma and vascular bundle cells in the basal region of internodes in elongated stems (Fig. 4A and 4C), but was not detected after hybridization using P23k sense probes (Fig. 4B). The basal region of internodes in elongated stems is known to be an area where cell wall deposition is active (Morrison et al. 1998), suggesting that P23k expression might be required for cell wall polysaccharide synthesis in the elongated stem.

Virus-induced gene silencing of P23k affects polysaccharide synthesis

The phenotype of P23k VIGS plants and the cellular expression pattern of P23k in the elongated stem prompted us to hypothesize that P23k contributes to intercalary elongation and tiller inhibition through modulating cell wall polysaccharide synthesis. To test this hypothesis, we histochemically compared the cell wall polysaccharide content in control and P23k VIGS barley with calcofluor, an authentic fluorescent dye that detects β-glucans (Fig. 5A). In P23k VIGS plants, a reduction in fluorescence intensity was observed compared with the control in transverse sections of the stem, suggesting that P23k VIGS causes a reduction in the amount of cell wall β-glucans.

Calcofluor stains β-glucans including cellulose, callose and (1,3;1,4)-β-D-glucan. To assess which type of polysaccharide synthesis was affected by P23k VIGS, we performed RT-PCR analyses with primers specific for polysaccharide synthesis-related genes. Interestingly, specific reduction in expression of the barley (1,3;1,4)-β-D-glucan synthase gene (HvCslF) was observed in the stem of P23k VIGS plants, while the expression of cellulose synthase (HvCesA) and callose synthase (HvGSL) were unaffected (Fig. 5B). These results indicate that P23k might be involved in (1,3;1,4)-β-D-glucan synthesis.

Discussion

Virus-induced gene silencing is a recently developed mRNA suppression technique used to characterize the function of plant genes. The generation of stable transformants is not required and the technique allows the characterization of phenotypes that might otherwise be lethal in stable transformants. Therefore, VIGS is particularly promising as a tool for the study of developmental and morphogenesis-related genes. Barley striped mosaic virus-based vectors have been used for VIGS in barley, and many genes have been successfully silenced in leaves (Holzberg et al. 2002, Hein et al. 2005, Scofield et al. 2005).

In our previous study, the P23k VIGS in barley leaves resulted in morphological changes involved in secondary wall deposition and vessel collapse, indicating a role for P23k in secondary wall development. However, the specific molecular mechanisms by which P23k influences cell wall polysaccharide synthesis remain unclear. The current study provides evidence that P23k silencing affects (1,3;1,4)-β-D-glucan synthesis, which may contribute to the cell expansion and differentiation processes in barley stems. Further studies are needed to elucidate the specific roles of P23k in cell wall biosynthesis and its implications for plant growth and development.
formation (Oikawa et al. 2007). In addition, histochemical analysis indicated that the distribution of P23k in leaves coincided with that of cell wall β-glucans. These results suggested that P23k might be involved in the synthesis of β-glucans and contributes to secondary wall formation in barley leaves. In the present study, we provide further evidence supporting the involvement of P23k in the synthesis of cell wall polysaccharides. First, the expression analyses showed that P23k mRNAs are localized in the sclerenchyma tissues where cell wall polysaccharides are abundant (Fig. 4). Second, the comparison of calcofluor fluorescence intensity showed that a significant decrease in the amount of β-glucans coincided with the silencing of P23k expression by VIGS (Fig. 5A). Third, additional tiller and spikelet formation and the inhibition of intercalary elongation in the newly formed spikelets were stimulated by P23k VIGS treatment (Fig. 3). Considering that the growth of inflorescence stems and leaves are highly influenced by cell wall synthesis (Somerville, 2006, Brown et al. 2007), the stunted intercalary elongation observed in P23k VIGS plants is consistent with the role of P23k in secondary cell wall synthesis. Barley striped mosaic virus infection alone did not cause these abnormal phenotypes. This is supported by the fact that no phenotypic alteration was observed in BSMV-infected VIGS-GFP experiments (Oikawa et al. 2007). Further control experiments may be required to evaluate the reliability of these results since we did not use an empty vector as a control in this VIGS experiment.

Plant cell walls are mainly composed of cellulose microfibrils and a diversity of polysaccharides collectively designated as hemicellulose. It is known that the type and content of hemicellulose differs among plants. In barley, (1,3;1,4)-β-D-glucan is a major component of hemicellulose in the primary walls and is also detected in secondary walls (Trethewey and Harris 2002). The (1,3;1,4)-β-D-glucan is unique to grasses (Gramineae), and is considered to act as a key component in both the construction and disassembly of cell wall architecture (Hoson et al. 2002, Buckeridge et al. 2004) in those plants. Carpita (1996) also reported that (1,3;1,4)-β-D-glucan is a key component in the regulation of development in grasses. Recently, cellulose synthase-like CslF and CslH genes were identified as key genes for the synthesis of cell wall (1,3;1,4)-β-D-glucan (Burton et al. 2006, Doblin et al. 2009). These authors generated transgenic Arabidopsis expressing CslF or CslH genes and confirmed (1,3;1,4)-β-D-glucan synthesis using specific monoclonal antibodies and enzymatic analysis. It provided direct evidence for the participation of rice CslF and CslH genes in (1,3;1,4)-β-D-glucan biosynthesis, as (1,3;1,4)-β-D-glucan is absent from the cell walls of wild-type Arabidopsis. However, with regard to CslF genes, the (1,3;1,4)-β-D-glucan content of cell walls in the transgenic Arabidopsis was considerably less than 0.1% (w/w). This result might indicate that other genes are necessary for the efficient biosynthesis of (1,3;1,4)-β-D-glucan regulated by CslF genes. Interestingly, the P23k gene is also specific to grasses, as with CslF genes. In addition, expression of the P23k gene is higher in barley than in other grasses, such as wheat and rice, in proportion to the (1,3;1,4)-β-D-glucan content in their endosperm walls (Stone and Clark 1992). Hence, we consider that P23k might participate in (1,3;1,4)-β-D-glucan biosynthesis.

The decreased β-glucan amount in P23k VIGS plants, as indicated by comparative calcofluor fluorescence intensity (Fig. 5A), and specific reduction of barley CslF gene expression in the same tissue (Fig. 5B) suggest that P23k might be involved in (1,3;1,4)-β-D-glucan synthesis. In rice seedlings, the molecular size and total amount of (1,3;1,4)-β-D-glucan affect coleoptile elongation (Chen et al. 1999). Sauter and Kende (1992) hypothesized that elevated levels of (1,3;1,4)-β-D-glucan contribute to elongation growth of internodes by increasing the extensibility of the cell wall in deepwater rice. We confirmed the similar distribution of CslF6 gene expression with P23k in barley seedlings (data not shown), which suggested that P23k expression might be involved in the regulation of (1,3;1,4)-β-D-glucan synthesis at the transcriptional level and that it contributes to morphogenetic regulation unique to cereals, such as internode elongation of the stem, although expression of P23k does not seem to be correlated with intercalary elongation necessarily.

In this study, we demonstrated the efficiency and robustness of the BSMV-based VIGS system during reproductive development by silencing P23k. Virus-induced gene silencing of P23k resulted in abnormal tiller formation and inhibition of intercalary elongation. The morphological data, together with the P23k expression data, suggest that P23k is involved in cell wall polysaccharide synthesis, especially the synthesis of (1,3;1,4)-β-D-glucan, which is unique to grasses. Further study of the cell wall chemistry of P23k VIGS barley will provide greater insight into the specific relationship between P23k and (1,3;1,4)-β-D-glucan metabolism.

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Literature Cited

