Journal of Experimental Botany, Vol. 58, No. 10, pp. 2617–2625, 2007
doi:10.1093/jxb/erm109 Advance Access publication 22 June, 2007

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Virus-induced gene silencing of P23k in barley leaf reveals morphological changes involved in secondary wall formation

Ai Oikawa[1], Abidur Rahman[1], Tetsuro Yamashita[2], Hideharu Taira[2] and Shin-ichiro Kidou[1,*]

[1] *Cryobiosystem Research Center, Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan*

[2] *Department of Agro-Science, Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan*

Received 24 February 2007; Revised 20 April 2007; Accepted 24 April 2007

Abstract

P23k is a monocot-unique protein that is highly expressed in the scutellum of germinating barley seed. Previous expression analyses suggested that P23k is involved in sugar translocation and/or sugar metabolism. However, the role of P23k in barley physiology remains unclear. Here, to elucidate its physiological function, BSMV-based virus-induced gene silencing (VIGS) of P23k in barley leaves was performed. Expression and localization analyses of P23k mRNA in barley leaves showed up-regulation of P23k transcript with increased photosynthetic activity and the localization of these transcripts to the vascular bundles and sclerenchyma, where secondary wall formation is most active. VIGS of the P23k gene led to abnormal leaf development, asymmetric orientation of main veins, and cracked leaf edges caused by mechanical weakness. In addition, histochemical analyses indicated that the distribution of P23k in leaves coincides with the distribution of cell wall polysaccharides. Considering these results together, it is proposed that P23k is involved in the synthesis of cell wall polysaccharides and contributes to secondary wall formation in barley leaves.

Key words: BSMV-based VIGS, *Hordeum vulgare* L., P23k, sclerenchyma, secondary wall formation, vascular bundles.

Introduction

Sugar, a major photosynthetic product that is generated by the process of polysaccharide synthesis, is a main component of plant cell walls. Accordingly, the growth and development of plants is heavily dependent on sugar metabolism and transport. Hence, a better understanding of the molecular mechanisms involved in both sugar metabolism and transport is essential to increase crop yields. Much of the sugar-related machinery, including the sucrose transporter (*OsSUT*; Hirose *et al*., 1997), hexose transporter (*OsMST*; Toyohuku *et al*., 2000), sucrose synthase (*RSuS*; Huang *et al*., 1996), cellulose synthase (*OsCesA*; Holland *et al*., 2000), and cell-wall invertase (*OsCIN*; Cho *et al*., 2005), were identified and analysed in rice, a major agricultural crop. However, the detailed molecular mechanisms of sugar metabolism and transport in crops remain unclear.

The expression pattern of a monocot-unique 23 kDa protein, P23k, has been identified and analysed in barley (*Hordeum vulgare* L. cv. Minorimugi). Expression analyses showed that P23k is expressed in the presence of the starch degradation products glucose and sucrose, and is localized to the scutellum and the vascular bundle where sugar transport is active. Based on these results, it was proposed that P23k plays a role in sugar translocation (Kidou *et al*., 2006). Furthermore, it was shown that JIP-23, a paralogue of P23k that was identified as a jasmonic acid- and methyl ester-induced protein (Andresen *et al*., 1992), is highly expressed in developing barley seeds (Oikawa *et al*., 2007). Interestingly, it was also localized to sugar transport-related tissues: the vascular bundle and the aleurone layer in developing seeds. Hence, it can be considered that both paralogous proteins, P23k and JIP-23, are involved in sugar translocation in barley, and that each protein functions at different developmental stages. However, analyses of these proteins have been limited to their expression and localization during germination and ripening. To explore a possible direct

* To whom correspondence should be addressed. E-mail: kidou@iwate-u.ac.jp

relationship between P23k and sugar metabolism, a loss of function analysis of P23k in photosynthesizing leaves is required.

In plants, the development of stable transformants with antisense or RNAi methods is an established methodology to address the functions of unknown genes. However, it is difficult to make transformants using standard methods in monocots, such as rice and barley, because the regeneration and selection of transformants takes a considerable amount of time. As an alternative approach, virus-induced gene silencing (VIGS) has recently been developed as an mRNA suppression technique to characterize the function of plant genes (Burch-Smith *et al*., 2004). It does not require the generation of stable transformants and 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 genes with transient regulatory roles. In dicots, VIGS using *potato virus X* (PVX)- and *tobacco mosaic virus* (TMV)-based vectors has been established and used for the analysis of genes involved in development (Burton *et al*., 2000; Ratcliff *et al*., 2001; Liu *et al*., 2004). On the other hand, *barley striped mosaic virus* (BSMV)-based vectors were used for VIGS in monocots, and some silencing of pathogen-related genes was reported in barley and wheat (Holzberg *et al*., 2002; Hein *et al*., 2005; Scofield *et al*., 2005). However, to date, no study of monocots has shown the significance of VIGS in studying genes related to plant morphology.

Here, for the first time, the efficiency and robustness of the BSMV-based VIGS system for studying plant morphology by silencing P23k is demonstrated. VIGS of the P23k gene resulted in a change in leaf morphology leading to strong asymmetries and cracks. Combining the loss of function and localization data, it is also proposed that P23k is involved in cell wall polysaccharide synthesis for secondary cell wall formation.

Materials and methods

Plant material and growth conditions

Barley (*Hordeum vulgare* L. 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 a 1/5 Hoagland No. 2 liquid medium (pH 5.5) and grown in a greenhouse.

Construction of pγ.P23k-ir and inoculation

The BSMV γRNA-based vector used in this study was described previously by Holzberg *et al*. (2002). For the construction of pγ.P23k-ir, two P23k cDNA fragments were amplified from *p23k-1* cDNA (GenBank accession number AB251338) using the following primer combinations (*Pac*I-P23k-F: 5′-CCTTAATTAA-GCTTGTGTGGCGAAGACG-3′ and *Eco*RI-P23k-R: 5′-GGAAT-TCTAGTAGACGAAACATATCCAT-3′; or *Not*I-P23k-F: 5′-TAAGAATGCGGCCGCTTGTGTGGCGAAGAC-3′; and *Eco*RI-P23k-R). Following subsequent purification and *Eco*RI digestion, the two PCR products were ligated. These were then digested with *Pac*I and *Not*I and inserted between the *Pac*I and *Not*I sites of pγ.bPDS4-as (Holzberg *et al*., 2002). pγ.FP-s and pγ.bPDS4-as (Holzberg *et al*., 2002) were used as controls of BSMV infection. Infectious BSMV RNAs were prepared from each clone by *in vitro* transcription using a high yield capped RNA transcription kit (mMESSAGE mMACHINE, Ambion). Two microlitres of each transcript, which included the BSMV RNAs α, β, and genetically modified γ, was combined with 36 μl of FES buffer (Pogue *et al*., 1998) and inoculated into the second developed leaf of barley plants at the three- or four-leaf stage.

RNA analysis by RT-PCR and in situ *hybridization*

To generate first-strand cDNA, 1 μg of total RNA was annealed with 2 μM of oligo-dT in a 20 μl reaction mixture, and extended using Moloney murine leukaemia virus (M-MLV) reverse transcriptase (TOYOBO) at 42 °C for 1 h. To confirm the successful inoculation of BSMV RNAs, 10 μM of random hexamers were used instead of oligo-dT. One microlitre of each RT reaction was used as the template in 20 μl PCR reactions containing 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 4 mM dNTPs, 0.2 units of Ex Taq polymerase (TAKARA), and 1 μM primers. The primers used for gene amplification were as follows: P23k (5′-GGTACGGTAACGGAATAGC-3′ and 5′-TCGCCACACAA-GCCTTTGATGTT-3′); JIP-23 (5′-GGTACGGTAACGGAATA-GC-3′ and 5′-AACTACACAAGCGTACATGGACG-3′); EF-1α (5′-TTCAACGTCAAGAACGTGGCT-3′ and 5′-ACACAAATA-ACCCAAGCGACTA-3′); BSMV (5′-GTGGTACCTTAACTA-CAAGTAC-3′ and 5′-CGATATGAGAAAGTTTCAGCAC-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 60 °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 by PCR amplification of the corresponding cDNA clone. For *in situ* hybridization, selected barley tissues were fixed for 5 h at room temperature in a fixing solution (3.7% formaldehyde, 5% acetic acid, 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 PLL-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 for *in situ* hybridization, a *p23k-1* cDNA fragment (53–551) was subcloned into a pSPT18 plasmid vector. P23k sense and antisense probes were generated by *in vitro* transcription using a DIG RNA labelling kit (Boehringer Mannheim). Hybridization signals were detected using a DIG nucleic acid detection kit (Boehringer Mannheim).

Protein analysis by immunoblot and immunohistochemical staining

For immunoblot analysis, total proteins were extracted using a buffer consisting of 50 mM Tris–HCl (pH 8.0), 100 mM KCl, 0.5 mM EDTA, 5 mM DTT, 1 mM PMSF, and 5% glycerol, and then subjected to 12% (w/v) SDS–PAGE following the procedure of Laemmli (1970). After transferring to a PVDF membrane, immunoblot analyses with an antibody against a peptide corresponding to the N-terminal region of P23k (MASGVFGTPISEKTVIATGE) were performed according to the manufacturer's (Amersham Pharmacia)

instructions. Total protein was detected using a Coomassie Brilliant Blue stain as a control. For immunohistochemical analysis, sections were prepared from embedded mature barley leaves. They were deparafinized with xylene and rehydrated through a graded ethanol series, and then treated with blocking buffer (1× PBS, 0.2% Tween 20, 0.1% NaN_3, 1% BSA) for 1 h at room temperature and washed five times with 1× PBS. After washing, sections were incubated in the anti-P23k antibody for 2 h, washed with 1× PBS, and then incubated in secondary antibody, anti-rabbit IgG alexa 546 (Funakoshi), for 1 h at room temperature. Finally, sections were mounted in a solution containing 1 M Tris–HCl (pH 9.5), 50% glycerol, 0.2% NaN_3, and 1% phenylenediamine, and observed by fluorescence microscopy. As a negative control, sections were treated with pre-immune serum instead of the anti-P23k antibody, and processed as above. For histochemical localization of polysaccharides, the same embedded sections used for immunohistochemical analysis were stained with a 0.005% aqueous solution of calcofluor white (fluorescent brightener 28; Sigma) or a 0.5% aqueous solution of toluidine blue O, and visualized with a fluorescent microscope (Olympus, Japan).

Results

Expression and localization of P23k mRNA in leaves

The expression and localization of P23k mRNA were examined in photosynthesizing barley leaves, in which sugar metabolism and sugar transport are active. Figure 1 shows the result of P23k gene expression in young barley leaves at three different developmental stages: first-leaf stage (seedling), third-leaf stage, and seventh-leaf stage. Also the gene expression of JIP-23, a paralogous protein of P23k, was analysed. JIP-23 gene expression was weakly detected in leaves at the first-leaf stage, but was not detected in leaves at the third- and seventh-leaf stages. By contrast, the level of P23k gene expression in leaves was higher at the third- and seventh-leaf stages than at the first-leaf stage. This result indicated that expression of the P23k gene is up-regulated with growth, depending on the photosynthetic ability of the leaves.

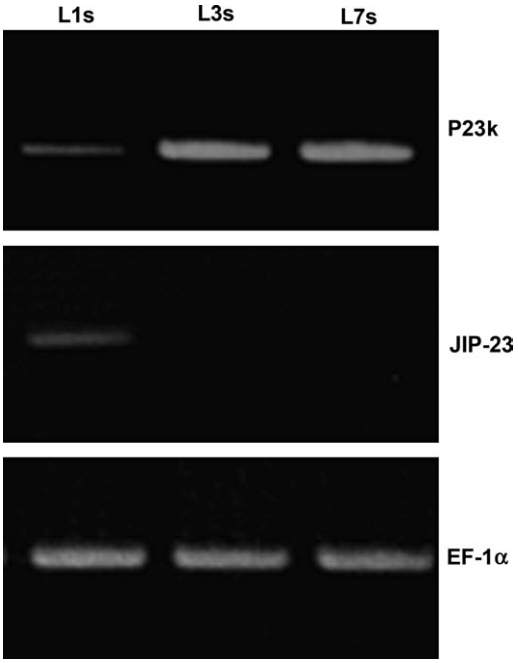

Fig. 1. RT-PCR analysis of P23k gene expression in barley leaves during the vegetative stage. JIP-23, a jasmonate-inducible protein, is a paralogous protein of P23k. EF-1α gene expression was analysed as a control. L1s, Upper developing leaves at the first-leaf stage; L3s, upper developing leaves at the third-leaf stage; L7s, upper developing leaves at the seventh-leaf stage.

To examine the localization of P23k mRNA in leaves, *in situ* hybridization was performed. The transverse section of developing (elongating) young leaves showed that P23k mRNA is mainly localized to vascular bundles of the main vein (Fig. 2A). However, in developed mature leaves, P23k mRNA is detected in the sclerenchyma located above the main vein (Fig. 2B) and leaf edge (Fig. 2C). The vascular bundles and sclerenchyma, where P23k mRNA was observed, are known to be the organs in which secondary wall formation is active. These results indicate that, in addition to sugar transport, P23k may also be involved in sugar metabolism for secondary wall formation.

Virus-induced silencing of P23k and its association with morphological changes in leaves

To examine the requirement for P23k in the vascular bundles and sclerenchyma, an attempt was made to down-regulate P23k gene expression by BSMV-based VIGS. Figure 3 shows a scheme of BSMV genomes (α, β, and γ) and the three modified γ genomes (pγ.P23k-ir, pγ.bPDS4-as, and pγ.GFP-s) used in the present study. Lacomme (2003) has reported that a short inverted repeat cDNA fragment is much more effective at achieving gene silencing than standard sense and antisense cDNA fragments. Therefore, to suppress P23k mRNA effectively, a 60 bp inverted repeat cDNA fragment of P23k UTR, pγ.P23k-ir, was used (Fig. 3). The specificity of the 60 bp sequence was confirmed by the absence of this sequence in rice genome by BLAST search, and by obtaining 60 bp PCR and RT-PCR products using barley tissue. As visual positive and negative controls, pγ.bPDS4-as and pγ.GFP-s were used as described earlier (Holzberg *et al.*, 2002).

To gain a better insight into the level of P23k gene silencing, the expression of P23k in leaves was analysed at both the transcriptional and translational levels. RT-PCR analyses revealed reduced expression of P23k in BSMV.γP23k-ir-infected leaves compared with mock leaves (Fig. 4A). The infection of leaves with BSMV.γP23k-ir was confirmed by amplification of the BSMV γ gene in the viral vector. Furthermore, immunoblotting using an anti-P23k antibody did not detect any P23k protein in BSMV.γP23k-ir-infected leaves (Fig. 4B). Taken together, these results indicate that VIGS of P23k

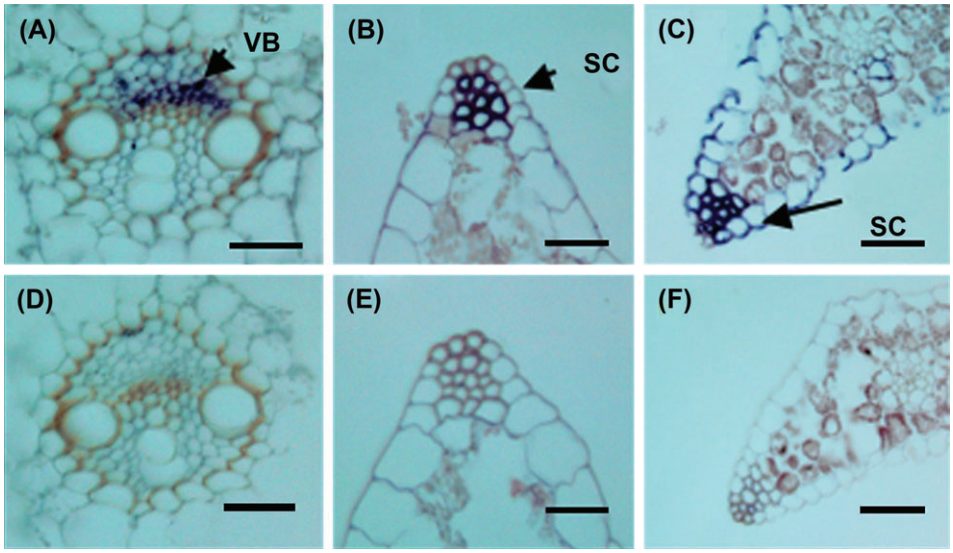

Fig. 2. Localization of P23k mRNA in transverse sections of leaves. *In situ* hybridization analysis using P23k sense and anti-sense probes was carried out to detect P23k mRNA. (A, D) Main vascular bundles (VB) in developing leaf. (B, C, E, F) Sclerenchyma (SC) above the main vein (B, E) and at the leaf edge (C, F) in developed leaf. Scale bar=100 μm.

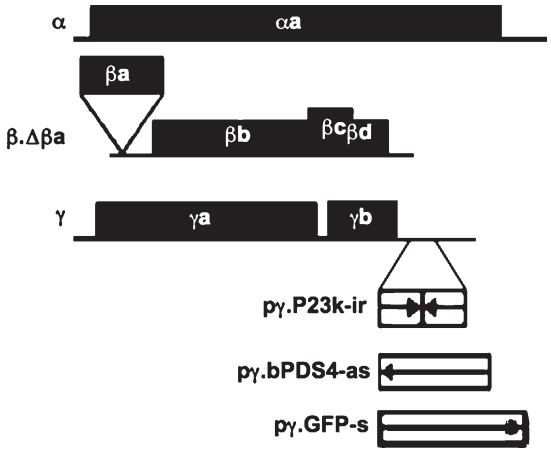

Fig. 3. Schematic representation of BSMV genome organization and BSMV constructs engineered to express inverted repeat P23k, PDS in an anti-sense orientation, and GFP in a sense orientation. The 60 bp sequence specific to barley *P23k-1* UTR was chosen for the inverted repeat of P23k. The genomic organization of the BSMV RNAs α, β, and γ was reported by Holzberg *et al*. (2002). RNAβ is a βa (coat protein) deletion mutation.

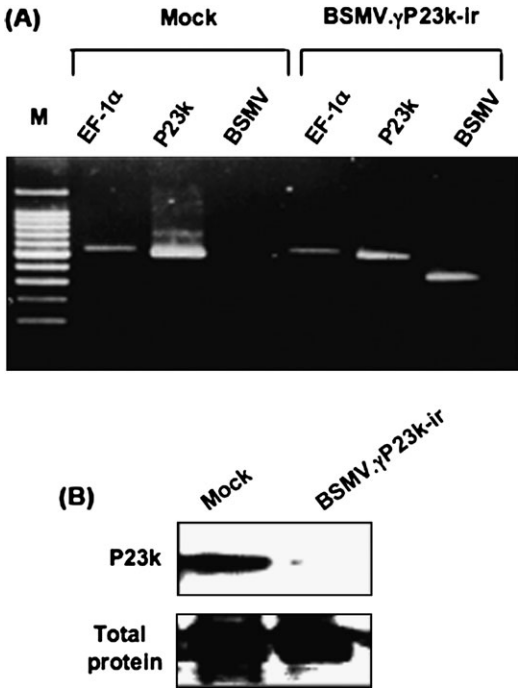

Fig. 4. Expression of P23k mRNA (A) and P23k protein (B) in BSMV.γP23k-ir systemic leaves. RT-PCR analysis by primers, for the amplification within P23k ORF outside the selected fragment for VIGS, was used for the detection of P23k endogenous mRNA. Western-blot analysis using an anti-P23k antibody was performed to detect P23k. The amounts of EF-1α and total protein were analysed as controls. These experiments were repeated three times with independent groups of leaves.

triggered by BSMV.γP23k-ir leads to the complete down-regulation of P23k expression.

Figure 5 shows the phenotypes of each BSMV-based VIGS response. Interestingly, strong asymmetries and many cracks were observed specifically in leaves infected with BSMV including γRNA from pγ.P23k-ir (BSMV.γP23k-ir) (Fig. 5A). These phenotypes were comparatively strong at the bottom side of the leaves in several developmental stages (from the third- to the seventh-leaf stages), and found to be fairly stable over the duration of the experiment (approximately 7–8 weeks after inoculation). The same phenotypes were observed in leaves when three antisense cDNA fragments within P23k ORF were used in VIGS constructs (data not shown). By contrast,

mock leaves and other control leaves, which were infected with BSMV including γRNA from pγ.bPDS4-as (BSMV.γPDS-as) or pγ.GFP-s (BSMV.γGFP-s), showed no abnormalities (Fig. 5B–D). Taken together, these results suggest that the leaf morphological abnormalities observed in the VIGS lines are due to the silencing of *P23k*.

Asymmetry and crack assessment in BSMV.γP23k-ir-infected leaves

BSMV.γP23k-ir-infected leaves showed asymmetric organ development. It was speculated that these asymmetries were triggered by the abnormal position of the main vein in a leaf. To confirm this hypothesis, a statistical analysis of leaf asymmetry was performed. Asymmetry was defined by the ratio of the lengths from the main vein to each leaf edge. The results were plotted on a graph for a variety of distances from the bottom of the leaf (Fig. 6). In BSMV.γGFP-s-infected leaves the ratio was one for the length of the leaf, as shown by the straight line. By contrast, much larger ratios, suggesting greater asymmetry, were seen in BSMV.γP23k-ir infected leaves, and the ratio varied along the length of the leaf as shown by the wavy line, being higher at the bottom of the leaf.

Also a statistical analysis of crack numbers with width was performed in BSMV.γP23k-ir-infected leaves. Five individual leaves were used for the analysis. An average of eight cracks was observed per leaf (Fig. 7A). On the other hand, the width of each crack was 2 cm on average (Fig. 7B). The number of cracks and their width varied greatly among these individual leaves. Thus, it was speculated that there might be a relationship between the number of cracks and their width, and a graph was constructed in which the longitudinal axis represents the number of cracks and the horizontal axis represents the width. As expected, an inverse correlation was observed, suggesting that cracks are caused by mechanical weakness (Fig. 7C).

Localization of P23k and cell wall polysaccharide

The effect of down-regulation of P23k expression on leaf vascular formation and the mechanical strength of leaves prompted the hypothesis that P23k is involved in the synthesis of cell wall polysaccharides for secondary cell wall formation. This hypothesis was tested by examining whether the localization of polysaccharides correlates with the localization of P23k. Transverse sections of elongating leaves were used for histochemical analysis. Immunohistochemistry with preimmune serum resulted in no signal, as shown in Fig. 8A, while P23k was apparently detected in the central part of the vascular tissue using an anti-P23k antibody (Fig. 8B). Cell wall polysaccharides were also detected in the central part of the vascular tissue by using calcofluor white, which stains cellulose, callose, and other β-glucans (Fig. 8C). Merged images showed that P23k is localized to the central part of vascular bundles, where

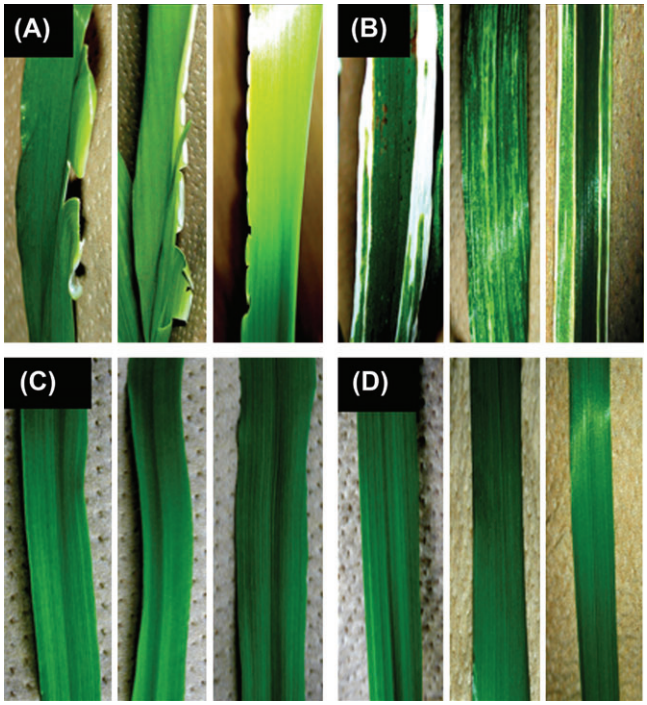

Fig. 5. BSMV-based VIGS of the P23k gene in barley leaves and phenotypic changes. Virus symptoms of upper developing leaves from the fifth- to the seventh-leaf stages were recorded at 20 d post-inoculation with BSMV.γP23k-ir (A), BSMVγPDS-as (B), BSMV.γGFP-s (C), or water as mock (D).

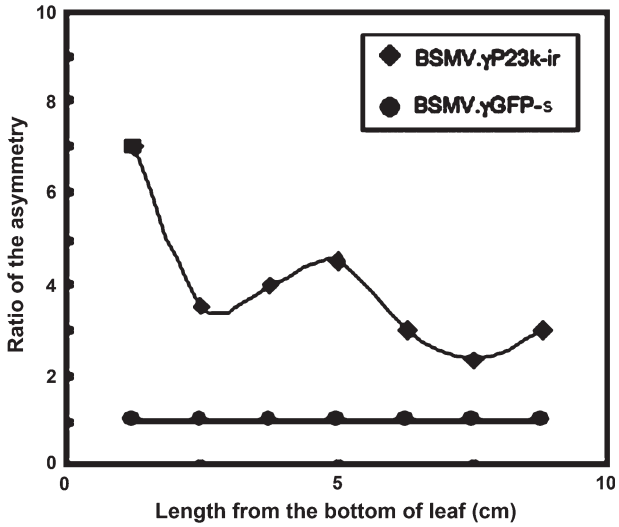

Fig. 6. Morphological assessment of asymmetric main vein orientation in BSMV.γP23k-ir systemic leaves. The length from the main vein to each leaf edge was measured. The asymmetry was defined by the ratio of left-side length to right-side length.

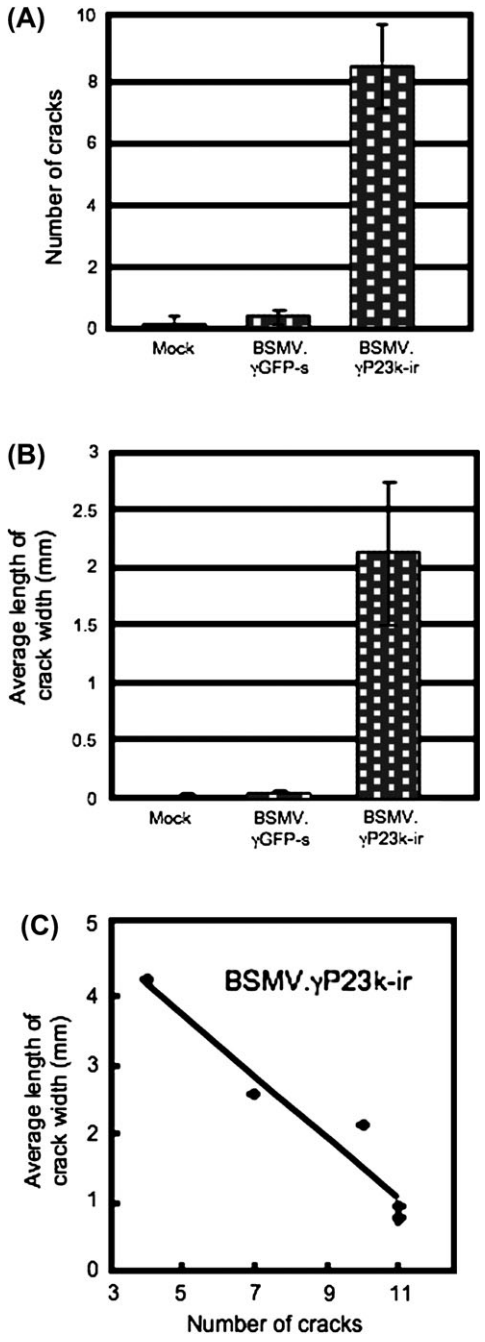

Fig. 7. Morphological assessment of cracked leaf edges in BSMV.γP23k-ir systemic leaves: (A) comparison of crack numbers around leaf edge; (B) comparison of the average crack width; (C) high correlation between the number of cracks and their width. The bars represent standard error and were obtained from five measurements using independent leaves.

cell wall polysaccharide is abundant (Fig. 8D). The presence of P23k and polysaccharides in vascular tissue, along with the abnormal leaf morphology induced by *P23k* gene silencing, suggest that P23k may contribute to the synthesis of polysaccharides for secondary wall formation.

Discussion

To examine the involvement of P23k in sugar translocation and/or metabolism, expression analysis of P23k was performed in photosynthetic leaves. RT-PCR analysis showed that the up-regulation of P23k mRNA was correlated with a rise in photosynthetic ability, and therefore sugar supply, in leaves (Fig. 1). This result, together with the up-regulation of P23k mRNA by sugars in germinating seeds (Kidou *et al*., 2006), suggests that P23k plays a role in sugar translocation and/or metabolism.

Sucrose transporters that contribute to sugar translocation are specifically expressed in the vascular bundles of rice (Matsukura *et al*., 2000) and wheat (Aoki *et al*., 2004). If P23k is directly involved in sugar transport, as well as these transporters, its mRNA should be observed in vascular bundles and down-regulation of P23k should lead to abnormal growth and development. Indeed *in situ* hybridization analysis (Fig. 2) showed that P23k mRNA is specifically localized to the vascular bundles of developing young leaves and to the sclerenchyma of developed mature leaves. Moreover, secondary wall formation is active in both the vascular bundle and the sclerenchyma. Based on this tissue-specific P23k expression, it was speculated that P23k may also be involved in sugar metabolism for secondary wall formation, rather than in sugar translocation only.

The necessity of primary and secondary cell wall syntheses for plant morphogenesis and mechanical strength has been revealed by mutant analyses using *Arabidopsis* and rice. The *rsw1 Arabidopsis* mutant of *AtCesA1*, a cellulose synthase gene involved in primary cell wall synthesis, shows reduced cellulose synthesis, widespread morphological abnormalities and the accumulation of non-crystalline β-1,4-glucan (Arioli *et al*., 1998). On the other hand, the *irx3 Arabidopsis* mutant of *AtCesA7*, which is involved in secondary cell wall synthesis, shows collapsed xylem cells due to a reduction in cellulose content and a defect in secondary cell wall formation (Taylor *et al*., 1999). In monocot rice, mutations in three *CesA* genes, *OsCesA 4*, *7*, and *9*, which are involved in cellulose synthesis in secondary walls, cause a reduction in cellulose content in the stem, a thinner cell wall in the cortical fibre cells around vascular tissues, and a decrease in mechanical strength; however, the phenotype of these mutants does not include collapsed xylem cells as seen in the *irx3 Arabidopsis* mutant (Tanaka *et al*., 2003). Moreover, the rice mutant *bc1* exhibits a similar phenotype to the above mutants, and the causative gene *BC1*, which encodes a COBRA-like protein, has been shown to be expressed in the vascular bundles and sclerenchyma where secondary cell wall synthesis is active (Li *et al*., 2003). Consistent with these results, barley with a loss-of function of P23k induced by

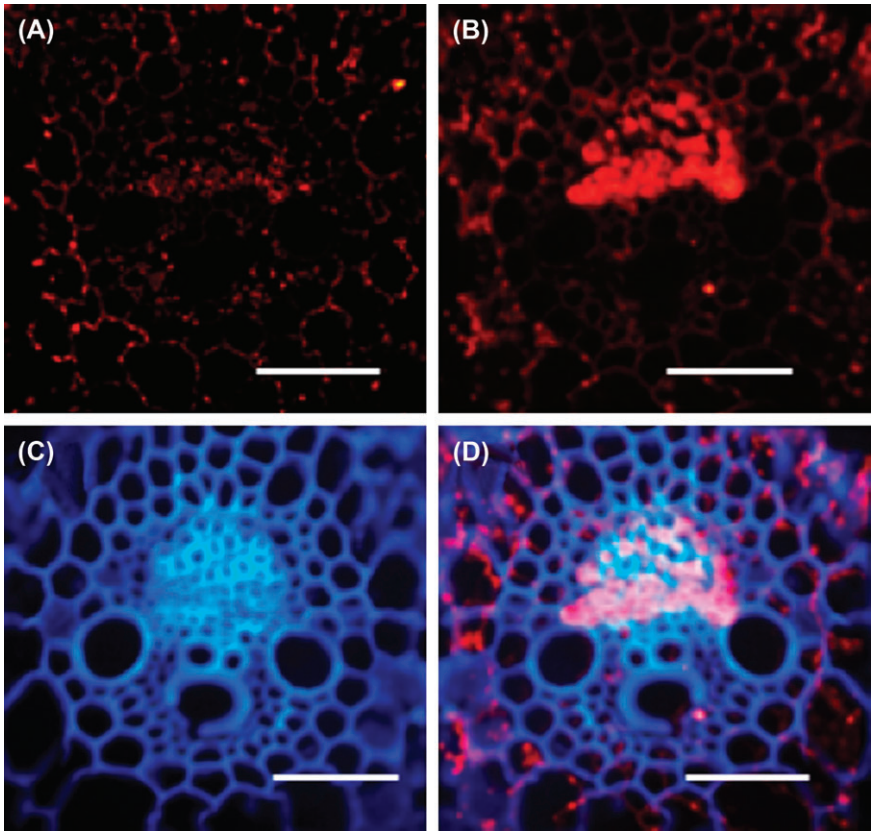

Fig. 8. Localization of P23k and polysaccharides in leaf vascular bundles (VB). Immunohistochemical analysis using pre-immune serum (A) and an anti-P23k antibody (B) was carried out to detect P23k proteins. Staining with calcofluor white (C) was carried out to detect polysaccharides. A merged image of those in (B) and (C) is shown in (D). Pink colour shows tissues in which P23k and polysaccharides are co-localized. Scale bar=100 μm.

BSMV-based VIGS also showed morphological abnormalities such as strong asymmetries and cracks in leaves (Figs 5, 6, 7). The observed change in the asymmetry ratio in the younger part of P23k VIGS leaves in Fig. 6 suggests that P23k plays a role in leaf development. This change in the leaves coincides with the expression pattern of P23k, which is found to be expressed in young vascular tissue of developing leaves (Fig. 2A). Similarly, the P23k expression in the sclerenchyma tissue of developed leaves coincides with the morphological change observed at the leaf edge (Fig. 2C). Taken together, these results indicate that P23k plays an important role in regulating leaf development and morphology.

Another explanation for this changed leaf morphology may be an altered mechanical strength of leaves. Indeed, statistical analysis of the phenotype induced by BSMV-based VIGS of P23k showed that leaf asymmetry is triggered by an abnormal orientation of the main vascular bundle, probably via transient mechanical weakness during leaf development (Fig. 6), and that cracks in the leaf edge are triggered by mechanical weakness in the sclerenchyma (Fig. 7). These results suggest the involvement of P23k in secondary wall formation in monocots.

The scutellum of germinating barley seeds, in which P23k is abundant, is active in polysaccharide synthesis, and the expression of P23k in the scutellum is highly dependent on the supply of glucose or sucrose, both of which are substrates for polysaccharide synthesis (Kidou *et al*., 2006). In addition, a high correlation was indicated in the localization of P23k and polysaccharides in leaves, as shown in Fig. 8, and a specific decrease in the amount of polysaccharides in BSMV.γP23k-ir infected stems has been confirmed by calcofluor white staining (A Oikawa *et al*., unpublished data,). Taken together, the present results suggest that P23k contributes to secondary wall formation via cell wall polysaccharide synthesis.

Based on the uniqueness of P23k protein to monocot cereals (Kidou *et al*., 2006), it is speculated that P23k plays a role in polysaccharide β-(1,3;1,4)-glucan synthesis, which is unique to monocot cereals. Indeed, JIP-23, a paralogous protein of P23k, is localized at the site of β-(1,3;1,4)-glucan synthesis in developing barley seeds (Wilson *et al*., 2006). The timing of JIP-23 expression and β-(1,3;1,4)-glucan synthesis also correspond. These data suggest the involvement of P23k and JIP-23 in barley β-(1,3;1,4)-glucan synthesis. The high homology between

P23k and JIP-23 (88% similarity at amino acid level) raises the possibility that P23k VIGS may also induce the silencing of JIP-23. However, the 60 bp sequence used to induce the silencing of P23k is specific only to barley P23k UTR, and was not present in the JIP-23 nucleotide sequence. Moreover, RT-PCR analysis in various developmental stages in Fig. 1 indicates that JIP-23 expresses only in the first-leaf stage but not in the mature leaves such as those at the fifth- to seventh-leaf stages chosen to perform VIGS for P23k.

Hoson (2002) reported that alterations in the amount of β-(1,3;1,4)-glucan in cell walls mainly affect cell elongation. The leaf abnormality observed following VIGS of P23k might be influenced by changes in the amount of β-(1,3;1,4)-glucan in cell walls in leaves. In fact, there are two lines of evidence supporting this hypothesis: (i) in the stem of P23k VIGS plants, a decrease in the intensity of the fluorescence signal in calcofluor white staining, which stains the polysaccharides [cellulose, callose, β-(1,3;1,4)-glucan] was observed; (ii) a specific reduction of barley β-(1,3;1,4)-glucan synthase gene (*HvCslF*; Burton *et al*., 2006) expression in the same tissue was observed, while the gene expressions for cellulose synthase and callose synthase were unaffected. These results suggest that P23k is possibly involved in β-(1,3;1,4)-glucan synthesis (A Oikawa *et al*., unpublished data).

With the aid of the recently developed technique VIGS, it has been demonstrated that monocot-unique P23k is involved in regulating the morphogenesis and mechanical strength of leaves. In addition, the localization study revealed that P23k is co-localized with cell wall polysaccharides in vascular bundles, indicating that P23k contributes to the synthesis of secondary cell walls. As far as is known, this is the first report to demonstrate the efficiency of a BSMV-based VIGS system to study morphology-related genes in monocots. Further studies using polysaccharide-abundant tissues such as stem and panicle will be required to clarify the mechanism by which P23k regulates secondary cell wall synthesis in barley.

Acknowledgements

We thank Dr GP Pogue and the Large Scale Biology Corporation for providing the BSMV γRNA based vectors for VIGS. We also thank Dr R Terauchi for his valuable advice about VIGS. This work was supported by a grant-in-aid from Iwate University and Iwate University 21st Century Center of Excellence Program Establishment of Thermobiosystem Research Program, Japan.

References

Andresen I, Becker W, Schlüter K, Burges J, Parthier B, Apel K. 1992. The identification of leaf thionin as one of the main jasmonate-induced proteins of barley (*Hordeum vulgare* L.). *Plant Molecular Biology* **19,** 193–204.

Aoki N, Scofield GN, Wang XD, Patrick JW, Offler CE, Furbank RT. 2004. Expression and localisation analysis of the wheat sucrose transporter *TaSUT1* in vegetative tissues. *Planta* **219,** 176–184.

Arioli T, Peng L, Betzner AS, *et al*. 1998. Molecular analysis of cellulose biosynthesis in Arabidopsis. *Science* **279,** 717–720.

Buckeridge MS, Rayolio C, Urbanowicz B, Aurelio MS, Carpita NC. 2004. Mixed linkage (1→3),(1→4)-β-D-glucans of grasses. *Cereal Chemistry* **81,** 115–127.

Burch-Smith TM, Anderson JC, Martin GB, Dinesh-Kumar SP. 2004. Applications and advantages of virus-induced gene silencing for gene function studies in plants. *The Plant Journal* **39,** 734–746.

Burton RA, Wilson SM, Hrmova M, Harvey AJ, Shirley NJ, Medhurst A, Stone BA, Newbigin EJ, Bacic A, Fincher GB. 2006. Cellulose synthase-like CslF genes mediate the synthesis of cell wall (1,3;1,4)-β-D-glucans. *Science* **311,** 1940–1942.

Burton RA, Gibeaut DM, Bacic A, Findlay K, Roberts K, Hamilton A, Baulcombe DC, Fincher GB. 2000. Virus-induced silencing of a plant cellulose synthase gene. *The Plant Cell* **12,** 691–706.

Cho JI, Lee SK, Ko S, *et al*. 2005. Molecular cloning and expression analysis of the cell-wall invertase gene family in rice (*Oryza sativa* L.). *Plant Cell Reports* **24,** 225–236.

Hein I, Barciszewska-Pacak M, Hrubikova K, Williamson S, Dinesen M, Soenderby IE, Sundar S, Jarmolowski A, Shirasu K, Lacomme C. 2005. Virus-induced gene silencing-based functional characterization of genes associated with powdery mildew resistance in barley. *Plant Physiology* **138,** 2155–2164.

Hirose T, Imaizumi N, Scofield GN, Furbank RT, Ohsugi R. 1997. cDNA cloning and tissue specific expression of a gene for sucrose transporter from rice (*Oryza sativa* L.). *Plant Cell Physiology* **38,** 1389–1396.

Holland N, Holland D, Helentjaris T, Dhugga KS, Xoconostle-Cazares B, Delmer DP. 2000. A comparative analysis of the plant cellulose synthase (*CesA*) gene family. *Plant Physiology* **123,** 1313–1324.

Holzberg S, Brosio P, Gross C, Pogue GP. 2002. Barley stripe mosaic virus-induced gene silencing in a monocot plant. *The Plant Journal* **30,** 315–327.

Huang JW, Chen JT, Yu WP, Shyur LF, Wang AY, Sung HY, Lee PD, Su JC. 1996. Complete structures of three rice sucrose synthase isogenes and differential regulation of their expressions. *Bioscience Biotechnology Biochemistry* **60,** 233–239.

Hoson T. 2002. Physiological functions of plant cell coverings. *Journal of Plant Research* **115,** 277–282.

Kidou S, Oikawa A, Sasaki N, Yasuda H, Yamashita T, Koiwa H, Kato K, Ejiri S. 2006. Identification of a 23 kD protein (P23k) related to the sugar supply in germinating seed. *Plant Biotechnology* **23,** 357–364.

Lacomme C, Hrubikova K, Hein I. 2003. Enhancement of virus-induced gene silencing through viral-based production of inverted-repeats. *The Plant Journal* **34,** 543–553.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Li Y, Qian Q, Zhou Y, *et al*. 2003. *BRITTLE CULM1*, which encodes a COBRA-like protein, affects the mechanical properties of rice plants. *The Plant Cell* **15,** 2020–2031.

Liu L, Nakayama N, Schiff M, Litt A, Irish VF, Dinesh-Kumar SP. 2004. Virus induced gene silencing of a *DEFICIENS* ortholog in *Nicotiana benthamiana*. *Plant Molecular Biology* **54,** 701–711.

Matsukura C, Saitoh T, Hirose T, Ohsugi R, Perata R, Yamaguchi J. 2000. Sugar uptake and transport in rice embryo: expression of companion cell-specific sucrose transporter

(*OsSUT1*) induced by sugar and light. *Plant Physiology* **124,** 85–93.

Oikawa A, Yamashita T, Taira H, Ejiri S, Kidou S. 2007. Jasmonate-induced 23 kD protein, JIP-23, is involved in seed development of barley. *Plant Biotechnology* **24,** 217–220.

Pogue GP, Lindbo JA, Dawson WO, Turpen TH. 1998. Tobamovirus transient expression vectors: tools for plant biology and high-level expression of foreign proteins in plants. In: Gelvin SB, Schilperoot RA, eds. *Plant molecular biology manual*. Dordrecht: Kluwer Academic Publishers, 1–27.

Ratcliff F, Martin-Hernandez AM, Baulcombe DC. 2001. Tobacco rattle virus as a vector for analysis of gene function by silencing. *The Plant Journal* **25,** 237–245.

Scofield SR, Huang L, Brandt AS, Gill BS. 2005. Development of a virus-induced gene-silencing system for hexaploid wheat and its use in functional analysis of the Lr21-mediated leaf rust resistance pathway. *Plant Physiology* **138,** 2165–2173.

Tanaka K, Murata K, Yamazaki M, Onosato K, Miyao A, Hirochika H. 2003. Three distinct rice cellulose synthase catalytic subunit genes required for cellulose synthesis in the secondary wall. *Plant Physiology* **133,** 73–83.

Taylor NG, Scheible WR, Cutler S, Somerville CR, Turner SR. 1999. The irregular xylem3 locus of Arabidopsis encodes a cellulose synthase required for secondary cell wall synthesis. *The Plant Cell* **11,** 769–780.

Toyofuku K, Kasahara M, Yamaguchi J. 2000. Characterization and expression of monosaccharide transporters (*OsMSTs*) in rice. *Plant Cell Physiology* **41,** 940–947.

Urbanowicz BR, Rayon C, Carpita NC. 2004. Topology of the maize mixed linkage (1→3),(1→4)-β-D-glucan synthase at the Golgi membrane. *Plant Physiology* **134,** 758–768.

Wilson SM, Burton RA, Doblin MS, Stone BA, Newbigin EJ, Fincher GB, Bacic A. 2006. Temporal and spatial appearance of wall polysaccharides during cellularization of barley (*Hordeum vulgare*) endosperm. *Planta* **224,** 655–667.