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Original Paper

Identification of a 23 kDa protein (P23k) related to the sugar supply in germinating barley seeds

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Abstract We identified a 23 kDa protein expressed abundantly in young barley seedlings; this protein was named P23k. P23k was shown to have evolved in barley and its allied species such as wheat, oat and rye, suggesting that P23k might be closely related to a specific biological trait of winter cereals. To determine the function of P23k, we examined the expression and localization of P23k and its mRNA in barley seedlings. P23k mRNA was markedly expressed in the scutellum of imbibed barley seeds, and was synchronously down-regulated with consumption of the starchy endosperm. In addition, ABA and sugar treatment of the scutellum showed that expression of P23k is highly dependent on the supply of glucose or sucrose, both of which are starch catabolites. Furthermore, immunocytochemical analysis showed that P23k is primarily localized around the membranes, where sugar translocation is active. These results indicate that P23k plays a role in sugar translocation in young barley seedlings.

Key words: Barley (*Hordeum vulgare*), germination, scutellum, sugar translocation.

Cereals have a unique organ called the scutellum that attaches to the embryo. Sugars and nitrogen compounds, endosperm degradation products, are absorbed by scutellar epidermal cells then metabolized into translocation products such as sucrose and glutamine in the scutellum. Thereafter, they are loaded into vascular bundles in the scutellum and transported to the embryo as an energy source for the germinating seed. Transporters involved in nutrient transport are therefore thought to exist in these tissues. In line with this, Matsukura et al. (2000) reported that the gene encoding rice sucrose transporter, *OsSUT1*, is expressed in the scutellum and phloem of germinating seeds.

The scutellum also plays a role as a storage organ. Many nutrient storage organelles such as amyloplasts, protein bodies and oil bodies exist in scutellar cells. They are hydrolyzed into sugars, amino acids and fatty acids followed by seed imbibition and used as an energy source or components of living organisms. In addition, many metal ions such as Mg^{2+} and Ca^{2+} also exist as phytin granules in the scutellum, and are hydrolyzed into ions and inositol hexaphosphate by phytase. Accordingly, many kinds of functional protein related to metabolism are abundantly expressed in these tissues. In line with this, carboxypeptidase (Ranki et al. 1994), alpha glucosidase (Tibbot and Skadsen 1996) and lipoxygenase (van Mechelen et al. 1999), all of which are essential for degradation of nutrients in the seed, are abundant in germinating barley seeds. Thus, the scutellum plays an irreplaceable role in supplying nutrients during germination and early seedling development.

P23k was initially identified as a candidate of vernalization-related protein in barley. In order to clarify the relationship between P23k and vernalization, we therefore attempted to analyze the function of P23k in barley vernalization. The results (data not shown) did not

Abbreviations: ABA, abscisic acid; cDNA, complementary DNA; DIG, digoxygenine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EST, expressed sequence tag; EtBr, ethidium bromide; IEF, isoelectric focusing; MeJA, methyl jasmonate; ORF, open reading frame; 3-*O*-MEG, 3-*O*-metyl glucose; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulphate.

Accession numbers: The nucleotide sequences of *p23k-1*, *p23k-2*, *p23k-3*, and *p23k-4* have been submitted to the DDBJ database with accession numbers AB251338, AB251339, AB251340, AB251341, respectively.

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agree with our hypothesis that P23k is involved in the vernalization process, but nevertheless, they did highlight some interesting findings about the relationship between P23k and germination.

In this study, we analyze the expression and localization of P23k and its mRNA in germinating barley seedlings to further understand its role. Moreover, we also investigate the expression patterns of the paralogous gene JIP-23 to determine the difference between it and P23K.

Materials and methods

Plant material, growth conditions and chemical treatments

Barley (*Hordeum vulgare* L. cv Minorimugi) seeds were surface sterilized for 30 min with 2% (w/v) NaClO then germinated between wet towels in the dark at 25°C. The germinated seeds were transplanted to a plastic net floated on 1/5 Hoagland No. 2 liquid medium (pH 5.5) and grown in a growth chamber at 25°C with a 16 h light and 8 h dark cycle. For embryo sugar treatment, the starchy endosperms were scraped away from the surface-sterilized seed. The seeds were then transplanted onto filter paper soaked in 1/5 Hoagland No. 2 liquid medium (pH 5.5) containing 100 mM glucose, sucrose or 3-*O*-metyl glucose. For ABA treatment, surface-sterilized seeds were transferred to filter paper soaked in water containing 10 μM ABA. For methyl jasmonate (MeJA) treatment, 1-day-old seedlings were floated on water containing 44 μM MeJA and kept for 2 days at 25°C under continuous light.

Extraction of proteins, two-dimensional gel electrophoresis and amino acid sequence analysis

Total proteins were extracted from barley seedlings by homogenizing the tissues in extraction buffer (50 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.5 mM EDTA, 5 mM DTT, 1 mM PMSF, 5% glycerol). The homogenate was centrifuged at 5,000×*g* for 10 min at 4°C then the protein concentration was determined using a Protein Assay Kit I (BIO-RAD). Two-dimensional gel electrophoresis was performed according to the method of O'Farrell and Goodman (1977). Total proteins (50 μg) were subjected to isoelectric focusing (IEF) with ampholine mixture (3.5–10 ampholine: 5–8 ampholine=1 : 4, Pharmacia) for 13 h at 400 V and then for 1 h at 800 V. The IEF gel was further run on 12% SDS-polyacrylamide gel and stained using a Silver Stain Kit II (Wako) according to the manufacturer's instructions. For amino acid sequencing of the 23 kDa protein, the two-dimensional gel was stained with Coomassie Brilliant Blue. The band for P23k was excised and digested with endoproteinase Lys-C (Wako) and the digested peptides were isolated using reverse-phase high-performance liquid chromatography. Amino acid sequences of each peptide were determined using an automated protein sequencer (Shimadzu) according to the manufacturer's instructions.

cDNA cloning and nucleotide sequencing

The barley (*Hordeum vulgare* L. cv Minorimugi) cDNA library (λZAP mRNA from 7-day-old seedlings) was used for isolation of cDNA encoding P23k. A cDNA clone encoding partial P23k sequences (Figure 1B) was used as a probe. It was obtained by an EST analysis of barley young seedlings (data not shown). Approximately 2×10^4 recombinants were screened by plaque hybridization then the hybridizing plaques were isolated. After three cycles of plaque purification, *in vivo* excision of the pBluescript SK(−) was performed using *Escherichia coli* SOLR. The nucleotide sequences of the cDNA clones were determined using an automated DNA sequencer (ALOKA) with a Thermo Sequenase Cycle Sequencing Kit (Amersham). Sequences were determined more than twice.

Northern blot analysis

Total RNA (10 μg) isolated from selected tissues of barley seedlings was electrophoresed in 1.4% formaldehyde agarose gel then transferred to nylon membranes (Hybond-N^+, Amersham). Hybridization was carried out at 65°C with an [α-^{32}P]-labeled *p23k-1* cDNA probe. The nylon membranes were washed at 65°C with 2xSSC, 0.1% SDS (2×30 min) and exposed to X-ray film with an intensifying screen at −80°C.

RT-PCR analysis

To generate first strand cDNA, 1 μg of total RNA was annealed with 2 μM of oligo-dT in 20 μl reaction mixture and extended using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (TOYOBO) at 42°C for 1 h. One μl of the RT reactions was used as a 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 unit of Ex Taq polymerase (TAKARA) and 1 μM of primers. The primers using for gene amplification were CF: 5′-GGTACGGTAACGGAATAGC-3′′ for P23k and JIP-23, P23k-RS: 5′-TCGCCACACAAGCCT-TTGATGTT-3′ for P23k, and JIP-23-RS: 5′-AACTACACA-AGCGTACATGGACG-3′ for JIP-23. 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 with 2% agarose gel electrophoresis and visualized by EtBr staining. The specificity of the primers was tested by PCR amplification of the corresponding cDNA clone.

In situ hybridization

Selected barley tissues were fixed for 5 h at room temperature with fixing solution (3.7% formaldehyde, 5% acetic acid and 50% ethanol). The fixed tissues were dehydrated in a series of ethanol and xylene and embedded in paraffin (Paraplast plus, Sigma). Embedded tissues were then sectioned at a thickness of 7 μm and placed on PLL-coated micro slide glass (Matunami). Sections were deparafinized with xylene and rehydrated through a graded ethanol series. They were subsequently pretreated with proteinase K (Berhringer Mannheim) at 37°C for 30 min, dehydrated in a graded ethanol series, and dried under vacuum for 1 h. For 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 Labeling Kit (Berhringer Mannheim). The

hybridization signals were detected using a DIG Nucleic Acid Detection Kit (Berhringer Mannheim).

Preparation of anti-P23k antibody and immunohistochemical analysis

A peptide corresponding to the N-terminal region of P23k (MASGVFGTPISEKTVIATGE) was synthesized using a peptide synthesizer. It was linked to a carrier protein, hemocyanin, and injected into a rabbit. Antiserum was then used as a polyclonal antibody for detection of P23k. For immunohistochemical analysis, sections were prepared from the same embedded barley tissues used for *in situ* hybridization. They were deparafinized with xylene and rehydrated through a graded ethanol series then treated with blocking buffer (1xPBS, 0.2% Tween20, 0.1% NaN_3, 1% BSA) for 1 h at room temperature and washed five times with 1XPBS. After washing, sections were treated with anti-P23k antibody for 2 h and then treated with secondary antibody, anti-rabbit IgG alexa 546 (Funakoshi), for 1 h at room temperature. Finally, the sections were mounted in solution containing 1M Tris-HCl (pH9.5), 50% glycerol, 0.2% NaN_3, and 1% phenylendiamine and observed with fluorescence microscopy. As a negative control, sections were treated with preimmune serum instead of anti-P23k antibody and processed as above.

Immunogold labeling and transmission electron microscopy

The scutellum from germinating seeds was fixed for 2 h at room temperature in solution containing 9% paraformaldehyde, 1% glutaraldehyde, 60 mM sucrose and 50 mM sodium cacodylate (pH7.4). The fixed tissue was washed three times with 60 mM sucrose and 50 mM sodium cacodylate solution (pH7.4) and dehydrated in a series of ethanol. It was then embedded in LR White resin (NISSIN EM) and sectioned for electron microscopy using an ultra microtome. For immunogold labeling, ultra thin sections of 80 nm were placed on a 100 mesh formvar-coated nickel grids and dried at room temperature. The grids were floated on a drop of blocking buffer (1xPBS, 0.2% Tween20, 0.1% NaN_3, 5% BSA) for 1 h at 37°C and then washed three times with washing solution (1xPBS, 0.2% Tween20, 0.1% NaN_3, 1% BSA). They were treated with anti-P23k antibody for 16 h at 4°C and then goat anti-rabbit IgG conjugated to 20 nm gold particles as the secondary antibody for 1 h at 37°C. After washing, the grids were stained with uranyl acetate and lead citrate solutions. Observations were carried out using a Hitachi H-800 transmission electron microscope at 80 kV. As a negative control, sections were treated with preimmune serum instead of anti-P23k antibody and processed as above.

Results

Identification of P23k and determination of its partial amino acid sequence

In using 2D-PAGE to search for barley proteins that disappear specifically during vernalization treatment (40 days at 5°C), a 23 kDa protein was identified and subsequently named a candidate vernalization-related protein, P23k (Figure 1A). Unfortunately, a subsequent study showed that the level of P23k decrease was independent of vernalization treatment (Figure 4C). However, it simultaneously revealed that P23k exists specifically at the seedling stage. This experimental result led us to hypothesize that P23k plays an important role in early seedling development. To verify this, we therefore attempted to identify P23k. The band for P23k was collected and degraded by endoproteinase Lys-C, and a purified peptide fragment was sequenced. The determined peptide sequence (LANSNLNSTDENYG-YVSK) showed 67% homology to a protein of Mr 23,000 polypeptide (accession no. P32024) (Andressen et al. 1992), a jasmonic acid (JA)- and JA methyl ester (MeJA)-induced protein isolated from the same barley cultivar (Figure 1B). Later, Hause et al. (1996) designated this Mr 23,000 polypeptide JIP-23. However, it did not show high similarity to any other protein sequences in the databank of Swiss-Prot.

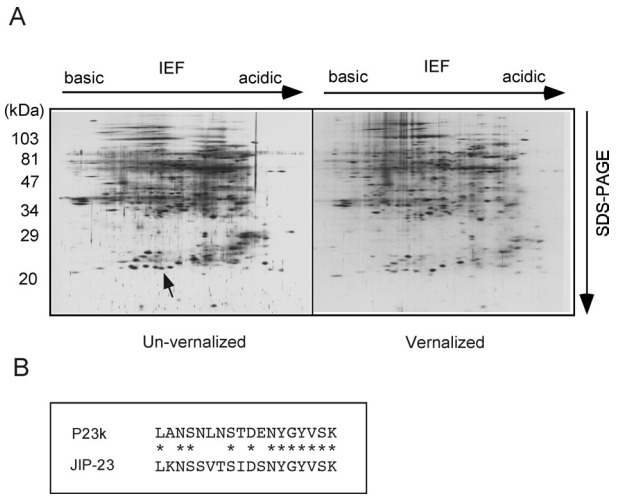

Figure 1. Identification of a 23 kDa protein, P23k. (A) Two-dimensional gel electrophoresis. Proteins were separated by IEF (pH 3.5–10) and 12.5% SDS-PAGE, and then visualized by silver staining. The arrowhead indicates the band corresponding to P23k. (B) The partial amino acid sequences of P23k and JIP-23. Homology analysis of the P23k sequence was carried out using BLAST.

Isolation of cDNA clones encoding P23k

Thirty positive clones were identified by plaque hybridization in about 5×10^4 recombinants. They were almost equally classified into four groups by partial sequencing of both termini. After classification, we selected the longest cDNA clones of each group (*p23k-1*, *p23k-2*, *p23k-3* and *p23k-4*, respectively) and determined their complete nucleotide sequences (Figure 2). There were some substitutions in their nucleotide and predicted amino acid sequences. The amino acid sequence similarity between P23k and JIP-23 was 88%. In addition, nucleotide sequence of *p23k-1* was identical with the exon 1 and 2 of Jip23b genomic clone

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p23K-1                                                                CAGCAATCGATCCATCTCTAAACTAGCAAAGCAA   34
p23K-2                                                                *GG**********                       14
p23K-3                                                                CT*GGT*GAA**GTA*GT*CAGG*********    32
p23K-4                                                                CCTAA*CT*GGT*GAA**GTA*GT*CAGG*********  38

p23K-1  ATGGCCTCAGGAGTGTTTGGTACCCCCATTTCGGAGAAGACGGTGATAGCCACTGGTGAGTATAAGGAACCCATTACTCAAAAGGATGTT  124
p23K-2  ***************************************************C*********************************  104
p23K-3  ***************************************************C*********************************  122
p23K-4  ***************************************************C*********************************  128

p23K-1  GCAGACTATGCCATGAAGATGATCAACGCCGGTGGTAAGGATATTAACGCACTAACCTTCGTCGACAATCTCAAAGAGCGGTACGGTAAC  214
p23K-2  ***************************************************A*********************A**********  194
p23K-3  *********A*************************************************A*********************A**********  212
p23K-4  *********A*************************************************A*********************A**********  218

p23K-1  GGAATAGCTGTAAAATGCCTCATCTACAATGCCACTGGTGCCACTTTGAGCTTGGCTAAGTACAACGATTGGCACGGCCATATCTATGAT  304
p23K-2  ******T*C*****************************C*********************************************  284
p23K-3  ***************************************************C*********************************  302
p23K-4  ***************************************************C*********************************  308

p23K-1  ACACCCTACCCATCAGATATTCAGAATGGGCAATGGGGTGCATTCCTCCACGTCCACCCAAGTGGTGCTGCGGTTGGTTCAGCTGGTGCC  394
p23K-2  **G*************************************************T*********************C*********  374
p23K-3  ***********G*****************C*************************T*********************C*********  482
p23K-4  ***********G*****************CC************************T*********************C*********  488

p23K-1  TACACCGAAATCCGTGAGGAAGGACACTACCCAAGTGTGGGAAGCTGGGGTTATATCTATGGTGTGAAGCTTGCAAATTCAAATCTCAAC  574
p23K-2  ************A*************************************************C*A*********************  554
p23K-3  ***************************A*************************************************G********  572
p23K-4  ***************************A*************************************************G********  578

p23K-1  TCTACTGATGAAAACTATGGATACGTTTCCAAGACTAATATCCGTGAAGGCTCTACCATGAACGCCCGTGGAGTTTTCCAGTTTCCCTAC  664
p23K-2  *********T*************************T*************************************************  644
p23K-3  ********T************T*************************************************A*******  662
p23K-4  ********T************T*************************************************A*******  668

p23K-1  TAGATCTCGTTATCAACATCAAAGGCTTGTGTGTGGCGAAGACGTTGCGGTGTATTTCCTAAGATCCGGTGTTCAAACTATGGATATGTTTC  754
p23K-2  *******T*****************************************C*************************************  734
p23K-3  ***************************************************G*******A********************A******  752
p23K-4  ***************************************************G*******A********************A******  758

p23K-1  GTCTACTATCTTTGTGGTGCATTTCCTTAGATCGGGTGCTTATGAATAATCAAGGAAGAAGACAATAAATGAGATATCATGTGTAATTTT  844
p23K-2  **************A*********A*************************************************************  824
p23K-3  ***************************A*************************************************G*****  842
p23K-4  ***************************A*************************************************G*****  848

p23K-1  ATATTATTTACAAGTCGTTTGTGTCAAGCTATCCCTCTTTTGTACCCACTACTGTTATCCCTAATAAATATCAAATTTATGATAAAAAAA  934
p23K-2  ***************************************************A*********************************G********  914
p23K-3  ***************************************************A*********************************G********  932
p23K-4  ***ATTAAAAAAAAAAAAA                                                                        868

p23K-1  AAAAAAAAAAAAAAAA                                                                          952
p23K-2  **********                                                                                924
p23K-3  ****************                                                                          949
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Figure 2. The nucleotide sequences of four kinds of *p23k* genes. Dots indicate the nucleotides that are identical to those of *p23k-1*. The initiation codon (ATG) and the stop codon (TAG) are underlined. The nucleotide sequences of *p23k-1*, *p23k-2*, *p23k-3*, and *p23k-4* have been submitted to the DDBJ database with accession numbers AB251338, AB251339, AB251340, AB251341, respectively.

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                10         20         30         40         50         60
P23k     MASGVPGTPI SEKTVIATGE YKE-PITQKD VADYAMKMIN AGGKDINALT FVDNLKERYG
JIP-23   ********** *AQ******* ***-****** ********** *****V**QK **********
Wheat    ********** *****L**** ***-****** ****T***** ********Q* **********
Rye                                                              ****
Oat
Rice     *-*T-F*V** TDE*LE*MSR *AGKS*S*V* R*RE**RL*H *E**NLD**Q HALG**AS**

                70         80         90        100        110        120
P23k     NGIAVKCLIY NATGATLSLA KYN----DWH GHIYDTPYPS DIQNGQWGAF LHVHPSGAAV
JIP-23   ********** *******N** ***----*** ********** ********** *********A
Wheat    ***S****** ******LN** N*K----*** ********** ********** **********
Rye      ********** *********V T**----*** ********** ********** *****R***A
Oat
Rice     D*VSAMV*V* *****A*E*V DGEGGSM**Y *YV*HEQP*A SF*****L** **A**TAQSI

               130        140        150        160        170        180
P23k     GSAGAVVYRT KIPSSSRSCD WLFSWTAPYI GD-NGVYTEI REEGHYPSVG SWGYIYGVKL
JIP-23   *********S ******S*** ******V*** *G-******* ********** **D***N***
Wheat    ********** *V***RS*** ******V*** *A-**I**** ********** **D***D***
Rye      ********** --SNDDT*** ******V**S *S-******* ***E****** **DF**NE**
Oat                   EAAGM** *****SI*** **-******* *****F*NY- -*****KQ**
Rice     *CEA*R*F*G RDVDGQVR-* FMVA*SL*WS ATQ*SA***V **KD*F*NY- -****KEE**

               190        200        210        220
P23k     ANSNLNSTDE -NYGYVSKTN IGEGSTMNAR GVFQ-----F PY
JIP-23   K**SVT*I*S -*******AD ***GT***** ***E-----* **
Wheat    E*A*L****K -TMDMFPRLT ---------- S*KA-----L *
Rye      E***A****G -*******VD ********** G**Q-----* **
Oat      QKAGRSC**N -**EH***VE ****T*V*** A**Q-----L **
Rice     EKAGKICT*Q TDKNCA*TVS V*GCTSSEFI A*L*HKFGPL *EE
```

Figure 3. Alignment of the amino acid sequences of barley P23k and its homologues. Amino acid sequences from barley (JIP-23, accession no. P32024), wheat (accession no. CV770628), rye (accession no. BE587635), oat (accession no. CN820096) and rice (accession no. AL731596) were aligned. Each amino acid sequence is represented by a standard single letter code. Gaps were introduced to obtain maximum similarity. Asterisks indicate identical amino acid residues with P23k.

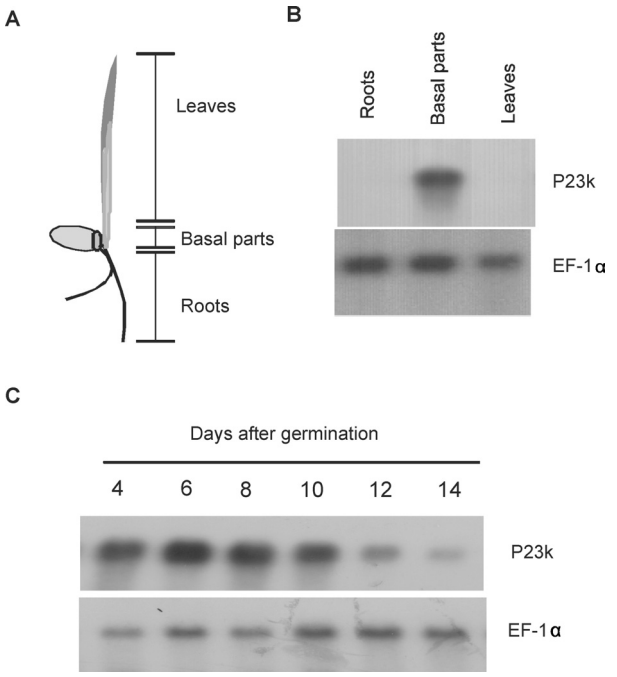

Figure 4. The spatial and temporal expression patterns of the P23k gene in barley seedlings. The areas used for RNA extraction are indicated in (A). Five-day-old seedlings were used for spatial expression analysis (B), and basal parts of the seedlings were used for temporal expression analysis (C). Northern blot analysis was carried out using 10 μg of T-RNA from barley. [α-^{32}P]-labeled *p23k-1* cDNA was used as a probe.

(accession no. X98124) that cloned by hybridization with JIP-23 cDNA (Müller-Uri et al. 2002). It was encoding a part (1-149) of the *p23k-1* ORF (1-210). Figure 3 compares the deduced amino acid sequences of *p23k-1* and its homologues derived from other plant species. cDNA clones encoding P23k homologues have only been isolated in wheat (accession no. CV770628), rye (accession no. BE587635) and oat (accession no. CN820096), all of which are closely related to barley; none have been reported in rice, which is also a poaceous plant species. Only one evolutionarily equivalent rice gene (accession no. AL731596) that shares about 30% similarity to P23k is registered in the DNA data bank. Moreover, no P23k homologue exists in *Arabidopsis thaliana*.

Spatial and temporal expression of the P23k gene in barley seedlings

To investigate the tissue specificity of P23k gene expression, 5-day-old barley seedlings were separated into leaves, basal parts and roots (Figure 4A). P23k mRNA was specifically expressed in basal parts including the scutellum and shoot apical meristem (Figure 4B). Temporal expression of P23k mRNA was examined using different stage of barley seedlings. Figure 4C shows that the level of P23k mRNA was kept constant in young seedlings up to 10 days after

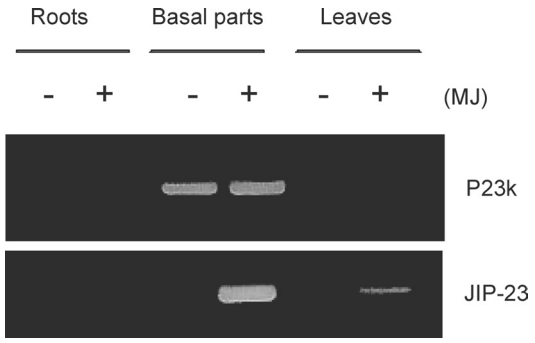

Figure 5. RT-PCR analyses of paralogous genes encoding P23k and JIP-23. T-RNA from 3-day-old seedlings treated with MeJA (44 μM) or untreated were used for analysis.

germination, but suddenly decreased in 12-day-old seedlings.

Effect of MeJA on expression of the P23k gene

In view of the sequence similarity between P23k and JIP-23, we examined whether P23k is also induced by MeJA. RT-PCR was used to differentiate expression of the two genes (Figure 5). MeJA had no significant effect on the expression of P23k. In addition, JIP-23 was not expressed under normal growth conditions.

Effect of starchy endosperm degradation products on expression of the P23k gene

Imbibed barley seeds were treated with 10 μM ABA for 1 day to investigate the dose-dependent effect of starchy endosperm degradation products on the levels of P23k mRNA. As shown in Figure 6, it was markedly down-regulated synchronously with decreasing levels of α-amylase mRNA. In addition, expression of the P23k gene was also induced after expression of the α-amylase gene.

In situ histochemical analysis of P23k and its mRNA

Localization of P23k mRNA and protein in the scutellum of barley seedlings was examined to speculate the function of P23k. As shown in Figure 7A, the signals of P23k mRNA were markedly localized in the scutellum and vascular bundles. They were not detected after hybridization using P23k sense probes (Figure 7B). On the other hand, the P23k protein was localized in cells of the radicle and plumule, in addition to the scutellum and vascular bundles

Effect of sugars on expression of the P23k gene

The main components of starchy endosperm degradation products are sugars such as glucose. Accordingly, we speculated that P23k expression in the scutellum might depend on sugars supplied by the endosperm after imbibition. To investigate this, we analyzed the effect of glucose, sucrose, 3-*O*-MEG and maltose on p23k mRNA

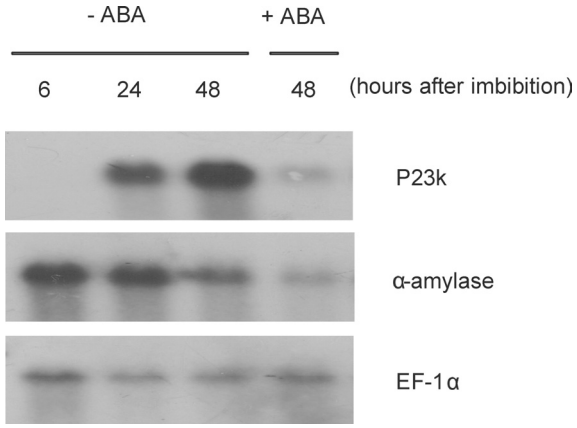

Figure 6. Effect of ABA on expression of P23k and α-amylase. Northern blot analysis was carried out using 10 μg of T-RNA from imbibed barley seeds. The imbibed seeds were treated with 10 μM ABA for one day. [α-^{32}P]-labeled *p23k-1* cDNA was used as a probe.

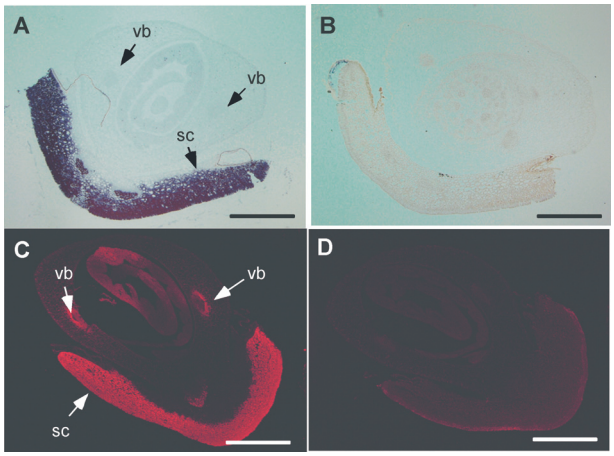

Figure 7. Localization of P23k mRNA and protein in the basal parts of young barley seedlings. *In situ* hybridization analysis using anti-sense (A) and sense probes (B) was carried out to detect P23k mRNA. Immunohistochemical analysis using anti-P23k antibody (C) and preimmune serum (D) was carried out to detect P23k protein. vb: vascular bundle, sc: scutellum. Scale bar is 0.5 mm.

expression in the scutellum. Embryos removed from the endosperm were placed on wet filter paper with one of the above sugars and treated for 2 days. Levels of P23k mRNA were examined by *in situ* hybridization. Figure 8A shows the level of P23k mRNA at a steady state before endosperm resection; expression disappeared completely at 2 days after endosperm resection (Figure 8B). However, in the presence of glucose (Figure 8C) or sucrose (Figure 8D), expression was maintained. In addition, sucrose treatment had a stronger effect on P23k gene expression than glucose treatment. On the other hand, the glucose analogue 3-*O*-MEG, which isn't metabolized into sucrose, did not maintain P23k gene expression (Figure 8E). We also investigated the effect of mannitol to show that the expression of P23k is not induced by osmotic pressure (Figure 8F).

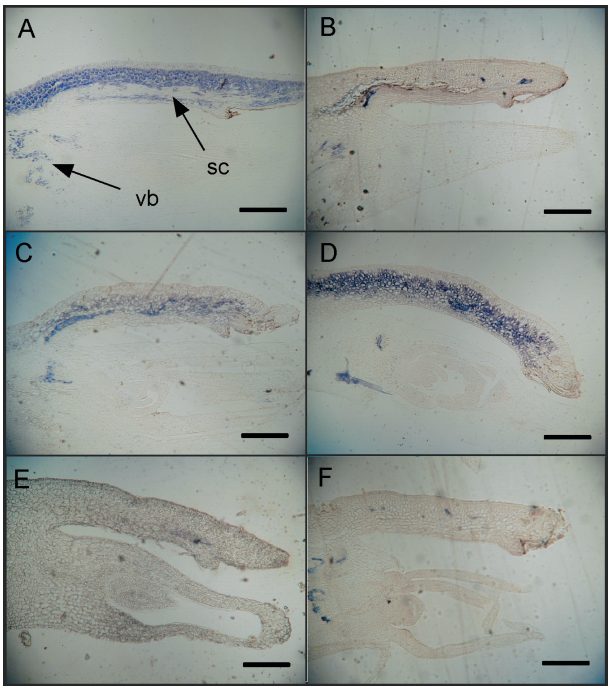

Figure 8. Effect of sugars on expression of the P23k gene in the scutellum of germinating barley seedlings. The scutellum was scraped away from the endosperm then transferred to filter paper including water (B), glucose (C), sucrose (D), 3-*O*-MEG (E) or mannitol (F) for 2 days. The scutellum of 3-day-old seedlings was used as a positive control (A). vb: vascular bundle, sc: scutellum. Scale bar is 1 mm.

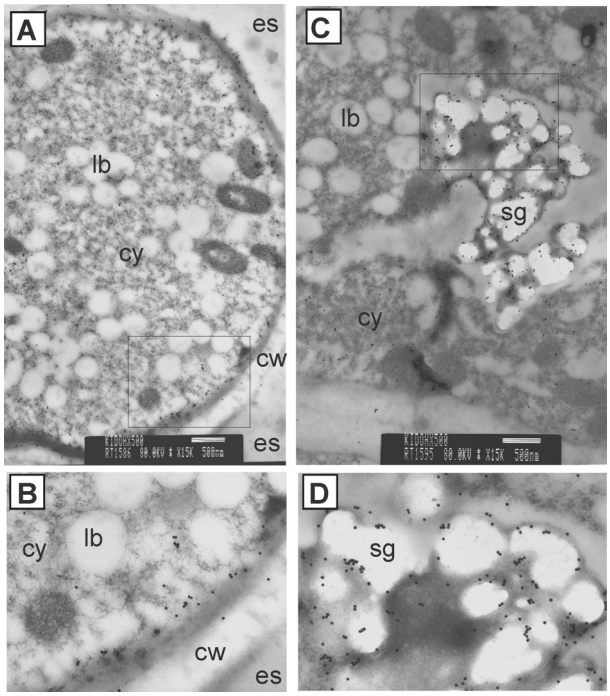

Figure 9. TEM immunolocalization of P23k in 3-day-old barley seedlings. (A, B); scutellar epidermal cells. (C, D); scutellar cells. Bars represent 500 nm. cy: cytoplasm, cw: cell wall, es: endosperm, lb: lipid body, sg: starch grain. Scale bar is 500 nm.

Intracellular localization of P23k in the scutellum

To elucidate the function of P23k, we analyzed the intracellular localization of P23k in the scutellum. Figure 9A, B show P23k localization in scutellar epidermal cells, which touch the endosperm. Signals were observed on the surface of the cells where the endosperm degradation products are absorbed. This localization was specific to scutellar epidermal cells; no localization was confirmed in internal cells of the scutellum. In internal cells, P23k was mainly localized around the surface of starch grains, which, in addition to the endosperm, are another sugar source (Figure 9C, D).

Discussion

P23k was initially obtained as a candidate of vernalization related protein

P23k was initially identified as a candidate protein thought to repress transition from the vegetative stage to reproductive stage in barley. Moreover, it was shown to disappear in vernalized barley (Figure 1A) and never reappear thereafter (data not shown). These findings supported the idea that P23k might be a key factor in regulation of vernalization in barley. However, in subsequent studies (data not shown), this hypothesis was denied, and the question as to why the P23k expression is specifically down-regulated by vernalization remained unknown. To address this question, we therefore attempted to analyze the expression and localization of P23k and its mRNA in germinating barley seedlings.

Identified P23k is a paralogous protein of a JA and MeJA-induced protein, JIP-23

The partial amino acid sequence of P23k showed similarity to the barley protein JIP-23, a JA and MeJA-induced protein found in barley leaf (Andresen et al. 1992). This result revealed that P23k is a paralogous protein of JIP-23. Interestingly, P23k did not show similarity to any other amino acid sequences registered in the data bank Swiss-Prot. Concurrently, it is suggested that these two paralogous proteins, P23k and JIP-23, evolved specifically in barley. The sequence similarity also suggested that P23k might be JA- and MeJA-induced as with JIP-23 (Hause et al. 1996). To examine this, we isolated cDNA clones encoding P23k from a barley cDNA library. Thirty positive clones were identified by plaque hybridization and equally divided into 4 kinds of sequence. All encoded the determined amino acid sequence of P23k, showing overall amino acid sequence identity of 98% (data not shown). The clone encoding JIP-23 was not obtained though the P23k probe used in the screening must cross hybridize with the genes encoding JIP-23. This result suggested that JIP-23 dose not express in barley seedling without JA- or MeJA treatment.

P23k have evaluated specifically in winter cereals including barley

The isolation of these P23k genes and subsequent sequence homology analyses provided us with some interesting information about the evolution and distribution of P23k. First, the gene encoding P23k seems to have been duplicated, resulting in at least four P23k genes in the barley genome. Hause et al. (1996) reported that there are seven distinct JIP-23 isoforms in barley. Sequence similarity between P23k and JIP-23 suggested that some of their isoforms must be P23k. Moreover, these data indicate that the genes encoding P23k and JIP-23 are commonly duplicated in the barley genome and constitute a multigene family. No such gene duplication was revealed in ort or rye by searching the DNA data bank. This suggests that these duplications occurred recently, after divergence from a common ancestor of barley, ort and rye. The gene duplication often occurs with genes that encode an essential protein. For example, in rice, translation elongation factor 1α, a key factor in protein synthesis, is transcribed from 4 genes (Kidou et al. 1998). Hause et al. (1998) reported that the expression of JIP-23 is also high in barley cultivars. The duplication might therefore be the result of breeding and selection of ancestral barley to form the present cultivars. In view of these findings, it is clear that both P23k and JIP-23 play an important role in the agricultural traits of barley. Second, the gene encoding P23k exists in only a few plant species, namely, barley, wheat, rye and oat. An orthologous gene showing low similarity to P23k was also found in rice (Figure 3); however, none could be found in the *A. thaliana* genome. These results indicate that P23k and JIP-23 specifically evolved in an original race of cereal plants, thereafter distributing within this group.

P23k and JIP-23 would play different roles in barley

The findings indicate that P23k and JIP-23 evolved from a common ancestral gene in barley, suggesting that MeJA might also induce P23k as well as JIP-23. To confirm this, RT-PCR analysis was performed. MeJA was shown to have no effect on P23k expression. Moreover, the gene encoding JIP-23 was not expressed in barley seedlings under normal growth conditions (Figure 5). These results indicate that these paralogous proteins, despite having similar structures, play different roles in barley. P23k is thought to be one of the key proteins in germination and early seedling development.

Expression of P23k gene is dependent on sugar supplied by the endosperm

Expression of the P23k gene was induced in the basal parts of young barley seedlings, and remained constant until the entire endosperm was consumed (Figure 4). Based on the result of the plaque hybridization, it is clear that all or almost signals were derived from the expression of P23k mRNAs. The result suggests that P23k expression might depend on sugar supplied by the endosperm. To confirm this, we investigated expression of P23k mRNA under ABA treatment, which causes the sugar supply from the endosperm to decrease with down-regulation of α-amylase (Varty et al. 1982, Jacobsen et al. 1991). As expected, gene expression of P23k was down-regulated synchronously with down-regulation of the α-amylase gene (Figure 6). This result suggests that expression of the P23k gene is dependent on the supply of starchy endosperm degradation products, sugars, which are degraded by α-amylase. However, a further study using P23k promoter is required to determine whether expression is directly induced by these sugars.

P23k would participate in the sugar supply

In situ hybridization and immunohistochemical analysis were used to investigate localization of the P23k protein and mRNA in the basal parts of young barley seedlings. Figure 7A shows the localization of P23k mRNA and Figure 7C shows that of P23k protein; both were markedly localized in the scutellum and vascular bundles. Hause et al. (1996) have reported by using polyclonal antibody to JIP-23 protein, which cross react with JA non-inducible JIP-23 isoform, that JIP-23 and its isoform was detected in sctellum and in phloem cells of the root and scutellar nodule. Judged by our results, they may detect mainly JA non-inducible JIP-23 isoform, P23k. Figure 8 shows the effect of different sugars on the expression of P23k in the scutellum; expression was shown to be dependent on degradation products of the endosperm, namely, glucose (Figure 8C) and sucrose (Figure 8D). These results strongly suggest that P23k participates in the sugar supply from seeds to growing tissues of young barley seedlings. In general, it is perhaps difficult to imagine that a species-specific protein like P23k could be related to a common physiological phenomenon such as sugar supply; however, it is possible to suggest that P23k is related to the sugar supply in cereals with specific organs such as a scutellum and starchy endosperm. The central players of the sugar supply, sucrose transporters (Chiou et al. 1996, Weschke et al. 2000) and hexose transporters (Weschke et al. 2003), have been identified in many plants. They are localized in the cell membrane and transport sugars from the apoplast to symplast. They also play a role in sugar transport in the cell, between the cytoplasm and each organelle. Accordingly, if P23k is found around the membranes, a role in sugar translocation might be possible. In the present study, P23k was shown to be primarily localized near the cytoplasmic membrane of scutellar epidermal cells, which lie adjacent to the endosperm (Figure 9A, B). In addition, it was also found around starch grains, which are rich in sugars, in

scutellar cells (Figure 9C, D). These results strongly suggest that P23k is involved in sugar translocation in the scutellum.

It is difficult to ascertain the precise function of P23k at present; however, all the data presented here suggest that P23k plays a role in the sugar supply. Elucidation of P23k function is therefore expected to be important in understanding the early growth of cereal plants such as barley. Thus, future studies are needed to further clarify the function of P23k in sugar supply, and accordingly, biochemical analysis to investigate the interaction between P23k and sugars are in progress.

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