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Short Communication

Jasmonate-induced 23kD protein, JIP-23, is involved in seed development of barley

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Abstract We previously showed the high expression of a 23 kD protein (P23k) in germinating barley seeds. In this study, we examined whether P23k is involved in supplying sugar to developing barley grains. RT-PCR analysis revealed that, although P23k expression was not detectable in developing grain, the 23 kD jasmonate-induced protein (JIP-23) exhibited a high level of RNA expression. JIP-23 belongs to the same protein family as P23k. Its remarkable expression was specifically confirmed at early development stage when sugar import into the filling grains is active. Furthermore, *in situ* hybridization and immunohistochemistry showed that JIP-23 is localized to active tissues in sugar import, such as vascular tissues, nucellar projections, endosperm transfer cells, and inter nucellar cells. Taken together, these results suggest that JIP-23 is related to sugar import into filling grain of developing barley seeds. Hence, we propose that jasmonate may regulate the development of barley seed.

Key words: Barley, grain filling, jasmonate, seed development, sugar import.

The quality and weight of grain, which stores sugars and proteins, are controlled by nutrient import during the grain-filling period. Understanding the mechanism of nutrient import is therefore important for the improvement of agricultural productivity.

We previously identified a 23 kDa protein, P23k, highly expressed in the scutellum of germinating barley (*Hordeum vulgare* L. cv Minorimugi) seeds and analyzed its characteristics in young seedlings (Kidou et al. 2006). The results showed that P23k is a paralogous protein of JIP-23, a jasmonic acid (JA) and methyl ester (MeJA)-induced protein identified in barley leaf (Andresen et al. 1992). Moreover, the expression of P23k was shown to be dependent on sugars and its localization was observed around membranes where sugar transport is active. Additionally, regardless of the high homology between P23k and JIP-23, P23k was not induced by JA and MeJA, and JIP-23 was not expressed at the young seedling stage. These results indicate that these two paralogous proteins have different roles in barley and suggest that P23k plays a role in sugar translocation. If this is correct, P23k should also be expressed in other organs such as the filling grain of developing seeds, which are active in sugar import. To test this hypothesis, we examined the expression and localization of P23k in barley filling grains. Moreover, to further clarify the functional difference between these two paralogous proteins, we also examined the expression of JIP-23 with P23k.

The filling grain was divided into three stages (Figure 1A): stage I (0～3 days after fertilization), stage II (4～6 days after fertilization) and stage III (7～12 days after fertilization). In the scutellum of germinating barley seed, P23k was expressed higher than JIP-23 (Kidou et al. 2006). Based on this result, we expected the same expression pattern observed in filling grains. To confirm this, RT-PCR analysis was performed. Gene-specific reverse primers were used to differentiate expression of the two genes encoding P23k and JIP-23. Surprisingly, P23k mRNA was not detected in the PCR condition described in the figure legend, and only JIP-23 mRNA was observed in the filling grains (Figure 1B). Additional reaction cycles were required to detect P23k mRNA (data not shown). In contrast, JIP-23 mRNA was not detected, and only P23k mRNA was observed in the scutellum. This result indicates that these two paralogous proteins have different roles in barley. It is possible that P23k acts on sugar transport in the scutellum of

Abbreviations: DIG, digoxygenine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EtBr, ethidium bromide; MeJA, methyl jasmonate; ORF, open reading frame; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulphate.
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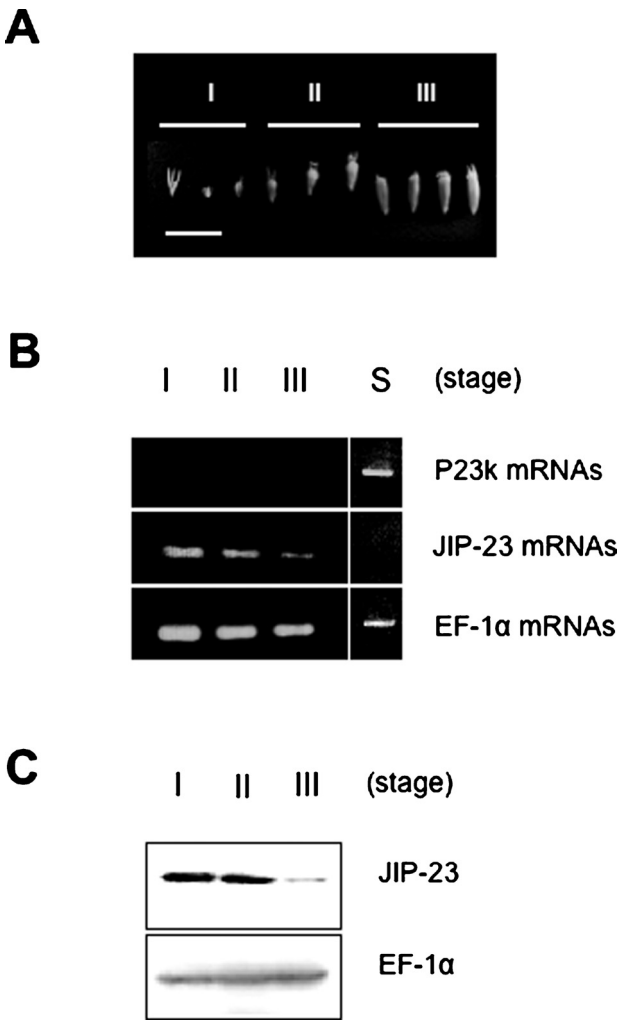

Figure 1. Expression of JIP-23 mRNA and protein during the grain-filling period of barley. (A) Morphology of the barley filling grains after fertilization. They were divided into three stages: stage I (0~3 days after fertilization), stage II (4~6 days after fertilization) and stage III (7~12 days after fertilization). Scale bar is 5 mm. (B) Expression patterns of paralogous genes encoding P23k and JIP-23 in filling grains (I~III) and scutellum of germinating barley seed (S). EF-1α was analyzed as a control. The primers used for the mRNA detection of P23k, JIP-23 and EF-1α were as follows: P23k (5′-GGTACGGTA-ACGGAATAGC-3′ and 5′-TCGCCACACAAGCCTTTGATGTT-3′); JIP-23 (5′-GGTACGGTAACGGAATAGC-3′ and 5′-AACTACACAA-GCGTACATGGACG-3′); EF-1α (5′-TTCAACGTCAAGAACGTGG-CT-3′ and 5′-ACACAAATAACCCAAGCGACTA-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 with 2% agarose gel electrophoresis and visualized by EtBr staining. (C) Expression patterns of P23k and JIP-23 proteins at the three stages. Immunoblot analysis was carried out using anti-P23k antibodies against a peptide corresponding to the N-terminal region of P23k (MASGVFGTPISEKTVIATGE) or anti-EF-1α antibodies.

germinating barley seeds, while JIP-23 acts on sugar import into the filling grain of developing barley seeds. EF-1α mRNA was also analyzed as a control in the RT-PCR analysis. Figure 1C shows the result of immunoblot analysis using an anti-P23k antibody, which can also detect JIP-23; the same expression pattern as with RT-PCR analysis was revealed. Remarkable expression was confirmed from stage I to stage II, but decreased at stage III. Similar expression patterns were observed in the case of the sucrose transporter HvSUT (Wesche et al. 2000), hexose transporter HvHXT (Wesche et al. 2003), and cell wall-binding invertase HvCWINV (Wesche et al. 2003), all of which are directly involved in sugar import into the developing grains. Wesche et al. (2000) also reported that an active sugar import into the filling grains is decreased at the stage corresponding to III. These data strongly suggest that JIP-23 is related to sugar import into filling grain of developing barley seeds. EF-1α was used as a control in the immunoblot analysis.

Figure 2 shows the localization of JIP-23 mRNA and protein in filling barley grains as determined by *in situ* hybridization (Figure 2A, B, C) and immunohisto-chemistry (Figure 2D, E, F, G, H). Figure 2I provides a schematic diagram of the transverse section of a filling grain of a developing barley seed. In cereals, sugars carried from source tissues via vascular tissues (VT) are imported to endosperm tissues via nucellar projections (NP), endosperm transfer cells (ET) and inter nucellar cells (IN) during the grain-filling period (Hoshikawa 1984). Thus, JIP-23 should localize in these tissues if it is related to sugar import. In stages I and II, JIP-23 mRNA strongly localized to IN, NP and VT (Figure 2A, B). However as a negative control, they were not detected after hybridization using sense probes (Figure 2C). The same localization was confirmed by immunohisto-chemistry (Figure 2D, E). In addition, the signals were slightly reduced at 12 days after fertilization (Figure 2G) in stage III, and almost completely disappeared at 15 days after fertilization (Figure 2H) with the decrease in sugar import (Wesche et al. 2000). No signals were detected in hybridization using preimmune serum as a negative control (Figure 2F). The association between JIP-23 localization and sugar import suggests that JIP-23 plays an important role in sugar import in the grain filling period of developing barley seeds.

Creelman and Mullet (1997) reported that jasmonates are essential for the development of sink tissues during, for example, fruit ripening, the production of viable pollen and root growth. Further, mRNA of allene oxide cyclase (AOC), the key enzyme of Jasmonate biosynthesis, accumulates abundantly in sink tissues notably flower buds, flower stalks and roots (Hause et al. 2000); expression was also shown to be induced by glucose. These data suggest that jasmonate biosynthesis may be induced by sugar imported to sink tissues and that newly synthesized jasmonates then may activate development of these tissues. JIP-23 is also induced by jasmonate treatment (Andresen et al. 1992), and is always detectable after an increase in the endogenous jasmonate level (Kramall et al. 2000). Therefore, Hause

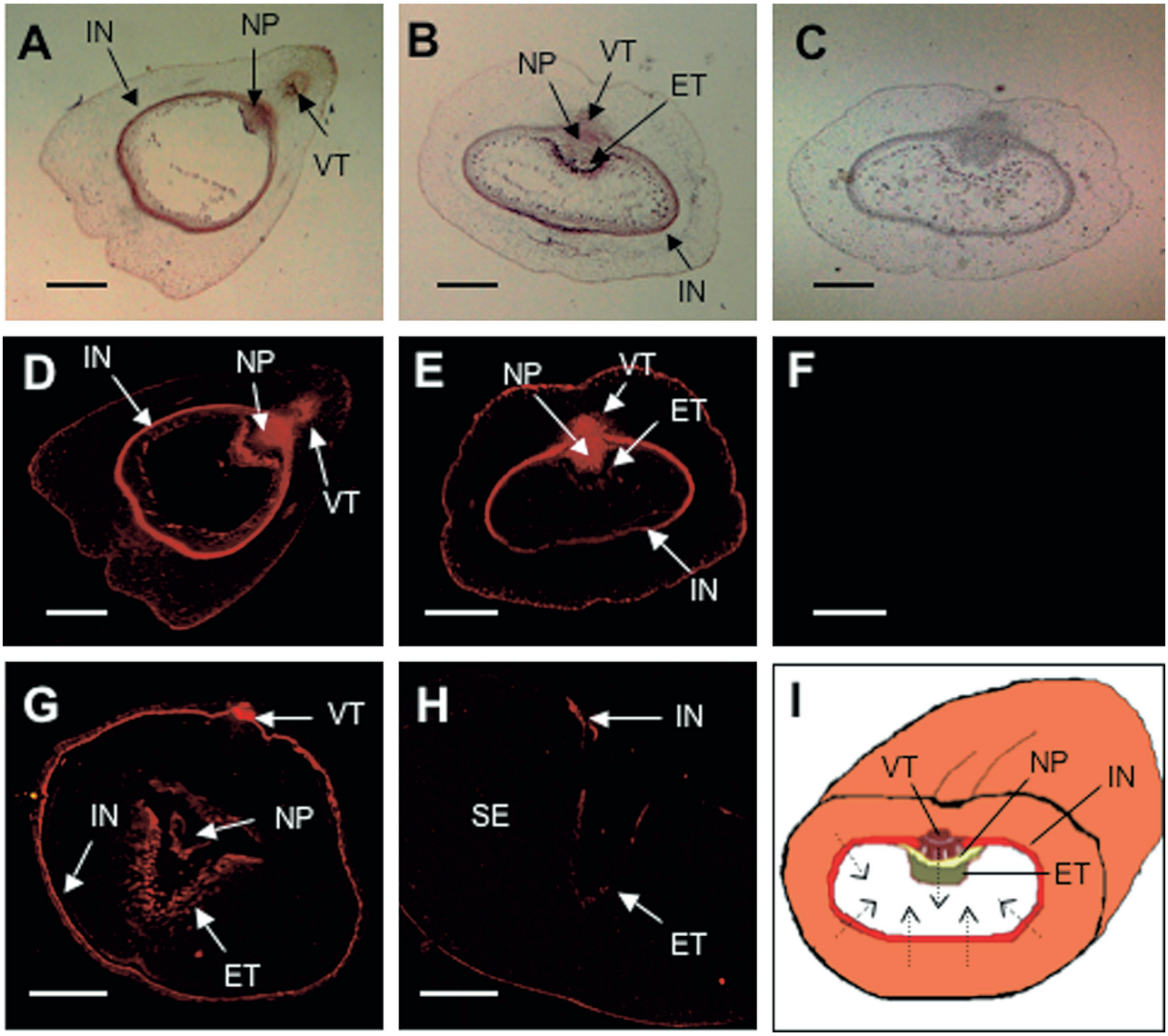

Figure 2. Localization of JIP-23 mRNA and protein in developing barley grains. *In situ* hybridization was carried out using a longitudinal section of grain at 3 days after fertilization (A) and a transverse section of grain at 6 days after fertilization (B). Hybridization using sense probe was carried out as a negative control (C). 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). Immunohistochemical analysis was carried out using a longitudinal section of grain at 3 days after fertilization (D), a transverse section of grain at 6 days after fertilization (E), a transverse section of grain at 12 days after fertilization (G) and a transverse section of grain at 17 days after fertilization (H). These sections were treated with anti-P23k antibody (1 : 500 dilution) for 1 h then treated with secondary antibody, anti-rabbit IgG alexa 546 (Funakoshi). Hybridization using preimmune serum was carried out as a negative control (F). (I) Schematic diagram of features related to sugar import in a filling barley grain. Lines show the direction of sugar import into the endosperm. IN: intergument, NP: nucellar projection, VT: vascular tissue, ET: endosperm transfer cells, SE: starch endosperm. Scale bar is 0.5 mm.

et al. (2002) indicates that increases in JIP-23 are a valuable indicator of endogenous jasmonate levels. Together, this report shows that the jasmonate-induced protein, JIP-23, is involved in sugar import at the grain filling stage, thus possibly contributing to seed development.

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