BIOLOGIA PLANTARUM 55 (1): 173-177, 2011

BRIEF COMMUNICATION

# Subcellular localization of rice hexokinase (OsHXK) family members in the mesophyll protoplasts of tobacco

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## Abstract

Hexokinase (HXK, EC 2.7.1.1) plays an important role in the metabolism and glucose signalling. To examine the characteristics of *HXK* gene family in rice, the subcellular localizations of ten hexokinases (*OsHXK1* - *OsHXK10*) were determined using OsHXK::GFP fusion proteins in tobacco mesophyll protoplasts. As was previously demonstrated, OsHXK4 was detected in the chloroplast stroma, OsHXK5 and OsHXK6 in the mitochondria, and OsHXK7 and OsHXK10 in the cytoplasm. In the present study, OsHXKs were clearly divided into three types (A, B, C) based on their N-terminal sequences. The new type-C HXKs in plants, OsHXK1, OsHXK7 and OsHXK8, which lack the plastidic transit peptide and the membrane anchor domain, were detected not only in the cytoplasm but also in the nucleus. The type-B HXKs, OsHXK2, OsHXK3, OsHXK9 and OsHXK10, which contained a membrane anchor domain, were distinctly localized in the mitochondria. These results suggest that OsHXKs localized in different cell compartments may be involved in the glucose signalling-related gene expression during growth and development of rice.

*Additional key words*: GFP, hexose, *Nicotiana tabacum*, *Oryza sativa.*

Hexokinase (HXK) is a dual-function enzyme that exists in all living organisms (Cárdenas *et al*. 1998). It not only phosphorylates hexoses to form hexose 6-phosphate, but also plays an important role in sugar sensing and signalling. Although plants have a relatively small family of HXK enzymes, their various localizations in cell compartments may be relevant to their different physiological functions in growth and development. Using cell fractionation procedures and activity measurements, HXK isoforms have been examined in cytoplasm, mitochondria, Golgi complex, chloroplast stoma, and membrane (Šindelářová and Šindelář 1988, Singh *et al*. 1993, Galina *et al*. 1995, Da Silva *et al*. 2001, Giege *et al*. 2003, Šindelář and Šindelářová 2003, Rezende *et al*. 2006). In recent years, several HXKs have been identified by their coding genes in some plant species, and they have been divided into two major categories, types A and B, based on their N-terminal sequences. Type-A HXKs are characterised by a chloroplast transit peptide, and type-B HXKs share a common hydrophobic membrane anchor domain (Olsson *et al*. 2003). To date, type-A HXKs, including NtHXK2, OsHXK4, LeHXK4 and AtHXK3, have been detected in the chloroplast stroma (Giese *et al*. 2005, Cho *et al*. 2006a, Kandel-Kfir *et al*. 2006, Karve *et al*. 2008). In these studies, the physiological roles of type-A HXKs were examined only for NtHXK2. As demonstrated, NtHXK2 was primarily in sink tissues, and it was likely involved in starch degradation rather than starch synthesis (Giese *et al*. 2005). Type-B HXKs have been examined to be associated with mitochondria in many plants (Damari-Weissler *et al*. 2006, 2007, Kandel-Kfir *et al*. 2006, Kim *et al*. 2006, Balasubramanian *et al*. 2007, Karve *et al*. 2008). In type-B HXKs, the role of AtHXK1 is believed to be in sugar sensing and regulating gene expression. Although type-B HXKs have been reported to be associated with mitochondria, AtHXK1 is also transported into the nucleus, where it forms a glucose signaling complex with the vacuolar $H^+$-ATPase B1 (VHA-B1) and the 19S

*Received* 17 April 2009, *accepted* 20 December 2009.

*Abbreviations*: cTP - chloroplast transit peptide score; GFP - green fluorescent protein; Glc - glucose; mTP - mitochondrial targeting peptide score; OsHXK - rice hexokinase; SP - secretory pathway score.

*Acknowledgements*: This work was supported by the National Natural Science Foundation of China (grant 30570148) and Project 973 from the Ministry of Science and Technology of China (grant 2005CB120803).

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regulatory particle of proteasome subunit (RPT5B). This complex modulates specific target gene transcription independent of glucose metabolism (Cho *et al*. 2006b). Thus, AtHXK1 would act as a glucose sensor to integrate the nutrient and hormone signals for governing the gene expression and plant growth in response to environmental cues (Moore *et al*. 2003).

In the monocotyledon rice, ten hexokinase genes have been isolated and characterised (Cho *et al*. 2006a). To date, subcellular localizations have been identified only for five OsHXK family members. Specifically, OsHXK4 and OsHXK7 were detected in the chloroplast stroma and cytoplasm, respectively (Cho *et al*. 2006a), OsHXK5 and OsHXK6 were recently found to be associated with the mitochondria (Cho *et al*. 2009), and OsHXK10 was found to be localised in the cytoplasm (Xu *et al*. 2008).

To further clarify the physiological roles of OsHXKs, it is necessary to investigate the subcellular distribution of the remaining OsHXK family members and to confirm the previous results. In the present study, the subcellular localizations for each member of the OsHXK family were systematically examined using green fluorescent protein (GFP) fusion constructs.

Ten rice (*Oryza sativa* L.) HXK cDNAs, from *OsHXK1* to *OsHXK10* (DQ116383-DQ116392), were previously cloned from the immature rice endosperms. To construct the chimeric *OsHXKs-GFP* gene, a two-step PCR strategy was used to attach the GFP to HXK C-terminus (Higuchi 1990). The coding regions of OsHXKs-GFP were obtained by overlapping PCR with *Eco*RI / *Xba*I for *OsHXK1*, *OsHXK2*, *OsHXK3*, *OsHXK4*, *OsHXK6*, *OsHXK8*, *OsHXK9* and *OsHXK10*, and with *Xho*I / *Kpn*I for *OsHXK5* and *OsHXK7*. The primers used for PCR amplification of the *OsHXK* coding regions were as follows: *OsHXK1*, 5'-GAGAATTCGGTTCAAAGCT TGTTCGATT-3' and 5'-TCCTCGCCCTTGCTCACC ATCGCCGCCATCTTCGGCAT-3'; *OsHXK2,* 5'-ATG AATTCCCATGAGGAAGGCGGCGGCGGCGGCGGT CGCGGCA-3' and 5'-TCCTCGCCCTTGCTCACCATG GCCATCGCGTCCGCCACCT-3'; *OsHXK3,* 5'-GAG AATTCAGGGGTGGAGGAGGAGAT-3' and 5'-TCC TCGCCCTTGCTCACCATTATGGGACCTCCTTGTT GCT-3'; *OsHXK4,* 5'-GAGAATTCATGTCCGCCGC CGCCGCCAT-3' and 5'-TCCTCGCCCTTGCTCACC ATCCTTGTAGAGATTTGAGCAG-3'; *OsHXK5,* 5'-GAGAATTCATGGGGAAGGCGGCGGCGGT-3' and 5'-TCCTCGCCCTTGCTCACCATGTCGATCTCGGCA TACTGGG-3'; *OsHXK6,* 5'-TAGAATTCATGGGGAA GGGGACGGTAGT-3' and 5'-TCCTCGCCCTTG CTCACCATCTCGACGCTAGCATACTGG-3'; *OsHXK7,* 5'-ATCTCGAGAAATGGTGGCGGCGGCCGTGGCGG CGGCGGA-3' and 5'-TCCTCGCCCTTGCTCACCA TATTCAGGTACTGAGAATGAGCAGC-3'; *OsHXK8,* 5'-GCGAATTCGTTTGTCTGAACCGAACTC-3' and 5'-TCCTCGCCCTTGCTCACCATTCGAGATTGAGA GGCTGCAA-3'; *OsHXK9,* 5'-TAGAATTCATGAGGA AGGCGGCGGCGCTGGCGT-3' and 5'-TCCTCGCCC TTGCTCACCATTGAACTATCTGCAGATTCAGC-3'; *OsHXK10,* 5'-GAGAATTCGGTGGGGTTCTCCT CCT-3' and 5'-TCCTCGCCCTTGCTCACCATCTCGG AGTTCTTCTGCCTAG-3'. The PCR products of the OsHXKs-GFP were cloned into the pRTL2 shuttle vector under the control of a double 35S promoter. All constructs were examined by DNA sequencing.

Mesophyll protoplasts were isolated from young tobacco (*Nicotiana tabacum* L.) leaves (Yoo *et al*. 2007). Typically, $2 \times 10^4$ protoplasts were transfected with 10 μg purified plasmid DNA using the polyethylene glycol 4 000 (*Fluka*, Buch, Switzerland) according to a previously developed protocol (Yoo *et al*. 2007). After the transformation with constructed plasmids, the protoplasts were incubated in the dark at 26 °C for 4 -24 h, and then they were observed by confocal microscopy.

Microscopic images were taken using an inverted laser scanning confocal microscope (*Fluoview 300*, *Olympus,* Tokyo, Japan). Hoechst 33342 was used at a final concentration of 5 μg $cm^{-3}$ (*Sigma*, St. Louis, USA). Fluorescence emission from the Hoechst 33342 dye was collected using a *BA 430-460* filter after excitation at 364 nm. GFP was excited at 488 nm, and fluorescence emission was collected using a *BA 505-525* filter. *MitoTracker* (400 nM; *CMXROS* from *Molecular Probes*) was excited at 543 nm, and the fluorescence emission was collected using a *BA 560-600* filter. The *BA 660* IF emission filter was used to observe the autofluorescence of chlorophyll. The confocal optical sections were examined in 0.5 μm increments.

The subcellular localizations of OsHXKs were predicted using the *TargetP* software (http://www.cbs.dtu.dk/services/TargetP; Emanuelsson *et al*. 2000). OsHXKs were divided into three types, A, B and C, based on their N-terminal sequences (Fig. 1). In the type-A OsHXKs, only OsHXK4 has a putative 37-amino-acid N-terminal cleavable chloroplast transit peptide (Nielsen *et al*. 1997), similar to those of NtHXK2, LeHXK4 and AtHXK3 (Giese *et al*. 2005, Kandel-Kfir *et al*. 2006, Karve *et al*. 2008). This indicated that OsHXK4 may be a type-A plastidic hexokinase (Olsson *et al*. 2003). The type-B OsHXKs, including OsHXK2, OsHXK3, OsHXK5, OsHXK6, OsHXK9 and OsHXK10, have predicted N-terminal membrane anchor domains, like those of AtHXK1, LeHXK1 and NbHXK1. Thus, it was confirmed that these OsHXKs may be type-B membrane anchor hexokinases (Olsson *et al*. 2003), previously shown to be associated with mitochondria (Damari-Weissler *et al*. 2006, 2007, Kim *et al*. 2006, Balasubramanian *et al*. 2007). Notably, a new type of HXK named type C was detected in rice; these OsHXKs, including OsHXK1, OsHXK7 and OsHXK8, lacked the plastidic transit peptide and the membrane anchor domain.

To explore the putative roles of OsHXKs, the subcellular localization of each member of the OsHXK family was examined using the plasmids in which the full-length OsHXKs were fused to green fluorescent protein (GFP). The constructs encoding the OsHXKs::GFP fusions were under the control of cauliflower mosaic virus 35S promoter, and they were transiently transformed into the protoplasts isolated from

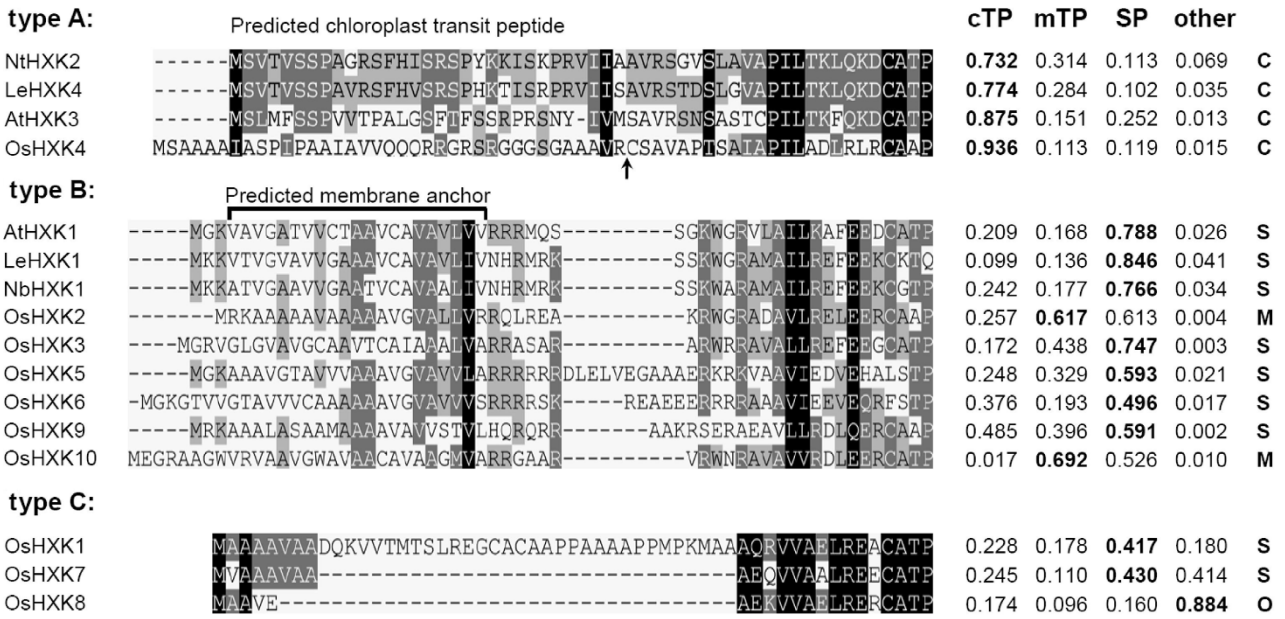


Fig. 1. Three rice hexokinase family members. Type-A hexokinases (HXKs) have predicted chloroplast transit peptides. Type-B HXKs have N-terminal membrane anchors. Type-C HXKs have neither predicted chloroplast transit peptides nor N-terminal membrane anchors. The N-terminal sequences of OsHXKs, NtHXK2, LeHXK4, AtHXK3, AtHXK1, LeHXK1 and NbHXK1 were aligned from the highly conserved enzymatic domain that starts at the *right side* of the figure. Comparisons of the N-terminal regions were performed using the subcellular prediction localization programme *TargetP*. *TargetP* scores and predictions are shown to the *right*, with the highest score for each protein shown in *bold*. cTP - chloroplast transit peptide score, mTP - mitochondrial targeting peptide score, SP - secretory pathway score, C - predicted chloroplast import, S - predicted secretory pathway, M - predicted mitochondrial import, O - other. Prediction of the membrane anchored segment was done using *TopPred2* (http://mobyle. pasteur.fr/cgi-bin/mobyleportal/portal.py?form=toppred). The *arrow* shows the predicted cleavage site of the chloroplast transit peptide of OsHXK4. Accession numbers of the proteins are: *Oryza sativa* (rice), OsHXK1-OsHXK10 (DQ116383-DQ116392); *Arabidopsis thaliana*, AtHXK1 (U28214), AtHXK3 (BT030472); *Nicotiana tabacum* (tobacco), NtHXK2 (AY553215); *Nicotiana benthamiana*, NbHXK1 (AY286011); *Lycopersicon esculentum* (tomato), LeHXK1 (AJ401153), LeHXK4 (DQ056862).

the mesophyll of tobacco. Protoplasts transformed with OsHXKs::GFP were stained with the mitochondria-selective probe, *MitoTracker*, or the nuclear area dye, Hoechst 33342. Chlorophyll autofluorescence was used as a chloroplast marker, and the fluorescence emissions from GFP, *MitoTracker* and *Hoechst 33342* were visualised by confocal microscopy.

The type-A OsHXK4 was also confirmed to be a chloroplast-localised enzyme (Fig. 2*A*), in agreement with the report of Cho *et al*. (2006a). For the type-B OsHXKs, the more signals from the GFP fusion with these enzymes (OsHXK2, OsHXK3, OsHXK5, OsHXK6, OsHXK9, OsHXK10) were examined using the mitochondria-selective probe *MitoTracker*. These OsHXKs were found to be associated with mitochondria (Fig. 2*B*). For the type-B OsHXKs, the localizations of OsHXK5 and OsHXK6 were recently reported (Cho *et al*. 2009). However, OsHXK10 was found to be localized in the cytoplasm by Xu *et al*. (2008). This result is different from that in the present study, in which the signal from OsHXK10 was strictly associated with the mitochondria. The signal from the OsHXK10-GFP fusion protein was examined in the present study with transient expression in rice cell suspension, indicating that OsHXK10 was localized to the mitochondria (data not shown). For the type-C OsHXKs, the signals from the GFP fusions with OsHXK1, OsHXK7 and OsHXK8 were detected with *Hoechst* and shown to be in the nucleus. Thus, the three OsHXKs were not only in the cytoplasm but also in the nucleus (Fig. 2*C*). However, OsHXK7 was found only in the cytoplasm of transgenic calli, leaf and root (Cho *et al*. 2006a). In the present study, it was demonstrated for the first time that some HXK proteins were observed in the nucleus *via* transient expression using GFP fusion constructs in a higher plant. However, it is required to confirm the nucleus localization of C-type HXKs because the free GFP tends to accumulate spontaneously in nucleus.

Hexokinase plays an important role not only in metabolism but also in glucose signalling. In the type-A HXK, only OsHXK4, targeted to the chloroplast stroma, is likely important when using hexose for starch degradation, as occurs in the plastids of rice sink organs (Olsson *et al*. 2003, Giese *et al*. 2005). To date, many studies have demonstrated that type-B HXKs are associated with mitochondria and may be involved in glycolysis and respiratory metabolism in plants. In *Arabidopsis,* HXKs located on the outside of the mitochondrial membrane

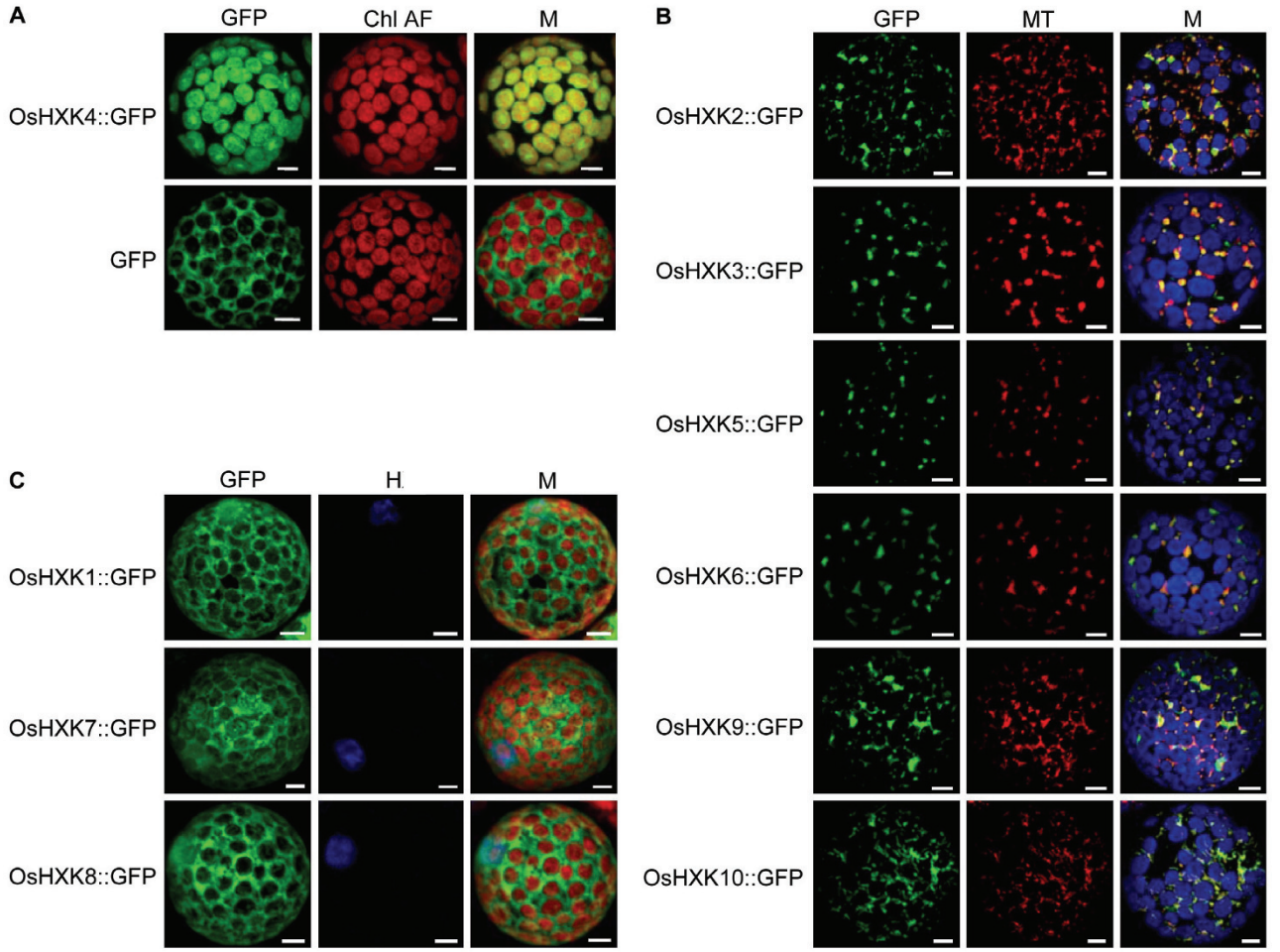


Fig. 2. Subcellular location of the OsHXKs::GFP fusion proteins in mesophyll protoplasts of tobacco. *A* - Type-A OsHXK (OsHXK4) examined in the chloroplast. Chlorophyll autofluorescence (ChlAF) was used as a chloroplast marker. The GFP signal is indicated in green, and chlorophyll autofluorescence is show in red (M). GFP is a control. *B* - Co-localisation of type B OsHXKs (OsHXK2, OsHXK3, OsHXK5, OsHXK6, OsHXK9, OsHXK10) with mitochondria. The GFP signal is indicated in green. Mitochondria were stained using *MitoTracker* (MT; red). The autofluorescence of the chlorophyll is artificially stained in blue. *C* - Type-C OsHXKs (OsHXK1, OsHXK7 and OsHXK8) localised to both the cytosol and nucleus. GFP signals are indicated in green. Hoechst-stained (H) nuclear areas are in blue. The autofluorescence of chlorophyll is in red. The *scale bar* represents 5 μm.

have been shown to be involved in the entire glycolytic metabolon, which allows pyruvate to be provided directly to mitochondria as a respiratory substrate (Giege *et al*. 2003). Consequently, it has been suggested that the type-B OsHXKs associated with mitochondria perhaps have some role in the micro-compartmentalisation of glycolysis. In the type-B HXKs, AtHXK1 has been found to be transported into the nucleus where it regulates the transcription of specific target genes (Cho *et al*. 2006b). Moreover, OsHXK5 and OsHXK6 have recently been reported to retain a dual targeting ability for the mitochondria and nucleus to function as part of a glucose sensor (Cho *et al*. 2009). Nevertheless, it remains unclear by which mechanism(s) the type-B HXKs associate/dissociate with the mitochondrial membrane and how they move into the nucleus under natural physiological conditions.

In the present study, OsHXK1, OsHXK7 and OsHXK8 of type-C HXKs were categorised as a novel type of HXK, found not only in the cytoplasm but also in the nucleus. To date, all reported HXKs, which belong to either type-A or type-B, have been in dicotyledons (Claeyssen and Rivoal 2007). By searching genomic databases, it was found that the sequence of ZmHXK (EU965740) is similar to that of the type-C OsHXKs, suggesting that ZmHXK may also belong to the type-C HXKs (data not shown). The type-C HXKs identified in this study may be a new type HXKs existed in rice and maize. For the type-C HXKs localized in the cytoplasm, their physiological roles may involve the phosphorylation of cytosolic hexoses (Koch 2004). For the type-C HXKs identified in the nucleus and cytoplasm, ScHXK2 in yeast was demonstrated to participate in an essential metabolic pathway and/or to induce gene expression (Randez-Gil *et al*. 1998, Rodríguez *et al*. 2001). Collectively, these findings suggest that the type-C OsHXK1, OsHXK7 and OsHXK8 may be involved not only in cytosolic hexose metabolism but also in gene expression associated with glucose signalling in rice plants.

Therefore, it is necessary to study the function of type A, B and C OsHXKs for understanding their roles in the metabolism and signal transduction during rice growth and development.

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