The synthesis of storage starch and protein accumulation are the main actions of endosperm organogenesis in terms of the economic importance to rice production. The enzymes for the starch synthesis pathway should work in concert to carry out the organogenesis of rice endosperm under the conduct of several regulatory factors. Although biochemical and genetic studies have identified many key enzymes for protein storage and the starch synthesis pathway, the regulatory mechanism conducting the enzyme activities is largely unknown.

SPK is a CDPK (calcium-dependent protein kinase), whose gene was specifically expressed in developing seeds, similarly to those of enzymes involved in storage starch biosynthesis (Kawasaki *et al*. 1993). *In situ* hybridization indicated that the SPK gene was exclusively expressed in the endosperm where a large amount of storage starch is accumulated (Fig. 1A). To determine the function of SPK, we created transgenic rice plants containing the antisense SPK gene. The transformant showed no obvious change in morphology during the vegetative stage and no difference in the timing of heading or flowering compared with the wild-type. No difference was shown in the amount of chloroplastic starch in the leaves.

However, the process of seed development was quite different in the transformants from that of the wild-type plants. Together with a delay in seed development, a defect in starch accumulation was evident in most immature seeds from the antisense transformants, and seeds from the transformants were filled with watery sap in which a large amount of sucrose is contained, whereas the immature seeds of the wild-type plants contained an abundance of starch in starch granules together with a small amount of sucrose, fructose and glucose at 2-weeks after flowering. This fact indicates that sucrose metabolism was hardly repressed in the watery seeds of the antisense transformants.

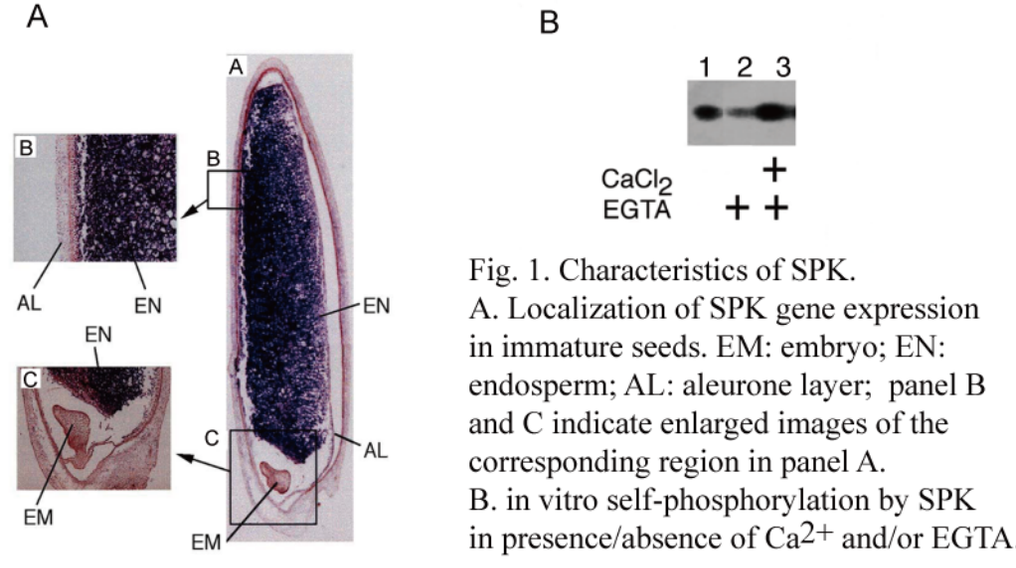

Fig. 1. Characteristics of SPK.
A. Localization of SPK gene expression in immature seeds. EM: embryo; EN: endosperm; AL: aleurone layer; panel B and C indicate enlarged images of the corresponding region in panel A.
B. in vitro self-phosphorylation by SPK in presence/absence of Ca^{2+} and/or EGTA.

To further elucidate the biochemical properties of SPK, a fusion protein consisting of glutathione-S-transferase (GST) followed by SPK was prepared. The purified GST-SPK fusion protein showed autophosphorylation activity. This activity was strongly inhibited by staurosporine, a broad-range protein kinase inhibitor, reduced in the presence of ethyleneglycol-bis-(b-aminoethylether)-N,N,N,N,-tetraacetic acid (EGTA) and restored upon addition of excess Ca^{2+} (Fig. 1B). Since no protein kinase activity was detected in assays examining the GST protein, it appears that the SPK has an activity as a CDPK.

Sucrose is a major end-product of photosynthesis and is transported into sink organs through the phloem. In the sink organs, sucrose not only plays a central role in plant growth and

development, but is also important as a starting material for the production of storage substances (Winter and Huber 2000). The phenotypic traits displayed by the antisense transformants strongly suggest that SPK is involved in regulation of the metabolic pathway from sucrose to storage starch in immature seeds.

Sucrose synthase catalyzes the initial step in storage-starch biosynthesis (Chourey *et al*. 1998). In maize leaves, sucrose synthase is phosphorylated by a CDPK to enhance its sucrose degradation activity (Huber *et al*. 1996). Rice has three isozymes of sucrose synthase, *Rsus1* to *Rsus3* (Su 1995). We prepared GST-sucrose synthase fusion proteins using artificial genes containing each of full-length cDNAs of the rice sucrose synthase genes, and examined them to be phosphorylated by SPK. As shown in Fig. 2A, the GST-sucrose synthase fusion protein was selectively phosphorylated by GST-SPK *in vitro*.

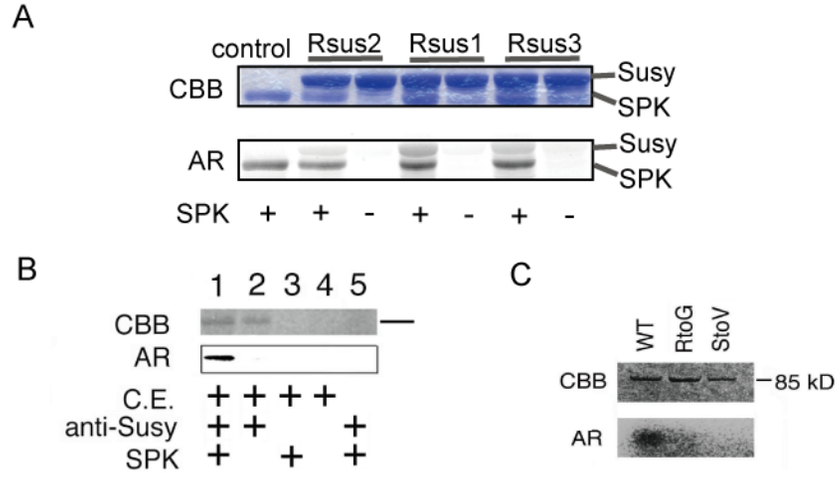

Fig. 2. Phosphorylation of susrose synthase by SPK.
A. *in vitro* phosphorylation of rice sucrose synthase isozymes. Each sucrose synthase was subjected to phosphoryation using γ-^{32}P-ATP by SPK. Upper panel shows the image of SDS-polyacrylamide gel electrophoresis by CBB staining (CBB). Lower panel shows the corresponding autoradiogram (AR). Reactions with/without SPK are indicated below the panel. Predicted molecuar sizes of sucrose synthases and SPK are indicate in the right. B. Phosphorylation of immunoprecipitated sucrose synthase (Rsus2). Reactions with/without crude extract of endosperm (C. E.), sucrose synthase antibody (Anti-Susy) and SPK (SPK) are indicated below the panel. C. Phosphorylation of mutated sucrose synthase (Rsus2). WT: wild-type sucrose synthase; RtoG: mutant protein with substitution of arginine to glycine in the consensus sequence; StoV: mutant with serine to valine in the consensus sequence..

When *in vitro* phosphorylation by the GST-SPK fusion protein was performed with a crude extract from an immature rice seed, four proteins of approximately 110, 85, 68 and 55 kDa in size, were principally phosphorylated. The sizes of the 85-kDa and 68-kDa proteins correspond to those of sucrose synthase and SPK, respectively. Immunoprecipitation of a crude extract of immature rice seeds using anti-GST-sucrose synthase antibody indicated that the 85-kDa protein was specifically phosphorylated *in vitro* by SPK (Fig. 2B). Thus, these results indicate that sucrose synthase is a potential target of SPK that is phosphorylated in immature seeds.

The consensus sequence recognized by serine/threonine protein kinases, RXXS, is conserved in rice sucrose synthases in their N-terminal regions, which is suggested to be required for

their activity (Huber *et al*. 1996). To identify the phosphorylation site in the rice sucrose synthase, site-directed mutagenesis was carried out. We prepared mutant proteins in which the arginine residue and the serine residue in the consensus sequence were substituted with a glycine residue and a valine residue, respectively. *In vitro* phosphorylation demonstrated that no phosphorylation occurred on these mutants, whereas the wild-type protein was efficiently phosphorylated (Fig. 2C). These results indicate that SPK may recognize the sequence RXXS in sucrose synthase and specifically phosphorylate the serine residue at this site. It also appears that SPK may serve to activate sucrose synthase in immature seeds.

Carbohydrates are synthesized by photo-assimilation in source organs and transported through sieve tubes into non-photosynthetic sink organs such as fruits, seeds, roots and tubers. Sucrose is a major compound transported to the sink organs through the phloem. During seed development, activity for the biosynthesis of storage starch is largely enhanced. In response to the increased requirement of sucrose in immature seeds, a large amount of sucrose is supplied through the phloem (Hayashi and Chino 1990).

SPK may function *in vivo* to activate the sucrose synthase in immature seeds. Indeed, we found that defect of SPK results in accumulation of a large amount of sucrose instead of storage starch. We also observed that sucrose flux in the phloem was also reduced (Asano *et al*. 2002; Shimada *et al*. 2004). These observations support the view that SPK may play a pivotal role *in planta* in the biosynthesis of storage starch.

The existence of several isoforms of sucrose synthase has been reported in many plant species. In rice, there are three known isoforms of sucrose synthase, *Rsus1*, *Rsus2* and *Rsus3*, are highly homologous. Among them, *Rsus1* and *Rsus3* are specifically expressed in aleurone layer and endosperm, respectively, whereas *Rsus2* is uniformly expressed (Wang *et al*. 1999). Sucrose synthase usually functions as the major enzyme that catalyzes the reversible conversion of sucrose in the presence of UDP to UDP-glucose and fructose (Weber *et al*. 1996). Recently, it has been suggested that this enzyme may catalyze the reaction for the conversion of sucrose and ADP to fructose and ADP-glucose (Baroja-Fernández *et al*. 2003).

We determined the effect of phosphorylation on these sucrose synthase activities. To examine this, the fusion proteins, GST-Rsus1, GST-Rsus2 and GST-Rsus3, were prepared. We confirmed that these proteins were phosphorylated by SPK. After the portion of GST was removed from these proteins, the sucrose synthase activity was determined *in vitro*. The activities of sucrose synthesis and cleavage reactions were determined by measurement of raised UDP and fructose according to Bieniawska *et al*. (2007). The sucrose synthase reaction accompanied with ADP was also analyzed. The activities of sucrose synthesis and cleavage were determined by measurement of raised fructose and ADP by the similar way. Activities on sucrose synthase with/without phosphorylation were compared.

On the reaction accompanied with UDP and UDP-glucose, it was observed that the K_m values and catalytic efficiencies of three isozymes of sucrose synthases were little altered after phosphorylated (Table 1). This result indicates that phosphorylation of sucrose synthase poorly affected the reaction accompanied with UDP and UDP-glucose. Regarding the activity with ADP and ADP-glucose, all of three sucrose synthases showed apparent activities of both sucrose cleavage and synthesis, although the activity was lower than those with UDP on average. When they were phosphorylated, the K_m values of sucrose cleavage reactions were significantly decreased along with increase of their catalytic efficiencies. This result indicates that the activity of sucrose cleavage with ADP was remarkably enhanced by phosphorylation, whereas the activity of sucrose synthesis was slightly altered (Table 1). Thus, it appears that phosphorylation of sucrose synthase greatly effects enhancement of the sucrose cleavage reaction accompanied with ADP.

Our results demonstrate that SPK promotes the efficiency of generation of ADP-glucose

from sucrose in endosperm as the fruit of phosphorylation of sucrose synthase. Storage starch is mainly produced from ADP-glucose (Martin and Smith 1995). Therefore, it is suggested that this reaction greatly contributes the supply of substrate for storage starch biosynthesis, and that loss of its function results in reduction of storage starch biosynthesis.

Table 1. Kinetic constants of sucrose synthase isozymes on the reactions accompanied with UDP or ADP.

		sucrose synthesis				sucrose cleavage			
		fru + UDP-glu —› suc + UDP		fru + ADP-glu —› suc + ADP		suc + UDP —› fru + UDP-glu		suc + ADP —› fru + ADP-glu	
	SPK	$Km^{UDP\text{-}G}$	*Kcat/Km*	$Km^{ADP\text{-}G}$	*Kcat/Km*	Km^{UDP}	*Kcat/Km*	Km^{ADP}	*Kcat/Km*
		(mM^{-1})		(mM^{-1})		(mM^{-1})		(mM^{-1})	
Rsus1	-	0.27	389	2.3	25	171	0.7	202	0.04
	+	0.39	271	7.8	18	112	0.6	110	0.07
Rsus2	-	0.33	336	3.1	22	62	1.3	1922	0.05
	+	0.32	409	5.3	15	86	1.1	61	0.13
Rsus3	-	0.33	66	4.4	4.6	111	0.4	572	0.04
	+	0.24	119	5.7	6.1	64	1.0	80	0.10

Reactions with/without phosphorylation are compared. "SPK" indicates with/without phosphorylation prior to sucrose synthase reaction. suc: sucrose; fru: fructose; glu: glucose; *Kcat/Km* indicates catalytic efficiency ($min^{-1}mM^{-1}$).

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