Ser162-Dependent Inactivation of Overproduced Sucrose-Phosphate Synthase Protein of Maize Leaf in Transgenic Rice Plants

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To investigate the role of Ser162 in phosphorylation-dependent regulation of maize sucrose-phosphate synthase (SPS) activities in rice, transgenic rice plants expressing wild-type or mutagenized maize SPS were produced. Our results indicate that Ser162 was responsible for overproduction-induced inactivation of SPS protein and for light/dark modulation of this protein in vivo.

Key words: Phosphorylation — Rice (Oryza sativa L.) — Sucrose-phosphate synthase (EC 2.4.14) — Transgenic plant.

Sucrose-phosphate synthase (SPS; EC 2.4.14) is a key regulatory enzyme of sucrose synthesis (Huber and Huber 1996). SPS activity is under complex regulation, involving allosteric effectors (glucose-6-phosphate; Glc6P and P) and site-specific, reversible seryl phosphorylation (Huber and Huber 1996). Two regulatory phosphorylation sites have been identified in spinach leaf SPS. Phosphorylation of Ser158, which corresponds to Ser162 in maize leaf SPS, inactivates the enzyme in the dark (Huber et al. 1995, McMichael et al. 1993). Phosphorylation of Ser424 activates the enzyme in response to osmotic stress (Toroser and Huber 1997). As these Ser residues are conserved among a number of higher plant species, it has been speculated that the regulation of SPS activity by the phosphorylation of these sites might be widespread among higher plants (Huber and Huber 1996, Toroser and Huber 1997).

SPS is phosphorylated or inactivated in the dark by several Ser/Thr kinases, and it is dephosphorylated or activated in the light by type 2A protein phosphatase (Huber and Huber 1996). In light/dark modulation, spinach and maize leaf SPSs show alteration in their affinity for substrates (UDPGlc and Fru6P) and allosteric effectors, but no change in maximum enzyme activity (Vmax; Lunn and Hatch 1997, Stitt et al. 1988). To assess the activation state of SPS, ‘non-selective’ assay conditions (assayed with saturating substrates minus P) and ‘selective’ assay conditions (assayed with limiting substrates plus P) have been widely used (Stitt et al. 1988). However, these assay conditions are chosen based on the properties of spinach leaf SPS only, and hence they might not be suitable for other SPSs with different kinetic properties from those of the spinach enzyme; uncritical use of these assay conditions for the SPSs from other species might mislead in the evaluation of the nature and degree of activation of SPS (Galtier et al. 1995, Lunn and Furbank 1999). For the assessment of the activation state of maize SPS, the use of Km for UDPGlc, instead of non-selective- and selective-activities has been proposed (Lunn and Hatch 1997, Stitt et al. 1988).

Phosphorylation-dependent inactivation of SPS proteins has been observed even in the light when the proteins are overproduced in transgenic plants (overproduction-induced inactivation; Galtier et al. 1995, Ono et al. 1999, Toroser et al. 1999). We previously showed that the affinity of SPS for UDPGlc decreased with the increase in Vmax in light-harvested leaves of transgenic rice plants expressing maize leaf SPS (Ono et al. 1999). A similar phosphorylation/inactivation phenomenon has been observed in transgenic tomato plants expressing maize leaf SPS (Galtier et al. 1995). As Ser158 in spinach leaf SPS (or Ser162 in maize leaf SPS) is the only regulatory site that is known to be involved in the inactivation of this enzyme, this phosphorylation site was suspected to be responsible for the inactivation of overproduced SPS proteins. This speculation led to a proposal that removal of this phosphorylation site could eliminate the inactivation (Stitt and Sonnewald 1995). Recently, Toroser et al. (1999) performed Ser158-to-Ala mutagenesis of spinach leaf SPS. They showed that Ser158 was responsible for the light/dark modulation of the enzyme in transgenic tobacco plants. They also showed that Ser158 was responsible for the light-independent, overproduction-induced inactivation.
of SPS proteins, although their results were unstable. Thus, the role of Ser158 in the overproduction-induced inactivation of spinach leaf SPS still remains to be clarified.

Sucrose translocation is a critical factor for the yield of rice. Previous investigations suggested that the elimination of phosphorylation-dependent inactivation of SPS could modify carbon partitioning, thereby improving the sucrose translocation. However, the effect of the removal of the phosphorylation site on SPS activity is still controversial. Moreover, the effects of constitutive activation of SPS on carbon partitioning are presumably different from species to species depending on metabolic backgrounds. To address these issues, we generated transgenic rice plants overproducing wild-type (WT) or Ser162-to-Ala mutagenized (S162A) maize SPS protein. For transgenic rice plants, we used a more accurate assessment of the SPS activities in transgenic Ser162-to-Ala mutagenized (S162A) maize SPS protein. For transgenic rice plants overproducing wild-type (WT) or metabolic backgrounds. To address these issues, we generated are presumably different from species to species depending on carbon partitioning, thereby improving the sucrose translocation of carbon partitioning in leaves.

Site-directed mutagenesis was performed by PCR. A BamHI/EcoRI fragment of maize leaf SPS cDNA (Ono et al. 1999) was subcloned into pBluescriptIISK+ (Strategene, La Jolla, CA, U.S.A.) and used as a template for the first round PCRs. The following primer sets were used for the first round PCRs: (1) SPS-S162AaF1, 5'-CGGTAGGTGCAGCGAAGTTCCTC-3', and M13 reverse primer 5'-GAGGAACTTCGCTGACCTTACCG-3; and (2) SPS-S162AbF1, 5'-GAGGAACTTCGCTGACCTTACCG-3', and M13 –20 primer 5'-GTAAAACGACGGCCAGT-3'. Products of the 2 PCRs were mixed and used as a template for the second round PCR. M13 reverse primers and M13 –20 primers were used for the second round PCR. All PCRs were performed for 15 cycles at 99°C for 20 s, 42°C for 1 min, and 72°C for 1 min. Products of the second PCR were digested with BamHI and Apal and subcloned into pBluescriptIISK+. To verify the amino acid change and to exclude additional mutations, the entire cDNA insert of the resulting plasmid was subjected to sequence analysis. After verification, a KpnI/BsrGI fragment was recloned into cDNA clone SPS14, yielding a complete maize leaf SPS coding region carrying the Ser162-to-Ala exchange.

A binary vector, pBE2113-GUS-Hygro, was constructed by inserting the hygromycin phosphotransferase gene downstream of the CaMV 35S promoter in pBE2113-GUS (Mitsuhara et al. 1996). To allow the expression of maize leaf SPS in transgenic plants, Apal (filled in)/SacI fragments of either unmodified or mutated SPS cDNAs were placed downstream of a HindIII/BamHI (filled in) fragment of rice chlorophyll a/b-binding protein promoter (photosynthetic tissue-specific promoter; Tada et al. 1991). The resulting DNA fragments were inserted at the HindIII/SacI site to replace the β-glucuronidase gene downstream of the CaMV 35S promoter in pBE2113-GUS-Hygro. These steps yielded pMPS (unmodified SPS) and pMPS-S162A (mutated SPS) constructs.

Agrobacterium-mediated transformation of rice (Oryza sativa L. cv. Nipponbare) was performed essentially as described by Toki (1997) using the two constructs. Hygromycin-resistant plants were regenerated, then maize-SPS-producing plants were selected by Western blotting experiments using maize-leaf-SPS-specific antibody (Ono et al. 1999, data not shown). Regenerated plants having no transgene were selected by PCR (data not shown) and used as untransformed control plants.

For subsequent experiments, three lines each of independent transformants expressing WT or S162A maize leaf SPS and one untransformed plant were selected and transferred to a greenhouse for seed production. Seeds of primary transformants were allowed to germinate in soil in 1/5,000 Wagner pots, and seedlings were grown in a naturally illuminated glasshouse maintained at 28°C day/24°C night. Fully expanded mature leaves were taken from 3-month-old plants at 14:00 h (light) or 22:00 h (dark; 4 h after sunset) and used for all experiments.

SPS activity was assayed as described by Ono et al. (1999). One hundred mg of samples were ground with a mortar and pestle and extracted with 800 μl of ice-cold buffer [50 mM HEPES-KOH (pH 7.5), 10 mM MgCl2, 1 mM EDTA, 1 mM ethylene glycol-bis(β-aminoethylether)-N,N,N',N’-tetraacetic acid, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 mM β-aminocaproic acid, 0.5% (v/v) bovine serum albumin, 0.1% (v/v) Triton X-100, 2% (w/v) polyvinylpyrroldione]. After centrifugation at 10,000 x g for 5 min, the supernatant was desalted by passage through a column of Sephadex G-25 (Pharmacia, Uppsala, Sweden). SPS activity was assayed by measuring the Fru6P-dependent production of UDP from UDPGlc (Lunn and ap Rees 1990). The reaction mixture (100 μl) contained 50 mM HEPES-KOH (pH 7.5), 10 mM MgCl2, various concentrations of UDPGlc (from 1–20 mM to 10–160 mM; five concentrations were selected for each sample), 8 mM Fru6P, 27.6 mM Glc6P and 20 μl of extract. A small portion (50 μl) of crude extract was taken for chlorophyll determination in 80% acetone (Arnon 1949). The apparent V_max and the K_m for UDPGlc were calculated from a Hanes plot.

Sucrose, starch and hexose (glucose and fructose) contents were measured enzymatically as described by Tobias et al. (1999).

In our previous transgenic rice plants, overproduced SPS proteins were inactivated in the light (their affinity for UDPGlc was reduced), while V_max remained high (Ono et al. 1999). To examine whether such inactivation occurred in this system, we analyzed the relationship between V_max and K_m for UDPGlc in light-harvested leaves from transgenic rice plants expressing WT maize leaf SPS (WTmaize plants, Fig. 1). In these plants, K_m clearly increased with the increase in V_max (Fig. 1). This indicates that the overproduced SPS proteins were inactivated. By contrast, only a small increase in K_m was observed in light-harvested leaves from transgenic rice plants expressing S162A maize leaf SPS (S162Am1 plants, Fig. 1). These results...
Overexpression of maize S162A SPS in rice clearly show that Ser162 is responsible for the overproduction-induced inactivation of the maize leaf SPS proteins in transgenic rice plants.

As described above, Ser162 is known to be responsible for light/dark modulation of maize leaf SPS. Thus, we analyzed the effect of the S162A mutation on the light/dark modulation of enzyme activity. Measurement of SPS activities revealed no difference in $V_{\text{max}}$ between light- and dark-harvested leaves in both the WT$_{\text{Zm}}$ and S162A$_{\text{Zm}}$ plants, or in maize and rice control plants (Fig. 2A). Meanwhile, the $K_{\text{m}}$ values for UDPGlc were found to be higher in the dark in maize and rice control plants, showing typical light/dark modulation of SPS activity. By contrast, the $K_{\text{m}}$ values in S162A Zm plants were constantly low, regardless of light conditions (Fig. 2B). This indicates that S162A SPS was insensitive to dark inactivation. These results indicate that Ser162 of maize leaf SPS, which corresponds to Ser158 of spinach leaf SPS, is responsible for the light/dark modulation in vivo. In WT$_{\text{Zm}}$ plants, the $K_{\text{m}}$ values were constantly high, regardless of light conditions (Fig. 2B). This indicates that the overproduction-induced inactivation of SPS proteins is light independent.

To examine the effects of the overproduction of WT or S162A maize leaf SPS on leaf carbon partitioning of transgenic rice plants, we measured sucrose, starch and hexose (glucose and fructose) contents using the same light-harvested leaves as used for the measurement of SPS activity (Table 1). Unexpectedly, we found no change in the contents of sucrose, starch, and sucrose/starch ratio in both the WT$_{\text{Zm}}$ and S162A$_{\text{Zm}}$ plants, despite increased SPS $V_{\text{max}}$ (Table 1). We also found no change in the contents of hexoses; they were very low (less than 2 $\mu$mol (g FW)$^{-1}$, data not shown) in all the plants.

Our transgenic studies indicate that the overproduced maize leaf SPS proteins in rice plants were inactivated independent of light, and that Ser162 was responsible for the inactivation. Similar Ser158-dependent inactivation has been reported for spinach leaf SPS overproduced in transgenic tobacco plants (Toroser et al. 1999); in this case, however, the inactivation was poorly reproducible. In this regard, our results for maize SPS overproduction in rice indicate that the inactivation (increase in $K_{\text{m}}$) of SPS activity was promoted as $V_{\text{max}}$ increased. Considering these observations, the poor reproducibility of the inactivation observed for spinach leaf SPS in transgenic tobacco plants is presumably due to the low $V_{\text{max}}$ of SPS activity in those transgenic tobacco plants. Thus, the overproduction-induced inactivation of SPS, which is Ser162/Ser158-dependent, seems to be a widespread phenomenon among plant species.

Inactivation of overproduced SPS proteins was not completely abolished in the S162A$_{\text{Zm}}$ plants (Fig. 1, 2B). Presumably, heterologous maize S162A SPS does not work properly under physiological cellular conditions in rice, irrespective of phosphorylation. Alternatively, Ser162-to-Ala mutation might change the kinetic property of the enzyme. Otherwise, phos-
phorylation of the ‘osmotic-stress-responsible’ regulatory site or non-regulatory sites of S162A maize SPS could also be responsible for the inactivation. Although the non-regulatory sites have not been assigned play a significant role in the regulation of SPS activity, it has recently been shown that phosphorylation of non-regulatory Ser229 of spinach leaf SPS, followed by binding of 14-3-3 protein, leads to inactivation of the enzyme in vitro (Toroser et al. 1998).

Overproduced WT maize SPS, as well as maize S162A SPS, in transgenic rice plants was insensitive to dark inactivation (Fig. 2B). Our results indicated that the overproduction-induced light-independent inactivation involves the phosphorylation of the same Ser residue as that responsible for the light/dark regulation of SPS. Thus, the transgene induced regulatory mechanism appears to have masked the inherent light/dark regulation of SPS.

Despite the very high SPS \( V_{\text{max}} \) in some transgenic rice plants, carbon partitioning in leaves did not change in either \( \text{WT}_{\text{Zm}} \) or \( \text{S162A}_{\text{Zm}} \) plants (Table 1). This is inconsistent with the results of our previous experiment, in which we found a positive correlation between the SPS \( V_{\text{max}} \) and the sucrose/starch ratio in \( \text{WT}_{\text{Zm}} \) plants (Ono et al. 1999). These conflicting results may be due to a difference in the stages of leaves used in the two experiments: we used mature source leaves at the vegetative growth stage in this experiment but flag leaves at the heading stage in the previous experiment. Perhaps, increased rates of sucrose synthesis were matched by increased sucrose export from the leaf, then a significant change in carbon partitioning could be missed by just measuring contents of sucrose, starch and hexose in the leaf. Determination of the export rates of photoassimilates using \( ^{13} \text{C} \) might help evaluate accurate effects of overproduction of WT and S162A maize SPS in rice.

In this paper, we showed that overproduction-induced inactivation of SPS can be suppressed by the elimination of regulatory phosphorylation site (Ser162). Although the effect of overproduction of mutagenized SPS protein on the carbon partitioning remains unclear, the properties of SPS in transgenic plants revealed in this study should be considered in any future genetic engineering of rice and other crops.

![Table 1](https://example.com/table1.png)

<table>
<thead>
<tr>
<th>Plant type</th>
<th>SPS ( V_{\text{max}} ) (( \mu \text{mol (mg Chl)}^{-1} \text{h}^{-1} ))</th>
<th>Carbohydrate content (( \mu \text{mol hexose (g FW)}^{-1} ))</th>
<th>Sucrose/starch ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sucrose</td>
<td>Starch</td>
</tr>
<tr>
<td>Control</td>
<td>41.9 ± 1.1</td>
<td>104.1 ± 9.1</td>
<td>9.2 ± 2.0</td>
</tr>
<tr>
<td>Untransformed</td>
<td>34.8 ± 1.0</td>
<td>106.7 ± 14.7</td>
<td>8.0 ± 2.4</td>
</tr>
<tr>
<td>( \text{WT}_{\text{Zm}} )</td>
<td>590.6 ± 32.8</td>
<td>113.6 ± 8.8</td>
<td>10.9 ± 2.7</td>
</tr>
<tr>
<td>( \text{S162A}_{\text{Zm}} )</td>
<td>524.1 ± 9.0</td>
<td>109.2 ± 7.2</td>
<td>8.8 ± 1.7</td>
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Values are mean ± standard error of the results from three different plants harvested in the light. The leaves used for these carbohydrate measurements were the same as those used for measurements of SPS activity shown in Fig. 1.

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