Note
Tryptophan Boost Caused by Senescence Occurred Independently of Cytoplasmic Glutamine Synthetase

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We examined to determine whether senescence-induced tryptophan levels are positively associated with levels of glutamine synthetase (GS1), the initial enzyme in tryptophan biosynthesis. We generated transgenic rice plants in which GS1 was suppressed by RNA interference technology. The transgenic line showed a dramatic decrease in GS1 protein and glutamine content, but the levels of tryptophan and mRNA of the key tryptophan biosynthetic genes upon senescence were comparable to those of the wild type.

Key words: glutamine synthetase; RNA interference; transgenic rice; tryptophan biosynthesis

The use of tryptophan is increasing worldwide due to increased demand for it, as a medicine and food additive. It has various biological effects in humans and other animals, including sleep-inducing, antidepressant, and calmative activities.1 Its biosynthetic pathway has been well studied in various organisms, and there are many reports on its production in microbes or plants through metabolic engineering.2,3 In plants, tryptophan biosynthesis is strictly controlled; anthranilate synthase, the first committed enzyme, is feedback-inhibited by micromolar concentrations of tryptophan, resulting in low levels of soluble tryptophan.4 Two recent studies found that feedback inhibition of tryptophan biosynthesis via anthranilate synthase is disarmed when plants are subjected to pathogen infection or senescence.5,6 For example, upon senescence, rice leaves begin to accumulate tryptophan at up to 1.6 mg per g fresh weight (fw) in parallel with the induction of anthranilate synthase transcripts.6

Glutamine synthetase (GS) catalyzes the condensation of NH4+ with glutamate to yield glutamine, and is found as various isozyme forms, of which GS1 is located in the cytosol and GS2 in the plastids. In senescing leaves, GS1 is induced and is responsible for the synthesis of glutamine, the major form of nitrogen used in remobilization into developing sink organs. The synthesis of glutamine in senescing organs is an essential step in nitrogen recycling.7 In addition to its major role in nitrogen remobilization, the increased glutamine levels in senescing organs may also play a role in the induced synthesis of tryptophan by providing excess substrate for anthranilate synthase, which requires two substrates: glutamine and chorismate.8 In this study, we examined the possible role of GS1 in tryptophan synthesis in senescence by generating transgenic rice plants in which GS1 expression was inhibited by RNAi technology.

Transgenic rice plants expressing the rice RNAi GS construct were generated by transforming wild-type rice (Oryza sativa cv. Dongjin) with the binary vector pANDA:RNAi GS1 (Fig. 1A).9 The transgene was under the control of the constitutive maize ubiquitin promoter. The transgenic cells were selected in the presence of hygromycin, because T-DNA carries the hygromycin phosphotransferase gene as a selectable marker. Six independent transgenic lines (T0) were generated and transferred to a field for the production of T1 seeds. Initially, the T1 transgenic rice seeds were screened in half-strength Murashige and Skoog medium containing hygromycin (50 mg/l) to check for the presence of the selectable marker gene. All seedlings resistant to hygromycin were selected, and were grown for 3 weeks. To determine whether the transgenic plants had suppressed levels of GS protein, we measured GS protein levels in healthy rice leaves by Western blot. In the plants, two types of GS are expressed: GS1 is a 40-kDa cytosolic protein, and GS2 is a 44-kDa chloroplast protein. They share 74% amino acid identity. As shown in Fig. 1B, the relative levels of GS2 protein in all the transgenic rice plants were similar to that found in the wild type, whereas GS1 protein levels varied significantly among the transgenic lines. In lines 1, 5, and 6, GS1 protein levels were not altered as compared to the wild type, while lines 3 and 4 did not produce detectable levels of GS1 protein, suggesting that RNAi functioned efficiently in these transgenic lines. Line 2 expressed a low level of GS1 protein, suggestive of successful suppression of GS1 by RNAi. The results of Western blot analysis clearly suggest that RNAi interference silenced the GS1 protein specifically (not the GS2 protein). To determine whether the suppression of GS1...
proteins was coupled with decreased levels of GS enzyme activity. We measured GS enzyme activity in both wild-type and transgenic lines. GS activity was measured in healthy leaves of wild-type and transgenic lines by a simple spectrophotometry method, as described elsewhere. GS enzyme activity was lower in line 2 and line 4, whereas line 1 was similar to wild type (Fig. 1C). This was closely consistent with the data observed GS protein levels.

The transcripts were analyzed by semi-quantitative RT-PCR to confirm that the RNAi GS functioned efficiently at the level of GS transcripts. GS1, expressed in the cytoplasm, consists of four isoenzymes that share amino acid identities of more than 80%. In contrast, GS2 is a single-copy gene expressed in chloroplasts. As for the senescence treatment, a group of 10 segments from 3-week-old rice leaves were transferred into a 50-ml polypropylene conical tube containing 10 ml of water without nutrients and were incubated for indicated durations. Upon senescence, tryptophan biosynthetic genes, such as anthranilate synthase (AS2) and tryptophan synthase (TS2), were induced as control genes for senescence-inducible markers (Fig. 1D). In addition, GS transcripts were differently regulated in the wild-type rice leaves. GS1-1 and GS2 were constitutively expressed in the wild-type leaves, whereas GS1-2 and GS1-3 were induced upon senescence. GS2 expression was suppressed when the rice leaves underwent senescence. Compared to the wild type, the GS1-RNAi transgenic lines showed decreased levels of GS transcripts, and all the GS1 isogenes were significantly suppressed, while GS2 expression was not suppressed. By contrast, the GS2 mRNA level of G4 at day 0 appeared to be rather higher than that of the wild type. The reason for this is not known. These data suggest that the GS1 RNAi transgene interfered specifically with the GS1 family, not with GS2. The absence of GS2 suppression in the GS1 RNAi transgenic rice plants is due to the relatively low level of sequence identity between the GS1 and GS2 gene.

Glutamine was measured using an L-glutamine Rapid Kit (Megazyme International Ireland, Wicklow, Ireland). Glutamine levels increased in both the wild-type and the transgenic lines upon senescence, but the fold increases were far less in the transgenic lines than in the wild type (Fig. 2A). To determine whether the transgenic lines suppressing GS1 protein expression show altered levels of tryptophan production upon senescence treatment, T1 transgenic rice leaves (lines 1, 2, and 4) detached from 3-week-old rice seedlings were subjected to senescence treatment as above, and the resulting samples were analyzed for tryptophan levels by HPLC. As shown in Fig. 2B, the tryptophan levels in the wild type increased dramatically on day 6, and peak levels of around 1.5 mg per g fw were found on day 8. Transgenic lines 1, 2, and 4 showed comparable levels of tryptophan regardless of the severity of GS1 suppression. In addition, the expression levels of AS2 and TS2 in the transgenic rice lines upon senescence were almost identical to those detected in the wild-type leaves (Fig. 1D). This implies that the induced synthesis of tryptophan in the

**Table 1. Primers Used in Semi-Quantitative RT-PCR Analysis in Rice Organs**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
<th>PCR cycle</th>
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<tr>
<td>AS2</td>
<td>5'-CACGGTGATGAGGAGGATTGG-3'</td>
<td>5'-GTTCTCGCATTCAGGCTTG-3'</td>
<td>25</td>
</tr>
<tr>
<td>T2r</td>
<td>5'-TGAGCTGCTGCTGGGCTG-3'</td>
<td>5'-TATACGACAAACACAGC-3'</td>
<td>25</td>
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<tr>
<td>GS1-1</td>
<td>5'-GAAACCTGAGGCTGCTGATC-3'</td>
<td>5'-CACCCAGTGGGCTGCTGAC-3'</td>
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</tr>
<tr>
<td>GS1-2</td>
<td>5'-CGGCTGGAGGACCAAGGAG-3'</td>
<td>5'-GGACGCTGCTTTCAGCCACA-3'</td>
<td>25</td>
</tr>
<tr>
<td>GS1-3</td>
<td>5'-GCATGGCGACCAAGGAG-3'</td>
<td>5'-CGACTCTTCCTCCATGGC-3'</td>
<td>28</td>
</tr>
<tr>
<td>GS2</td>
<td>5'-GGGAGGGGAGGGGAGGG-3'</td>
<td>5'-GCTGGGGGAGGGGAGGG-3'</td>
<td>28</td>
</tr>
<tr>
<td>UBQ5</td>
<td>5'-CCGACTACAACATCCAGAAGGAG-3'</td>
<td>5'-AACGGGAGCTGCTACCAGC-3'</td>
<td>25</td>
</tr>
</tbody>
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transgenic line is preceded by induction of key biosynthetic genes, as in the wild type, and this suggests that GS1 is not involved in the induction of tryptophan synthesis upon senescence. Although GS1 was induced in parallel with tryptophan biosynthetic genes upon senescence, GS1-mediated glutamine synthesis in senescing leaves did not affect for tryptophan synthesis, indicating that the glutamine boosted in senescing leaves is destined exclusively for the export of leaf nitrogen via the phloem toward developing leaves.

In conclusion, GS1 induction upon senescence is not metabolically coupled with the induction of tryptophan synthesis. This is the first report to describe the uncoupled relationship between tryptophan boost and glutamine synthesis upon senescence.

Acknowledgments

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References