Endosperm-Specific Expression of Serotonin N-Hydroxycinnamoyltransferase in Rice

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Abstract Serotonin N-hydroxycinnamoyltransferase (SHT) is a key enzyme in the synthesis of feruloylserotonin (FS) and 4-coumaroylserotonin (CS). These serotonin derivatives show strong antioxidant activity, making them valuable for both nutritional and pharmacological use in humans. Ectopic expression of SHT under the control of the endosperm specific-glutelin and prolamin promoters from rice was produced via Agrobacterium-mediated transformation. SHT expression was confirmed by Southern blot analysis, followed by Northern blotting and SHT enzyme activity analyses using total RNA and protein, respectively, extracted from transgenic seeds. The glutelin A3 (GluA3) promoter produced low SHT mRNA expression in rice seeds, whereas the prolamin promoter expressed high levels of SHT mRNA. In spite of the ectopic expression of SHT in rice seeds, both transgenic genotypes accumulated levels of serotonin derivatives similar to those found in wild-type rice. Furthermore, our data suggest that serotonin, rather than phenylpropanoid-CoAs, is the rate-limiting substrate in the biosynthesis of serotonin derivatives in SHT-overexpressing transgenic rice seeds.

Keywords 4-coumaroylserotonin · Endosperm-specific promoter · Feruloylserotonin · Serotonin N-hydroxycinnamoyltransferase · Transgenic rice

Introduction

Serotonin derivatives such as feruloylserotonin (FS) and 4-coumaroylserotonin (CS) are present in the seeds of various plants [1–4], in the twigs of diseased bamboo [5], and in pepper flowers [6]. Serotonin derivatives have various beneficial health effects, including antioxidant activity [7] and antitumor activity [8]. In plants, serotonin derivatives are believed to act as phytoalexins in defense against plant pathogens [4, 5]. The synthesis of serotonin derivatives is catalyzed by serotonin N-hydroxycinnamoyltransferase (SHT) using various phenylpropanoid-CoA thioesters and aromatic amines as substrates [9]. The first SHT-transgenic plant was generated by introducing the pepper SHT gene into the rice genome [10]. Ectopic expression of the pepper SHT gene under the control of the constitutive maize (Zea mays) ubiquitin promoter resulted in a nine-fold increase in the level of serotonin derivatives in transgenic rice seeds compared with wild-type seeds [11]. However, the levels of serotonin derivatives in ubiquitin-SHT transgenic seeds were too low to improve nutritional value. Owing to its ubiquitous expression [12], the maize ubiquitin promoter also drives transgene expression in seeds, including the embryo and endosperm, although its expression strength is significantly lower than that of seed-specific promoters derived from seed storage protein genes [13]. The promoters commonly used for seed-specific expression in rice come from the glutelin, globulin, and prolamin genes, which are major proteins in rice seeds [14].

As an initial work to produce transgenic rice with better nutritional value, we expressed the SHT gene under the control of the endosperm-specific glutelin A3 (GluA3) and prolamin promoters to determine whether seed-specific overexpression of SHT enhances the synthesis of serotonin derivatives.
Materials and Methods

Vector Construction and Agrobacterium-Mediated Rice Transformation

The maize ubiquitin promoter in this binary vector (pGA1611) was substituted with the GluA3 promoter [15] or the prolamin promoter (GenBank Accession no. D63901). To construct these vectors, the pepper SHT cDNA (GenBank Accession no. AF329463) and promoter regions were amplified using the appropriate primer sets (for SHT, 5′-ATCAAGCTTATGGCTTCTGCTCCTCAA-3′ and 5′-GTGGAGCTCCTAACAGCTTCCTGCACC-3′; for glutelin, 5′-AGTGGATCCAGAAGAAAGATAATAACC-3′ and 5′-GATAAGCTTAATGCTTTTGTGAGAAAT-3′; for prolamin, 5′-AGTGGATCCCTACATCGGCTTAGGTGT-3′ and 5′-TCAAAAGCTTAATTGTGGAAGGG), and were ligated into the pGA1611 binary vector as described previously [10]. The resulting construct featured the SHT gene between the endosperm-specific promoter and the nopaline synthase terminator. The pGA1611:GluA3-SHT and pGA1611:prolamin-SHT vectors were transformed into A. tumefaciens LBA4404. Rice transformation was performed as previously described [16].

Southern and Northern Blot Analyses

Five micrograms of genomic DNA digested with HindIII restriction endonuclease were used for Southern blot analysis. Total RNA (10 μg) was isolated from mature transgenic and wild-type rice seeds as previously described [10]. The DNA or RNA bands were hybridized with a radiolabeled SHT cDNA probe (Prime-It Kit; Stratagene).

Fig. 1 Schematic of the binary vector pGA1611 T-DNA used for plant transformation and molecular confirmation of transgene insertion. a Endosperm-specific expression of SHT under the control of the rice glutelinA3 (GluA3) promoter. b Endosperm-specific expression of SHT under the control of the rice prolamin promoter. GluA3, rice glutelin A3 promoter; SHT, serotonin N-hydroxycinnamoyltransferase; Tnos, nopaline synthase terminator; CaMV 35S, cauliflower mosaic virus 35S promoter; HPT, hygromycin phosphotransferase; TiA6-7, TiA6-7 terminator. e Southern blot analysis for GluA3-SHT transgenic lines. f Southern blot analysis for prolamin-SHT transgenic lines. WT Untransformed wild type; 1–19, transgenic lines (T1). e RT-PCR analysis of GluA3-SHT transgenic lines; M molecular weight marker (BstEII-digested Lambda DNA); U4 transgenic rice expressing SHT under the control of the maize ubiquitin promoter; 4–5, transgenic lines. f Northern blot analysis of prolamin-SHT transgenic lines. WT Untransformed wild type; 1–18 transgenic lines.
Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR analysis was performed with total RNA (2 μg) isolated from mature rice seeds expressing the GluA3-SHT transgene. First-strand cDNA was synthesized by using oligo (dT)\textsubscript{15} primers (Promega, Madison, WI, USA) and Super-Script III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The SHT and rice 18S actin transcripts were amplified by PCR using the appropriate primers.

SHT Enzyme Activity Measurement

Mature rice seeds were employed for enzyme assay. Sample preparation and assay conditions were followed as previously described [10].

Feruloylserotonin (FS) and 4-coumaroylserotonin (CS) Levels in Rice Seeds

The level of total serotonin derivatives (CS + FS) in T\textsubscript{2} mature transgenic rice seeds was quantified by HPLC as previously described [10].

Results and Discussion

Expression of Serotonin N-Hydroxycinnamoyltransferase in Transgenic Rice Plants

Two dozen independent transgenic lines (T\textsubscript{0}) transformed with GluA3-SHT or prolamin-SHT (Fig. 1a,b) were generated \textit{in vitro} by using the standard \textit{A. tumefaciens}-mediated rice transformation protocol [16] and then transferred to a greenhouse for the production of T\textsubscript{1} seeds. Among the GluA3-SHT lines, 12 transgenic T\textsubscript{1} lines were transgenic lines. d Feruloylserotonin (FS) plus 4-coumaroylserotonin (CS) content in seeds from prolamin-SHT transgenic lines (mean±SE, \(n=3\)). Seeds were dehusked, methanol-extracted, and subjected to HPLC. WT Untransformed wild type; 1–15 transgenic lines.
were detected in the GluA3-SHT lines. Line 4 expressed more SHT mRNA than line 5, but overall SHT expression was much greater in the ubiquitin promoter line. The level of RT-PCR product in seeds from the ubiquitin promoter line U4 was much higher than that from the GluA3-SHT lines. These data suggest that SHT mRNA was expressed at very low levels in seeds from the GluA3-SHT lines. In contrast, SHT mRNA was expressed abundantly in seeds from the prolamin-SHT lines (Fig. 1f). Transgenic SHT mRNA was detected in total RNA from the seeds of all T2 prolamin-SHT transgenic lines but not from the seeds of wild-type rice. Expression varied significantly among the transgenic lines: line 1 showed the highest expression, lines 2, 4, 5, and 16 showed moderate expression, and the remaining lines showed low expression. Two SHT-hybridizing bands were detected on the Northern blot. The lower band appeared to be a degradation product of SHT, suggesting that SHT mRNA was unstable in seeds from the prolamin-SHT transgenic lines.

**Measurement of SHT Enzyme Activity in Seeds**

SHT enzyme activity was measured in protein extracts from immature GluA3-SHT and prolamin-SHT T2 transgenic seeds. Among the GluA3-SHT lines, line 4 showed a two-fold increase in SHT activity compared with wild-type activity, whereas all other lines expressed SHT enzyme activity levels that were similar to or lower than wild-type activity (Fig. 2a). The levels of SHT enzyme activity in the GluA3-SHT transgenic lines were consistent with the levels of SHT mRNA expression. Among the prolamin-SHT transgenic lines, lines 1 and 4 showed a four-fold increase in SHT enzyme activity ranging 2.8 to 2.9 pkat mg\(^{-1}\) protein, and lines 8 and 15 showed a three-fold increase compared with wild-type activity (0.7 pkat mg\(^{-1}\) protein; Fig. 2b). The remaining lines showed SHT enzyme activities that were similar to or higher than wild-type activity. The high levels of SHT enzyme activity observed in the prolamin-SHT transgenic lines were consistent with the levels of SHT mRNA expression in the corresponding seeds.

**HPLC Analysis of Serotonin Derivatives in Transgenic Seeds**

To determine whether the ectopic expression of SHT in rice endosperm increases the synthesis of FS and CS in T2 rice seeds, homozygous T2 seeds from the GluA3-SHT and prolamin-SHT lines were dehusked and extracted separately with methanol; these extracts were analyzed to quantify CS and FS (Fig. 2c,d). The total CS + FS content did not increase in the GluA3-SHT transgenic seeds compared with wild-type seeds. The prolamin-SHT transgenic lines (lines...
Responses of Substrate Treatments in the Synthesis of CS and FS in Rice Seeds

Even though SHT mRNA was constitutively expressed at a relatively high level and SHT enzyme activity was high in seeds from the prolamin-SHT transgenic lines, the synthesis of CS + FS was not increased. No significant difference in the quantity of CS + FS was observed between the transgenic and wild-type plants. To determine whether the lack of CS + FS synthesis was the result of substrate deficiency in the prolamin-SHT transgenic seeds, we measured the CS + FS content in dehusked seeds after imbibition [17] in 2 mM serotonin or 2 mM ferulic acid for 2 days. The CS + FS content increased ten-fold in both wild-type and transgenic seeds after imbibition in either substrate (Fig. 3), but an increase in CS + FS was more evident after treatment with serotonin than ferulic acid. In addition, the CF + FS increase in the transgenic lines was two-fold that in wild-type plants after serotonin treatment. Although ferulic acid treatment increased the amount of CS + FS in imbided seeds, serotonin was a much more powerful inducing agent than ferulic acid. In addition, no additive induction occurred in the presence of both serotonin and ferulic acid, confirming that serotonin was the most significant factor. These data indicate that serotonin is a rate-limiting substrate in the synthesis of CS + FS in transgenic seeds expressing the prolamin-SHT transgene as also observed in transgenic leaves expressing the ubiquitin-SHT transgene [10].

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References