Enhanced neutraceutical serotonin derivatives of rice seed by hydroxycinnamoyl-CoA:serotonin N-(hydroxycinnamoyl)transferase

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Abstract

Serotonin derivatives such as p-coumaroylserotonin and feruloylserotonin, a family of plant polyphenol compounds, have been implicated in an array of biological activities including antioxidative activity, but neither their production nor identification has been reported in crop plants. Here, we report the detection and enhanced synthesis of serotonin derivatives in rice seeds. We identified that wild-type rice seeds synthesized a basal levels of serotonin derivatives with around 30 ng g \(^{-1}\) seed weight. However, transgenic rice expressing the pepper hydroxycinnamoyl-CoA:serotonin N-(hydroxycinnamoyl)transferase (SHT) produced on average 274 ng g \(^{-1}\) seed weight which was nine-fold higher than wild-type. In addition, production of serotonin derivatives increased upon chemical treatments such as trans-cinnamic acid and tyramine in both wild-type and transgenic rice by two- to three-fold. The DPPH radical scavenging activities of transgenic rice were higher than that of wild-type and showed concentration dependent of serotonin derivatives. These results demonstrate that overexpression of SHT provides the feasibility of engineering increased neutraceutical serotonin derivative levels in plants using this gene.

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1. Introduction

Plants produce a diverse mixture of chemicals referred to as secondary metabolites, many of which can contribute to the promotion of good health. Recently, these nutritive and health-beneficial chemicals have increasingly been used as dietary supplements known as neutraceuticals which include vitamins, health-beneficial secondary metabolites, and minerals [1].

Serotonin derivatives are polyphenol compounds and include p-coumaroylserotonin (CS) and feruloylserotonin (FS) that have often been isolated from the seeds of Carthamus tinctorius [2], Echinochloa utilis [3], Amorphophallus konjac [4], Leuzea carthamoides [5] and Centaurea nigra [6]. Recently, serotonin derivatives have also been found in the twigs of the diseased bamboo as antifungal compounds [7] as well as in the roots of rice [8].

CS and FS have first identified as antioxidant compounds from safflower (Carthamus tinctorius) oil cake [9], and also exhibit various activities, e.g., cathartic effect [10], inhibitory activity on proinflammatory cytokine production [11], anti-tumor promoting activity [12] and the effect of lowering the plasma cholesterol [13]. Particularly, it was reported that both CS and FS exhibit stronger antioxidant activities than butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) [3,14,15].

Serotonin derivatives are synthesized from two substrates: hydroxycinnamoyl-CoA thioesters and serotonin, which is in turn synthesized by the transfer of hydroxycinnamic acids from hydroxycinnamoyl-CoA esters to serotonin (Fig. 1). This reaction is catalyzed by hydroxycinnamoyl-CoA:serotonin N-(hydroxycinnamoyl)transferase (SHT) [8]. SHT was first cloned from pepper (Capsicum annuum) as a tyramine N-(hydroxycinnamoyl)transferase (THT) [16], and further rediscovered to be SHT activity through the study of transgenic rice plants and kinetic analysis of recombinant pepper SHT [8]. In our prior
data, the transgenic rice plants harboring the pepper SHT produced serotonin derivatives in leaves only upon treatments of several substrates into the roots, suggesting that serotonin derivatives biosynthesized in the roots are transported to the shoot tissues by the treatment of several chemical compounds such as tyramine and trans-cinnamic acid [8].

In this study, we hypothesized that the transport of serotonin derivatives from the roots to the shoots would be knotted to the transport of serotonin derivatives into seeds. Therefore, we examined seed contents of serotonin derivatives in wild-type and transgenic rice plants over-expressing the SHT, the optimum concentration of chemical treatments for enhancing serotonin derivatives, and the antioxidant activity of transgenic rice plants. This is the first report on serotonin derivatives production and on possible modulation of serotonin derivatives levels in rice seeds by the ectopic expression of SHT.

2. Materials and methods

2.1. Transgenic rice plants growth, substrate treatments and product analyses

The T4 homozygous transgenic rice plants expressing a pepper hydroxycinnamoyl-CoA:serotonin N-(hydroxycinnamoyl)transferase (SHT) were from T2 transgenic rice plants of Jang et al. [8]. T2 transgenic rice seeds homozygous for the introduced SHT gene were further self-pollinated to obtain T4 seeds. These T4 or wild-type seeds were sterilized in 70% EtOH for 30 s, then in a commercial solution of NaClO (2%) for 20 min. The seeds were then rinsed several times with sterilized distilled water and sown in an 0.5% Murashige and Skoog medium in the presence or absence of 50 μM trans-cinnamic acid, and incubated at 25 °C under continuous light for indicated time points. The rice leaves were ground to power in liquid nitrogen and extracted with 3 ml of methanol as described previously [8]. The samples passed through Sep-Pak cartridges were subjected to HPLC for analyzing serotonin derivatives. The seed contents of serotonin derivatives were analyzed using T4 seeds grown in the paddy field treated once, with or without 1 mM various substrates (2000 ml) onto the paddy soil at 40 days after anthesis.

2.2. Measurement for SHT activity

Rice leaves were homogenized in a mortar and pestle at 4 °C with a 100 mM sodium phosphate buffer (pH 7.5) and 14.4 mM mercaptoethanol. The homogenates were centrifuged for 10 min at 12,000 × g, and the supernatants were employed as a crude enzyme solution. Unless otherwise indicated, the 10 μl crude enzyme solution were assayed in a total of 10 μl of 1 mM feruloyl-CoA, 10 μl of 10 mM serotonin and 70 μl of a 100 mM Tris–HCl buffer (pH 8.5). After a 10 min incubation at 30 °C, the reaction was stopped with 20 μl acetic acid. The mixture was filled up to 500 μl with methanol, and a 10 μl aliquot was subjected to HPLC analysis. Protein concentration was determined by the Bradford method using the Bio-Rad protein assay dye (Hercules, CA, USA).

2.3. Radical scavenging activity using DPPH method

Leaf extracts passed through Sep-Pak cartridges for HPLC analysis described above were employed in order to circumvent the problems associated with the complicated interactive effects of various chemical components in the whole leaf extracts. The 50 μl leaf extracts were adjusted to 100 μl by adding MeOH. Nine hundred microliters of a 0.15 mM methanolic solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) was added to Eppendorf tubes and shaken vigorously. The tubes were allowed to stand at 27 °C for
20 min. The control was prepared as above without any extract. Changes in the absorbance of the samples were measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following numerical formula: radical scavenging activity (%) = (control OD – sample OD/control OD) × 100. Additionally, the known concentrations of CS and FS mixtures from leaf extracts were analyzed for their radical scavenging activities as for reference.

3. Results and discussion

3.1. SHT enzyme activities in T₄ homozygous transgenic rice

The pepper SHT was introduced into the rice genome under the control of the maize ubiquitin promoter [8]. The SHT expressing transgenic plants employed for biochemical and seed content analyses of serotonin derivatives were the homozygous T₄ generation in order to avoid the possible gene silencing occurred in the T₃ generation, even though T₂ and T₁ generation plants were not silenced [17]. To investigate expression of SHT gene in T₄ generation, we measured the SHT enzyme activity in all independent transgenic lines and wild-type by using 15-day-old leaves grown in half-strength Murashige and Skoog medium. The levels of SHT activities ranged from 25 to 60 pkat (mg protein)⁻¹ in transgenic lines, but showed less than 1 pkat (mg protein)⁻¹ in both wild-type and transgenic control (Fig. 2). A highest SHT activity was detected in line 14 with a 60 pkat (mg protein)⁻¹, and then followed by line 10 and other lines. The level of SHT enzyme activity of line 14 in T₄ generation was almost equivalent with the one measured in T₂ generation with a 78 pkat (mg protein)⁻¹ [8]. The overall patterns of SHT activities among T₄ transgenic lines were closely comparable to those measured in T₁ generation, suggesting that the transgene was stably and functionally expressed in the rice after generations.

3.2. Quantification of p-coumaroylserotonin and feruloylserotonin in dehulled rice seeds

T₄ transgenic seeds homozygous for SHT transgene were dehulled and subjected to HPLC analysis for measuring the levels of serotonin derivatives (Fig. 3). A very low amount of serotonin derivatives was detected in the seeds of wild-type and transgenic control with around 30 ng g⁻¹ seed weight. In contrast, the transgenic seeds exhibited 5–18 times higher serotonin derivative levels compared with wild-type seeds. Total serotonin derivatives (CS + FS) accumulated in the seeds of transgenic lines at levels fluctuating between 165–562 ng g⁻¹ seed weight. Transgenic line 14 showed highest levels of serotonin derivatives with 562 ng g⁻¹ seed weight (CS: 213 ng and FS: 349 ng). Even though p-coumaroylserotonin was detected slightly higher than feruloylserotonin in most transgenic lines except line 14 and wild-type tested, no preferential production of either CS or FS was observed in general except lines 14 and 16. This phenomenon is ascribed to the intrinsic characteristics of SHT enzyme with the similar Vₘₐₓ/Kₘ toward feruloyl-CoA and p-coumaroyl-CoA. The levels of serotonin derivatives in the transgenic seeds averaged around 274 ng g⁻¹ seed weight. The mechanism by which the transgenic SHT rice under the control of the maize ubiquitin overproduces serotonin derivatives in the seeds is not clear thus far, but this is caused either by the seed expression of SHT transgene driven by the ubiquitin promoter [18] or by the transport of serotonin derivatives synthesized in the roots into the seeds [8].

3.3. Effect of substrate treatments on the accumulation of serotonin derivatives in the seeds

Application of several amine or phenolic chemicals onto the roots grown in vitro was known to induce the production of serotonin derivatives in the shoots because of the facilitation of serotonin derivative movement from the roots to the shoots [8]. To test whether the chemical application into the rice roots grown in the paddy field had a similar
effect on an increased synthesis of serotonin derivatives in the seeds, we applied once several chemicals as an aqueous solution (2000 ml per plant) at 40 days after anthesis. Rice seeds harvested 30 days after treatment with 1 mM chemicals responded with the enhanced accumulation of serotonin derivatives (Table 1). In wild-type treated with tyramine, serotonin derivative levels increased to more than three-fold above the level detected in wild-type seeds without tyramine treatment. In a similar manner as shown in the wild-type, transgenic seeds also produced higher amounts of serotonin derivatives when rice plants were treated with 1 mM chemicals. On average, serotonin derivatives in the transgenic rice plants challenged with chemicals increased to a level approximately double that of the untreated transgenic plants. Among several chemicals tested, tyramine seemed to be the best inducer, but other chemicals such as trans-cinnamic acid and p-coumaric acid had the similar effects for inducing the synthesis of serotonin derivatives in the transgenic seeds. The inducible production of serotonin derivatives in the seeds by chemical treatments is well matched with the data reported in the rice leaves [8]. Thus, the induced accumulation of the serotonin derivatives in the seeds may be in support of our hypothesis that the serotonin derivatives transport from the roots to the seeds via the leaves. In addition, the chemical treatments around day 11 on which leaf senescence was about to take place. On day 34, leaves were completely senesced and the levels of CS and FS decreased down to 3 and 5 µg g⁻¹ fresh leaves. In the absence of trans-cinnamic acid, serotonin derivatives were not detected even in leaves of transgenic rice [8]. Compared to the transgenic rice, only trace amounts of serotonin derivatives were detected in the wild-type leaves with around 1.5 and 2.5 µg g⁻¹ fresh leaves of CS and FS, respectively, on day 11. The synthesis pattern of serotonin derivatives was quite similar between the transgenic and wild-type showing the same peak accumulation on day 11 and the decline thereafter. A comparison of the level of serotonin derivatives in the seeds (998 ng g⁻¹ seed weight) with that found in the leaves (360 µg g⁻¹ fresh leaf weight) challenged with trans-cinnamic acid revealed that a very small portion of serotonin derivatives about 0.3% was mobilized from the leaves to the roots or synthesized in the seeds. Therefore, it would be intriguing to know whether SHT gene fused to the seed specific promoter such as glutelin promoter leads to the enhanced synthesis of serotonin derivatives in the seeds.

### 3.5. Antioxidant activities of transgenic rice plants measured by DPPH

Since CS and FS have been reported to show strong antioxidant activities [14,15], we checked 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities of transgenic rice plants. According to the report of Baek et al. [14], FS showed lower RC₅₀ (estimated concentration to reduce 50% of control absorbance) value with a 6.6 µg than those of other antioxidants such as butylated hydroxytoluene (BHT; RC₅₀ = 12.2 µg) and butylated hydroxyanisole (BHA; RC₅₀ = 7.4 µg). Because of a low level of serotonin derivatives in the transgenic seeds, we have decided to utilize the leaf extracts to measure the radical scavenging activities from the transgenic and wild-type. The leaf extracts were from the samples used for CS and FS analysis in Fig. 4. The leaf extracts of wild-type showed the low levels of activities which quenched 5–20% of DPPH radicals (or RSA activities) (Fig. 5A). In contrast, the leaf extracts of transgenic rice showed the high activities to quench DPPH radicals ranging from 10 to 93%. The DPPH radical scavenging activities of leaf extracts are shown in Table 2.
scavenging activity was closely associated with the levels of serotonin derivatives. The leaf extracts on day 11 showed the greatest activity to quench DPPH radicals followed by days 7 and 15. The mixtures of CS and FS from leaf extracts exhibited a 5.1 μg/fresh weight C50 value which is comparable with the value reported previously (Fig. 5B) [14].

According to the key role played by the absolute amounts of serotonin derivatives on radical scavenging activity, seed extracts from five grams of transgenic seeds which contains approximately 5 μg serotonin derivatives (CS + FS) will quench 50% of DPPH radicals. In conclusion, the results described here have implications for the genetic improvement of the phytochemicals of plant products. Although the exact function of CS and FS in plants is not well defined, the generation of substantial increases in amounts of antioxidant serotonin derivatives by overexpression of SHT may lead to increased resistance of plant tissues to both biotic and abiotic stresses [7,19]. This resistance may play important roles in crop productivity and increased storage life of seeds.

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References


