Senescence-Induced Serotonin Biosynthesis and Its Role in Delaying Senescence in Rice Leaves1[C][W][OA]

Kiyoon Kang, Young-Soon Kim, Sangkyu Park, and Kyoungwhan Back*

Department of Biotechnology, Interdisciplinary Program for Bioenergy and Biomaterials of Graduate School, Agricultural Plant Stress Research Center, Chonnam National University, Gwangju 500–757, South Korea

Serotonin, which is well known as a pineal hormone in mammals, plays a key role in conditions such as mood, eating disorders, and alcoholism. In plants, although serotonin has been suggested to be involved in several physiological roles, including flowering, morphogenesis, and adaptation to environmental changes, its regulation and functional roles are as yet not characterized at the molecular level. In this study, we found that serotonin is greatly accumulated in rice (Oryza sativa) leaves undergoing senescence induced by either nutrient deprivation or detachment, and its synthesis is closely coupled with transcriptional and enzymatic induction of the tryptophan biosynthetic genes as well as tryptophan decarboxylase (TDC). Transgenic rice plants that overexpressed TDC accumulated higher levels of serotonin than the wild type and showed delayed senescence of rice leaves. However, transgenic rice plants, in which expression of TDC was suppressed through an RNA interference (RNAi) system, produced less serotonin and senesced faster than the wild type, suggesting that serotonin is involved in attenuating leaf senescence. The senescence-retarding activity of serotonin is associated with its high antioxidant activity compared to either tryptophan or chlorogenic acid. Results of TDC overexpression and TDC RNAi plants suggest that TDC plays a rate-limiting role for serotonin accumulation, but the synthesis of serotonin depends on an absolute amount of tryptophan accumulation by the coordinate induction of the tryptophan biosynthetic genes. In addition, immunolocalization analysis revealed that serotonin was abundant in the vascular parenchyma cells, including companion cells and xylem-parenchyma cells, suggestive of its involvement in maintaining the cellular integrity of these cells for facilitating efficient nutrient recycling from senescing leaves to sink tissues during senescence.

Serotonin (5-hydroxytryptamine) is a ubiquitous monoamine that plays multiple roles as a neurotransmitter, hormone, and mitogenic factor and mediates a series of activities in various animal cells (Frazer and Hensler, 1999). In plants, serotonin has been found in a wide range of plant species (Roshchina, 2001) since its discovery was first reported in the fruit of the cowhage (Mucuna pruriens) plant (Bowden et al., 1954). Similar to the multiple roles played by serotonin in animal cells, serotonin has also been implicated in an array of physiological functions in plants that are purportedly related to growth regulation, flowering, xylem sap exudation, ion permeability, and plant morphogenesis (Csaba and Pal, 1982; Odjakova and Hadjiivanova, 1997; Murch et al., 2001; Roshchina, 2001).

Serotonin is predominantly distributed in reproductive as opposed to vegetative organs. For example, Griffonia simplicifolia leaves were found to contain 0.007 μg/g fresh weight (FW) serotonin, but seeds harbored 2,000 μg/g seeds (Fellows and Bell, 1970). In addition, serotonin levels are known to increase as fruits ripen in many species, including tomato (Solanum lycopersicum), although the inverse is true of the fruit of pineapple (Ananas comosus; Udenfriend et al., 1959; Foxy Parrat, 1961). Apart from enriched serotonin accumulation in fruits, serotonin accumulates in the stinging nettle (Urtica dioica) (Collier and Chesher, 1956) and in the pods of cowhage (Bowden et al., 1954), in which serotonin is suggested to play a protective role against predators.

One interesting study on serotonin synthesis and its possible biological function was reported for walnut (Juglans regia) seeds, in which serotonin is mainly accumulated during the process of fruit abscission (Bergmann et al., 1970). This abscission period is accompanied by proteolysis and deamination of amino acids giving rise to ammonium accumulation in walnut seeds. To circumvent the toxic accumulation of ammonia, Gln synthetase assimilates ammonia together with Glu via the synthesis of Gln, which directly serves as a substrate for Trp synthesis. However, this hypothesis has not been corroborated by further enzymatic or molecular analysis.

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* Corresponding author; e-mail kback@chonnam.ac.kr.

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Serotonin biosynthesis occurs via two enzymatic steps (Fig. 1A). The first committed enzyme is tryptophan decarboxylase (TDC), which catalyzes the conversion of Trp into tryptamine. The terminal enzyme is tryptamine 5-hydroxylase (T5H), which hydroxylates the C-5 position of tryptamine to form serotonin (Kang et al., 2007a). TDC serves as a bottleneck point regulating serotonin biosynthesis since TDC expression is very low or negligible, while T5H is constitutively expressed in healthy rice (*Oryza sativa*) plants. Due to the low expression of TDC in rice plants, serotonin levels in leaves and seeds were reported to be around 0.3 μg/g FW and 0.12 μg/g seeds, respectively, whereas transgenic rice plants overexpressing TDC produced 25- and 11-fold higher serotonin in the leaves and seeds, respectively, than the wild type (Kang et al., 2007b).

Although an exact role for serotonin in plants remains to be elucidated, it is tempting, by way of extrapolation, to think that serotonin synthesis is closely associated either with ripening or maturation of plant organs or with the accumulation of ammonia, which occurs predominantly during the process of plant senescence (Peeters and Van Laere, 1992). One approach to testing this possibility is to examine the levels of serotonin upon senescence in rice plants because they are known to harbor at least two functional TDC genes of which TDC1 is functionally implicated in synthesizing serotonin (Kang et al., 2007b).

In contrast to the previous reports showing the predominant production of serotonin in reproductive organs, this report describes the enormous induction of serotonin synthesis in senescing rice leaves, which is characterized by chlorophyll loss, membrane lipid peroxidation, increased reactive oxygen species (ROS), and induced senescence-related genes. It further shows that the induction of serotonin accumulation is coordinately regulated with the induction of the entire set of Trp biosynthetic mRNAs and is proportional to the induction of TDC protein. Furthermore, the accumulation of serotonin is believed to play a protective role against ROS, leading to a delay in the process of senescence as demonstrated by analyses of transgenic rice plants, such as *TDC* overexpression and *TDC* RNA interference (RNAi) lines.

**RESULTS**

**Synthesis of Serotonin in Attached Leaves of Rice Seedlings upon Senescence Induced by Nutrient Deprivation**

When 8-d-old rice seedlings were grown in water with no nutrients, the plants began to undergo the senescence process and turn yellow at 16 d. By day 26, most of the existing leaves had turned yellow and were dry and twisted, whereas most stems and roots remained healthy (Fig. 1B). In response to the process...
of senescence, a high level of serotonin accumulated in senescing rice leaves (Fig. 1C). In healthy leaves, serotonin levels were typically below 0.5 μg/g FW. However, as rice plants aged, serotonin synthesis began at 11 d (10 μg/g FW) in leaves and reached up to 75 μg/g FW at 16 d, at which time the leaves began to turn yellow. Serotonin continued to accumulate in leaves until day 26, at which time the serotonin content was around 350 μg/g FW. In roots, serotonin accumulated as the plants aged, but its level at 26 d was 9-fold lower compared to that in leaves. Serotonin was also observed in stems and reached a peak level of 20 μg/g FW, which was 2-fold lower than that in roots. Although serotonin levels varied among tissues, serotonin was abundantly synthesized in senescent rice tissues, and the induced synthesis of serotonin was closely paralleled by the appearance of symptoms of senescence. In contrast, the rice seedlings grew without showing senescence symptoms and did not show any increases in serotonin synthesis in the leaves either in the presence of half-strength Murashige and Skoog solution without Suc or on soil-based compost (data not shown).

Biochemical and Molecular Changes in Senescing Rice Plants

To examine whether rice seedlings exposed to nutrient-free water go through a typical senescence process, several biochemical and molecular indices related to the senescence syndrome were investigated (Fig. 2). First, chlorophyll content gradually decreased over time. In 3 d, the leaf chlorophyll content decreased by 25% and remained at that level until 11 d later. Thereafter, the chlorophyll content dropped dramatically to 50% and 10% of that in the initial nonsenescent leaves at 21 and 26 d, respectively. In contrast, stem chlorophyll levels declined by 25% after 3 d, but this level of chlorophyll was maintained until 26 d, suggesting that no dramatic senescence had occurred in the stems compared to the leaves (Fig. 2A). Next, we measured ROS and malondialdehyde (MDA), which are characteristic symptomatic indicators of senescence in plants (Fig. 2, B and C). Upon senescence, ROS levels in leaves began to increase at 11 d and thereafter increased rapidly. Likewise, MDA levels in leaves increased in parallel with ROS levels. In contrast, stems and roots showed no significant increases in either ROS or MDA levels. To further confirm the senescence process at the molecular level in our in planta system, we performed northern-blot analysis using representative senescence-associated genes, such as Osl2 and Osl139, which were induced in rice leaves upon senescence (Lee et al., 2001). As shown in Figure 2D, the Osl2 transcript was rarely detectable before senescence but was gradually induced in response to senescence. Higher levels of Osl2 transcripts were detected after 21 d. Compared to Osl2, the level of Osl139 transcripts was relatively low but increased as senescence proceeded. Taking all the data together, rice seedlings underwent senescence in our in planta rice seedling system.

Induction of TDC in Parallel with Serotonin Accumulation

Serotonin is consecutively synthesized from Trp by two enzymes. TDC is the first committed enzyme, which catalyzes the conversion of Trp to tryptamine, followed by catalysis of tryptamine to produce serotonin by T5H (Kang et al., 2007a). TDC is the rate-limiting enzyme for serotonin biosynthesis and exists in at least two functional copies in the rice genome (Kang et al., 2007b). To determine whether serotonin accumulation upon nutrient-deficient induced senescence is closely associated with induction of TDC mRNA, we performed independent northern-blot analyses with two TDC cDNAs as probes (Fig. 3, A and B). The TDC1 mRNA transcript was not detected in healthy tissues even after 6 d of senescence treat-
By day 11, the level of the TDC1 transcript began to increase, and rapid increase was observed in leaves after 16 d. The increase in TDC1 transcripts was proportional to the accumulation of serotonin upon senescence. Unlike leaves, stems and roots showed no significant induction of the TDC1 transcript in response to senescence, which was consistent with low serotonin levels and the lesser degree of senescence symptoms compared to leaves. In marked contrast, the TDC2 transcript was rarely detectable in all senescing tissues, including leaves, suggesting that TDC1 played the major role in serotonin biosynthesis when rice plants were challenged with senescence. To see whether the transcriptional induction of TDC1 is related to high enzyme activity, we measured TDC enzyme activity in rice leaves upon senescence (Fig. 3C). TDC enzyme activity increased 16-fold by 16 d after the senescence treatment relative to that of the control at day 0. In comparison, the levels of T5H, the terminal enzyme for serotonin biosynthesis, were not altered during the entire period of senescence. Thus, the induction of TDC is likely to be most responsible for the accumulation of serotonin in senesced leaves of rice.

### TDC Protein Is Maximally Expressed in the Fully Senesced Leaves

To examine the relative levels of the TDC1 protein in senescing leaves of rice seedlings, we took the 16-d-old senescing rice leaves induced by nutrient deprivation and dissected them into three parts: the tip (fully senesced), middle (partially senesced), and base (barely senesced) to measure the level of expression of the TDC1 protein and the level of serotonin. As shown in Figure 4, expression of the TDC1 protein was highest in the fully senesced tip, followed by the middle and base. Although the polyclonal antibodies raised from TDC1 protein also show cross-reactivity to TDC2 protein, it is clear that the immune-reacted bands identified by the TDC1 antibodies predominantly correspond to TDC1 protein because the TDC2 mRNA was not significantly induced upon senescence (Fig. 3). The relative levels of the TDC protein were closely associated with the level of serotonin. The tip contained the highest level of serotonin at 270 μg/g FW, whereas the middle and base parts contained 152 and 30 μg/g FW, respectively. In particular, Trp, the substrate of the TDC enzyme, coordinately increased up to 400 μg/g FW in the fully senesced tip, which corresponded to a level 1.5-fold higher than serotonin. These data clearly suggested that TDC expression is abundant in the senesced tissues of rice leaves and is strongly induced in parallel with the high production of serotonin as well as Trp as the rice leaves undergo senescence. In addition, the effects of exogenous applications of serotonin on leaf senescence were inves-
tigated by measuring chlorophyll, ROS, and MDA. As shown in Supplemental Figure S1, treatment with 500 μM serotonin showed 2-fold higher chlorophyll content than the untreated leaves at 26 d. Both ROS and MDA levels decreased significantly in serotonin-treated leaves compared to untreated leaves. In addition, TDC enzyme activity was 3-fold lower in serotonin-treated leaves (500 μM) than in untreated leaves at 26 d, suggestive of retarded senescence caused by serotonin (Supplemental Fig. S2).

Accumulation of Serotonin and Plant Hormonal Effects upon Senescence of Detached Leaves of Rice

In Figures 1 to 4, we show the course of serotonin synthesis as well as its effects on senescence (Supplemental Figs. S1 and S2) using an in planta system. To further verify the mechanisms of serotonin biosynthesis and its physiological roles and to simplify the experiment, we next used leaves detached from 4-week-old rice seedlings and measured the levels of serotonin in response to senescence. As shown in Figure 5, serotonin began to be synthesized on day 4, produced 984 μg/g FW on day 6, and peaked on day 8 with 1,634 μg/g FW (Fig. 5A). The maximum level of serotonin in the detached leaves was 4.7-fold higher than that found in the attached leaves upon senescence, indicating that the detachment of the leaves had a more dramatic effect on serotonin synthesis than was observed for the attached leaves.

The effects of plant hormones, such as zeatin and abscisic acid (ABA), on serotonin synthesis in response to senescence were investigated. ABA treatment accelerated serotonin synthesis, producing 450 μg/g FW after 4 d, while control leaves produced only 16 μg/g FW serotonin and showed a maximum synthesis of serotonin (720 μg/g FW) at 6 d, followed by gradual decrease. In contrast to ABA, zeatin treatment delayed serotonin synthesis, and its levels were far lower compared to those of the untreated control leaves. These data on the changes in serotonin levels upon hormonal treatment were consistent with the known roles of zeatin and ABA, which play inhibitory and stimulatory roles in senescence, respectively. In addition, the Trp content also increased greatly in parallel with the serotonin levels upon senescence (Fig. 5B). The relative levels of Trp during the entire time course of senescence were higher in the ABA-treated leaves than in the untreated control. Similarly, zeatin-treated leaves showed lower levels of Trp synthesis than those detected in the untreated and ABA-treated leaves. The relative levels of serotonin in the detached leaves upon

**Figure 5.** Effects of plant hormones on the induced synthesis of serotonin, Trp, and TDC protein during senescence of detached rice leaves. A, Effects of zeatin and ABA on serotonin levels. B, Effects of zeatin and ABA on Trp levels. C and D, Immunoblot analysis of the TDC protein (C) and phenotypes (D) in response to zeatin and ABA treatments. The apical 15 cm of the third leaf from 4-week-old rice plants was used. A group of 10 segments was transferred into a 50-mL polypropylene conical tube containing 10 mL of water supplemented with either zeatin (5 μM) or ABA (5 μM) and incubated under the same growth conditions described above. Data represent the means ± so of two replicate samples.

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hormonal treatment were closely coupled with the relative levels of TDC protein expression (Fig. 5C). For example, the untreated control began to show a detectable level of TDC protein at 6 d and reached peak expression at 8 d, whereas the ABA-treated leaves showed a fast induction of TDC at 4 d and reached a maximum at 6 d. TDC expression levels were higher in the untreated control leaves relative to those of the ABA-treated leaves, accounting for the higher levels of serotonin synthesis in the untreated control leaves. Zeatin treatment suppressed the induced expression of the TDC protein and led to the low levels of serotonin synthesis compared to the untreated control. Taken together with the induced accumulation of serotonin upon senescence in the in planta system, these results clearly indicate that serotonin accumulation is directly associated with the process of senescence (Fig. 5D).

Regulation of Trp Biosynthetic mRNAs and Induction of Anthranilate Synthase Enzyme Activity upon Senescence of Detached Leaves of Rice

The results from the detached leaves show that serotonin accumulation in response to senescence is accompanied by the induced accumulation of free Trp. To test whether Trp biosynthetic genes are induced upon senescence, five Trp biosynthetic genes were selected for characterization. Figure 6A shows that the mRNAs encoding the enzymes anthranilate synthase (ASα1 and ASβ2), phosphoribosylanthranilate transferase (PAT), indole-3-glycerolphosphate synthase (IGPS), and tryptophan synthase (TSα) were induced upon senescence. The induction patterns differed. The mRNAs encoding the ASα1, ASβ2, and PAT enzymes were induced after 4 d upon senescence treatment, whereas the mRNA transcripts for IGPS and TSα were shown to be induced at 6 d. In addition, Gln synthetase was also induced at 6 d and reached its maximum level at 8 d. All mRNA transcripts maintained their maximum levels until 10 d except IGPS, which showed a maximum level at 6 d and decreased thereafter. The induction of AS mRNAs was also observed in rice leaves infected with the conidia of Bipolaris oryzae (Ishihara et al., 2008) or in wounded leaves of rice (Tozawa et al., 2001). However, the induction of ASα1 was not observed at all, and the ASα1 transcript was suppressed upon pathogen infection. Thus, ASα1 seems to play a key role either in senescence-induced Trp or serotonin synthesis in rice plants. To determine whether the induction of AS mRNA is functionally associated with the induction of the AS protein, we measured AS enzyme activity during the time course of senescence. As shown in Figure 6B, AS enzyme activity was very low prior to senescence but was induced after 4 d upon commencement of senescence and showed a maximum activity of 6 pkat/mg protein followed by a gradual decrease after 8 d. Note that the maximum AS activity preceded the peak synthesis of serotonin.

Serotonin Biosynthesis in Rice Plants

Serotonin-Overproducing Transgenic Rice Leaves Lead to a Delay of Leaf Senescence

The role of serotonin in senescence was further investigated by performing a gain-of-function analysis. We used transgenic rice plants overexpressing the rice TDC1 gene under control of the maize (Zea mays) ubiquitin promoter. These TDC1 transgenic rice plants produced 25-fold higher serotonin in leaves than the wild type (Kang et al., 2007b). From several transgenic lines, we selected three transgenic lines of which two lines (10 and 14) exhibited overexpression of the TDC protein, whereas line 18 had TDC expression levels similar to the wild type, 18fold lower than the wild type due to the overexpression of the TDC protein (Fig. 6A). Serotonin concentrations in leaves were 16 μg/g FW after 4 d (Fig. 7B). During senescence, the serotonin concentration increased in both the wild type and transgenic lines as senescence proceeded, but the relative levels of serotonin were always higher in transgenic lines than in the wild type due to the overexpression of TDC. In contrast, Trp levels were lower in the transgenic lines (10 and 14) than in the wild type because the Trp was

Figure 6. Regulation of the Trp biosynthetic pathway upon senescence of detached rice leaves. A, Expression of Trp biosynthetic mRNAs. B, Measurement of AS enzyme activity. Experimental conditions were the same as described in Figure 5 except for the hormonal treatments. A, AK072053; ASβ1, AK059358; PAT, AK066714; IGPS, AK069031; TSα, AK066734; TDC1, AK069031; TDC2, AK110353; GS (Gln synthetase), AK243037.
converted into serotonin more efficiently in the transgenic lines than in the wild type by TDC overexpression (Fig. 7C). During the period of senescence, transgenic lines 10 and 14 showed delayed senescence compared to the wild type, except line 18, when judged by phenotype (Fig. 7D). These phenotypic differences were further confirmed by biochemical analyses measuring MDA and chlorophyll content. The transgenic lines with the high levels of serotonin exhibited less MDA production than the wild type (Fig. 7E). Accordingly, the loss of chlorophyll upon senescence was slower in the transgenic lines than in the wild type (Fig. 7F). The results were consistent with those obtained with the exogenous serotonin treatment.

Suppression of TDC by RNAi Produces Low Serotonin and Promotes Senescence

To further examine the function of serotonin in vivo, we employed RNAi interference to silence the expression of TDC1, which is a rate-limiting enzyme for serotonin synthesis. A transgene TDC1 was controlled by a maize ubiquitin promoter, and 20 independent transgenic lines were generated through Agrobacterium tumefaciens-mediated transformation (Fig. 8). Among them, two lines (RNAi-11 and RNAi-16) of T$_1$ generation were further selected for examining the loss-of-function effects of TDC1 on serotonin synthesis. Four-week-old mature leaves of rice plants were detached and the levels of serotonin measured upon senescence. TDC1 RNAi lines showed that serotonin synthesis decreased markedly during senescence. For example, the wild type and vector control produced around 900 mg g$^{-1}$ FW serotonin 6 d after senescence, whereas the RNAi lines (T$_1$), such as RNAi-11 and RNAi-16, only produced 125 and 207 mg g$^{-1}$ FW serotonin, respectively (Fig. 8B). Accordingly, these RNAi lines exhibited a rapid senescence relative to the wild type. Chlorophyll concentrations were 1.5-fold less in the RNAi lines than in the wild type after 8 d,
confirming that serotonin itself plays a direct role in delaying senescence in rice leaves (Fig. 8D). In contrast to serotonin levels, Trp levels did not dramatically change in the RNAi lines, although the RNAi lines had lower levels of Trp than the wild type or vector control, especially after 6 d (Fig. 8C). Note that the RNAi lines (T1) were not different phenotypically from the wild type in both the vegetative and reproductive stages, suggesting that serotonin is not directly involved in primary metabolism, but rather in secondary metabolism acting as a metabolite to delay senescence. In rice plants, serotonin is further metabolized into serotonin derivatives, such as feruloylserotonin (FS) and 4-coumaroylserotonin (CS), in reactions catalyzed by serotonin N-hydroxycinnamoyl transferase (SHT). These serotonin derivatives are acknowledged to be strong antioxidant compounds. Thus, the role of these serotonin derivatives in delaying senescence could not be ruled out. In an attempt to verify the effects of serotonin derivatives on senescence, we used pepper (Capsicum annuum) SHT-overexpressing transgenic rice plants that produce high levels of serotonin derivatives (Jang et al., 2004) and compared them to the wild-type senescence symptoms. As shown in Figure 9, the SHT transgenic line produced high levels of serotonin derivatives, whereas the levels of serotonin and Trp were similar to those of the wild type. The resulting senescence severity was slightly lower in the SHT transgenic line than in the wild type, possibly due to the high levels of serotonin derivatives in the SHT transgenic line; this suggests that serotonin derivatives are also slightly involved in delaying senescence but are not a determining factor for delaying senescence compared to serotonin in rice plants. The levels of serotonin derivatives in the TDC transgenic line were lower than those of the wild type, whereas serotonin levels were the inverse. The reason for the low levels of serotonin derivatives in the TDC line appears to be due to the low levels of endogenous rice SHT enzyme activity as a result of the delayed senescence of the TDC transgenic line. To test whether the endogenous rice SHT enzyme is also induced upon senescence, we measured SHT enzyme activity. As expected, the SHT enzyme activity was 2-fold lower in the TDC transgenic line than in the wild type (data not shown), suggesting the induction of rice SHT enzyme activity upon senescence as observed for TDC. In addition, the preferential production of CS rather than FS was manifested in the wild type as well as in the TDC transgenic line, whereas FS was the major serotonin derivative found in the SHT transgenic line, consistent with a previous report (Jang et al., 2004). These differ-

Figure 8. Biochemical and physiological analyses of TDC RNAi transgenic lines on the senescence of detached rice leaves. A, Schematic diagram of gene cassettes in the T-DNA of the TDC RNAi binary vector. B, Analysis of serotonin levels. C, Analysis of Trp levels. D and E, Chlorophyll levels (D) and phenotype (E) on 8 d. Detached rice leaves (4 weeks old) were subjected to senescence as described in Figure 5. WT, Wild type; VC, vector control; TDC-10, TDC-overexpressing transgenic line (T4); RNAi-11 and RNAi-16, TDC1 RNAi transgenic lines (T1). Data represent the means ± SD of five replicate samples. Different letters indicate significant differences according to Duncan’s multiple range test (P < 0.05).
ences in the production profiles of serotonin derivatives are attributable to the intrinsic features of the pepper and rice SHT enzymes, the latter of which has not as yet been characterized in detail. Accordingly, the results of in vitro antioxidant activity showing 2-fold higher radical scavenging activity of serotonin rather than serotonin derivatives (Fig. 9E) are clearly consistent with the results from the transgenic TDC and SHT lines, corroborating our observation that a strong relationship exists between serotonin accumulation and senescence retardation.

Immunohistochemical Localization of the TDC Protein and Serotonin in Senesced Leaves of Rice

The spatial distribution of the TDC protein and serotonin in the cross section of rice leaves was examined by immunohistochemical localization using TDC and serotonin polyclonal antibodies (Fig. 10). TDC protein and serotonin were not stained in control leaves but were clearly observed 7 d after senescence, which was consistent with the results of previous analyses (Fig. 5). Although all of the mesophyll cells except the epidermal cells were thoroughly stained, signals for the TDC protein were abundant in vascular parenchyma cells, whereas bundle sheath cells and the metaxylem were not stained (Fig. 10, C and F). The companion cells were also clearly stained, but the signal intensity was not high compared to that in the xylem parenchyma cells. In contrast, serotonin was strongly stained in companion cells at a level of intensity similar to the vascular parenchyma cells (Fig. 10, D and G). These data suggest that serotonin may play an important role in maintaining the cellular integrity of vascular bundles with its high antioxidant activity during the process of senescence.

DISCUSSION

This study characterized the mechanisms by which senescence triggers and coordinates serotonin synthesis through the biosynthetic machinery of the Trp pathway. Furthermore, we investigated the functional
roles of serotonin during senescence via the analyses of serotonin-overproducing and -deficient transgenic rice plants. Senescence is a genetically controlled process that plays an important role in the recycling of nutrients from old leaves to young productive leaves and developing seeds. A myriad of developmental and environmental factors regulating senescence are characterized by a loss of chlorophyll and degradation of macromolecules, such as protein, lipids, and RNA (Kim et al., 2007).

While most genes are inactivated during senescence, particular sets of genes, referred to as senescence-related genes, are activated and participate in catabolic activities. The senescence-related genes include genes whose products are related to pathogen defense mechanisms. This indicates that defense-related genes play a role in leaf senescence as well as in pathogen infection. For example, microarray analyses have shown that many genes involved in the biosynthesis of secondary metabolites are up-regulated (Gregersen and Holm, 2007); however, the role of these secondary metabolites during senescence in leaves has not been investigated in great detail, except for their possible intrinsic role in protection against pathogen attacks. Recently, serotonin, a Trp-derived secondary metabolite, was found to be employed in rice as either a substrate to synthesize serotonin derivatives, such as FS and CS, or incorporated directly into the cell wall upon pathogen attack (Jang et al., 2004; Ishihara et al., 2008). In this study, we found that serotonin as a secondary metabolite was accumulated in abundance upon leaf senescence and that it played an important role in alleviating the process of senescence in detached and attached leaves of rice.

**Coordinated Regulation of the Trp Biosynthetic Pathway Genes and Serotonin Accumulation in Senesced Rice Leaves**

During leaf senescence, the intensive breakdown of various macromolecules, such as proteins by the induction of proteases, is followed by an increase in free amino acids, such as Gln and Asn, which serve as long-distance transport forms of organic nitrogen (Hayashi and Chino, 1990). In addition to these amino acids, other amino acids, including Trp, were reported to increase in concentration during senescence in tobacco (Nicotiana tabacum) flowers and in the detached leaves of oats (Avena sativa) and Arabidopsis (Arabidopsis thaliana) and were presumably subjected
to remobilization for developing vegetative and reproductive tissues (Soudry et al., 2005). In plants, free Trp levels are tightly regulated via allosteric inhibition of AS by micromolar Trp levels, which cause the plant cells to maintain a low level of free Trp (Radwanski and Last, 1995). An increase in free Trp has been observed either in plants possessing a mutated AS or in plants challenged by stress conditions, such as wounding and pathogen infection. For example, transgenic rice plants expressing a mutant AS produced an enormous amount of free Trp, independent of the induction of Trp biosynthetic enzymes (Dubouzet et al., 2007). In addition, we found that free Trp levels are also heightened during senescence in rice leaves and that the induced synthesis of Trp occurs via the coordinated up-regulation of Trp biosynthetic genes encoding AS, PAT, and TS enzymes. The induction of Trp biosynthetic enzymes was also observed in pathogen-infected rice and Arabidopsis leaves, where serotonin and camalexin accumulated in parallel with coordinated induction of Trp biosynthetic transcripts (Zhao and Last, 1996; Ishihara et al., 2008). In pathogen-infected rice leaves, the elevated synthesis of Trp occurred upon induction of the AS genes, of which ASα2, ASβ1, and ASβ2 were up-regulated upon pathogen treatment and ASα1 was down-regulated (Ishihara et al., 2008). In addition, ASα1 mRNA was not induced in suspension cultures of rice cells in response to elicitor treatment (Tozawa et al., 2001). Unlike pathogen or elicitor treatments, senescence-induced Trp synthesis was followed by the induction of ASα1 as well as ASβ2, suggesting that ASα1 plays a pivotal role in the enhanced synthesis of Trp as well as serotonin in a senescence-specific manner. As for serotonin synthesis, the key enzyme is TDC, which catalyzes the conversion of Trp into tryptamine. TDC of rice exhibits a high (0.75 mM) K_m toward Trp and thus functions efficiently in the presence of high levels of Trp, termed a Trp feast (Kang et al., 2008). As expected, the induction of TDC took place at the same time as the Trp feast upon senescence. In addition to the induction of Trp biosynthetic genes, Gln synthetase was also induced upon senescence, but its induction occurred at a later stage of senescence compared to that of other Trp biosynthetic genes. In contrast to the attached leaves that displayed a gradual senescence, the detached rice leaves exhibited a rapid onset of senescence symptoms and a dramatic increase in serotonin content. This increase reflects not only the severity of senescence but also the lack of a source-to-sink connection for the detached leaves compared to the attached leaves. In concert with the induced serotonin levels, the synthesis of serotonin-derived metabolites, such as FS and CS, were also shown to accumulate 8 d after senescence.

Role of Serotonin in Senescence

The report on Trp-overproducing transgenic rice demonstrated that Trp seems to be a stable primary metabolite suitable for either nutrient remobilization or storage in senescing plant tissues (Dubouzet et al., 2007). This brings up the issue of why plants synthesize serotonin and what the beneficial effects of serotonin synthesis are as compared to plants unable to make serotonin. Although several different roles have been proposed, the function of serotonin is not yet clear. In this study, we found that Trp levels were significantly induced upon senescence and that the increased Trp was readily converted into serotonin by the induction of TDC. Thus, it seems reasonable to think that serotonin, rather than Trp, is a preferable mediator of senescence. This hypothesis is further supported by the sl mutant in rice, which is known to be controlled by a single recessive mutation and lacks serotonin synthesis (Ueno et al., 2003; Ishihara et al., 2008). During senescence, the plant cells must maintain life functions and protect themselves against the damage imposed by increasing levels of ROS.

A representative secondary metabolite participating in scavenging ROS generated during senescence is tocopherol. As a strongly lipid-soluble antioxidant, tocopherol is known to be synthesized exclusively in chloroplasts and to protect the tissues from photosynthesis-derived ROS (Munné-Bosch, 2005). Ascorbic acid is proposed to scavenge ROS (Takahama and Oniki, 1997) and has been shown to delay senescence in the Arabidopsis mutant vitamin c-1 (Barth et al., 2004). However, antioxidant activities of tocopherol and ascorbic acid are lower than that of chlorogenic acid (Rice-Evans et al., 1997). Serotonin plays a role as an antioxidant by scavenging ROS and shows strong in vitro antioxidant activity compared to Trp and chlorogenic acid. The antioxidant activity of serotonin far exceeds that of Trp, tryptamine, and serotonin derivatives. This suggests that serotonin relieves the accumulation of the toxic metabolite tryptamine and maintains the reducing potential of cells through its powerful antioxidant activity in the senesced leaves. The in vitro antioxidant activity of serotonin was further verified in transgenic rice plants producing either high (TDC overexpression lines) or low levels of serotonin (TDC RNAi lines) in which the serotonin-rich plants showed a phenotype of delayed senescence and the serotonin-deficient plants showed an accelerated senescence. In addition to reporting the serotonin synthesis upon senescence in plants, these results indicate that serotonin plays a practical role in delaying senescence by scavenging ROS efficiently. Although our results exhibited a clear correlation between serotonin and senescence symptoms, it is not clear if these findings are the result of the action of serotonin or whether factors other than serotonin could be involved in delaying senescence (e.g. Trp-derived oxidation products or serotonin-derived metabolites). Also, we cannot rule out the possible involvement of Trp in indole-3-acetic acid biosynthesis upon senescence, as indole-3-acetic acid is known to be involved in retarding senescence in detached senescing leaves.
of Arabidopsis (Noh and Amasino, 1999; Cohen et al., 2003).

Furthermore, the preferential expression of TDC within these vascular cells in parallel with serotonin production may be coupled with the enriched Trp that was induced upon senescence, although Trp biosynthesis occurs in the plastids (Radwanski and Last, 1995). In particular, companion cells and xylem parenchyma cells showing strong signals for serotonin were also reported to be the major site of the Gln synthetase enzyme, which catalyzes the conversion of Glu to Gln, a major long-distance transport form of organic nitrogen during the process of senescence (Sakurai et al., 1996). Companion cells and xylem parenchyma cells play key roles in the regulation of phloem loading (Van Bel, 1993). Therefore, it is highly likely that serotonin, with its high antioxidant activity, may play an important role in maintaining the cellular integrity of xylem parenchyma and companion cells by protecting them from the oxidative damage caused by the process of senescence and thus facilitate efficient nutrient recycling from senescing leaves into sink tissues. Finally, increased synthesis of serotonin was also observed in rice leaves challenged with pathogenic infection. The levels are similar to those found during senescence of attached rice leaves, and synthesis was also accompanied by a marked increase in the Trp content with a rapid induction of anthranilate synthase enzyme activity and corresponding mRNAs (Ishihara et al., 2008). However, the functional and physiological roles of serotonin between pathogenic infection and senescence appear to be different. Specifically, the serotonin accumulated upon pathogenic infection is incorporated into the cell walls leading to strengthening of the wall, whereas serotonin synthesized upon senescence is found in the soluble fraction of senescent tissues, especially in the vascular bundle cells, leading to its role in delaying senescence.

**MATERIALS AND METHODS**

**In Planta Senescence in Rice Seedlings**

Seeds of wild-type rice (*Oryza sativa*) were surface-sterilized and sown in half-strength Murashige and Skoog media in a plant growth room at 28°C with a 16-h-light/8-h-dark cycle for 8 d. A group of 10 seedlings was transferred into 50-mL polypropylene conical tubes with their roots exposed to water containing no nutrients. Senescence was visible after 16 d. Rice tissues were harvested at specified time points and subjected to further analysis. The data were analyzed by two to five replicates, and then the Duncan’s multiple test was also accompanied by a marked increase in the Trp content with a rapid induction of anthranilate synthase enzyme activity and corresponding mRNAs (Ishihara et al., 2008).

**RNA Gel Blot Analysis**

Total RNA (10 μg) was isolated from leaves of transgenic or wild-type rice plants using TRI reagent (Sigma-Aldrich). Northern-blot analysis was performed as described previously (Kang et al., 2007b). A series of cDNA clones were provided by the National Institute of Agrobiological Sciences (http://www.nrgc.dna.affrc.go.jp/). These cDNAs were used in this study include Gln synthetase (AK243037) and senescence-related marker genes, such as Osl2 (AK102306) and Osl139 (AK073816). All of these cDNAs were radiolabeled using a Prime-It Kit (Stratagene) and used for hybridization during northern-blot analysis.

**Construction of the TDC1 RNAi Binary Vector and Transgenic Rice Plants**

*Agrobacterium tumefaciens*-mediated transformation was used to generate TDC1 RNAi transgenic rice plants. The rice TDC1 gene was amplified with the following primers: 5’-CTGGATACCATCTAGATGACCATGACGAGCCAC-3’ (KpnI and SpeI sites underlined) and 5’-GGATCCGAGCTCCTCCGTATTATGGATATGAC-3’ (SpeI and SalI sites underlined). The PCR product of 519 bp was digested with either SpeI and SpeI or with KpnI and BamHI, gel purified, and ligated into the same restriction sites within the pTCK303 binary vector (a kind gift from Dr. Kang Chong of the Institute of Botany, Chinese Academy of Sciences, Beijing, China). The resulting TDC1 genes were now arranged in the order of an antisense TDC1, a rice intron, and sense TDC1 fragments between the maize (*Zea mays*) ubiquitin promoter and the nos 3’-terminator in the pTCK303 binary vector (Fig. 8A). After verifying the DNA sequence, the pTCK303-TDC RNAi binary vector was transformed into *Agrobacterium LBA4404*. Rice transformation was performed as previously described (Lee et al., 2000).

**Immunoblotting**

A polyclonal mouse antiserum raised against the purified recombinant rice TDC1 protein was employed for immunoblot analysis (Peptron). Rice leaves (0.2 g) were homogenized in a mortar and pestle with 1 mL of homogenization buffer: 80 mM Tris-HCl (pH 7.0), 20% (w/v) glycerol, 10 mM sodium metabisulfite, 10 mM sodium ascorbate, 1% (w/v) polyvinyl pyrrolidone, 5 mM β-mercaptoethanol, and 2 mM EDTA for the extraction of total soluble proteins. After centrifuging for 10 min at 13,500 g, the supernatant extracts were used as total soluble proteins. Proteins were separated by 10% SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. Immunodetection was performed according to standard procedures (Boehringer Mannheim).

**Measurements of Trp, Serotonin, and Serotonin Derivatives**

Rice tissues (0.25 g) frozen with liquid nitrogen were homogenized to a fine powder using a mortar and pestle. Methanol was added to the powder, and the mixture was passed through a filter (Millipore GF/C). Water (100 μL) was added to a 400-μL aliquot of the filtrate, and the mixture was passed through a Sep-pak Light C18 cartridge (Waters) that was equilibrated with 80% methanol. The cartridge was washed with 500 μL of 80% methanol and...
the effluent concentrated in a vacuum centrifuge. The resulting residue was dissolved in 40 μL of 50% methanol. This solution was analyzed by reversed-phase HPLC (Shimadzu) for quantification of serotonin and Trp. Compounds were separated on an Atlantis C18 column (3.9 × 150 mm; Waters) with an isocratic elution profile of 5% (v/v) methanol in water containing 0.3% trifluoroacetic acid at a flow rate of 0.8 mL/min. The elution of compounds was detected at 280 nm. The methanol extracts were passed through a Sep-Pak Silica cartridge (Waters) for analysis of serotonin derivatives. The fraction eluted in chloroform:methanol (30:1) was evaporated to dryness and dissolved in 0.5 mL of methanol. Detection of serotonin derivatives was measured at 320 nm under the same HPLC conditions described above.

Activity Measurements of AS, T5H, and TDC Enzymes

AS activity was measured as described previously (Bücker et al., 1995; Matsuoka et al., 2002). In brief, tissue extracts containing 20 μL Gln were incubated with 1 μM chorismate in 0.1 M citrate/phosphate buffer (pH 7.5) at 30°C for 1 h; the reaction was terminated by boiling for 5 min. After centrifugation at 12,000g for 10 min, the supernatants were subjected to HPLC for quantification of anthranilate. Compounds were separated on a Waters Atlantis C18 column (3.9 × 150 mm) with an isocratic elution profile of 45% methanol in water containing 0.3% (v/v) phosphoric acid at a flow rate of 0.8 mL/min. The anthranilate was determined via fluorescence detection (λex 340 nm, λem 400 nm). T5H and TDC activities were measured as described previously (Kang et al., 2007a, 2007b).

Radical Scavenging Activity Using the 1,1-Diphenyl-2-Picrylhydrazyl Method

The radical scavenging activity of a series of compounds, including Trp and serotonin, was measured using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method as described previously (Kang et al., 2005). Radical scavenging activity was expressed as a percentage of inhibition and was calculated using the following numerical formula: percentage of radical scavenging activity = (control OD – sample OD/control OD) × 100.

Immunohistochemical Localization of TDC Proteins and Serotonin

For immunolocalization, rice leaves were fixed in 0.05% glutaraldehyde and 4% paraformaldehyde in 50 mM sodium phosphate buffer (pH 7.0), dehydrated in ethanol, and embedded in paraffin. Tissues were sliced into 7-μm-thick transverse sections. The deparaffinized sections were incubated with mouse antiserum against rice TDC at a dilution of 1:7,000 or serotonin (Alpha Diagnostic) at a dilution of 1:100, respectively. According to the manufacturer’s instructions, the primary antibodies were detected with the Dako LSAB 2 System (DakoCyto) and colorized using 3-amin-9-ethylcarbazole for TDC or enhanced diamino benzidine for serotonin, respectively. Control experiments using preimmune sera were unreactive.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Effects of serotonin on chlorophyll, ROS, and MDA levels in attached leaves during senescence of rice seedlings.

Supplemental Figure S2. Effects of serotonin on Os/l2 gene expression and TDC enzyme activity in attached leaves during senescence of rice seedlings.

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LITERATURE CITED


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Serotonin Biosynthesis in Rice Plants


