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Senescence Induced Serotonin Biosynthesis and Its Role in Delaying Senescence in Rice Leaves

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ABSTRACT

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 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 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.
INTRODUCTION

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 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 fw (fresh weight) 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, although the inverse is true of the fruit of Ananas comosus (pineapple) (Udenfriend et al., 1959; Foy and 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 Mucuna pruriens (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 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,
glutamine synthetase assimilates ammonia together with glutamate via the synthesis of glutamine, which directly serves as a substrate for tryptophan synthesis. However, this hypothesis has not been corroborated by further enzymatic or molecular analysis.

Serotonin biosynthesis occurs via two enzymatic steps (Fig. 1A). The first committed enzyme is tryptophan decarboxylase (TDC), which catalyzes the conversion of tryptophan 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 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-fold 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 tryptophan 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 RNAi lines.

RESULTS

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

When 9-day-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 fresh weight (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 less 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
sucrose or on a 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 non-senescent 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. 2B, 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 Tryptophan Decarboxylase in Parallel with Serotonin Accumulation**

Serotonin is consecutively synthesized from tryptophan by two enzymes. Tryptophan decarboxylase (TDC) is the first committed enzyme, which catalyzes the conversion of tryptophan to tryptamine, followed by catalysis of tryptamine to produce serotonin by tryptamine 5-hydroxylase (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. 3A, B). The *TDC1* mRNA transcript was not detected in healthy tissues even after 6 d of senescence treatment. 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-day-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, tryptophan, 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 tryptophan as the rice leaves
undergo senescence. In addition, the effects of exogenous applications of serotonin on
leaf senescence were investigated by measuring chlorophyll, ROS, and MDA. As shown
in Supplemental Figure S1, treatment with 500 μM serotonin showed two-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 three-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 1–4, we show the course of serotonin synthesis as well as its effects on
senescence (Supplemental Figs. S1, 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 tryptophan content also increased greatly in parallel with the serotonin levels upon senescence (Fig. 5B). The relative levels of tryptophan 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 tryptophan synthesis than those detected in the untreated and ABA-treated leaves. The relative levels of serotonin in the detached leaves upon 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 Tryptophan 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 tryptophan.
To test whether tryptophan biosynthetic genes are induced upon senescence, five
tryptophan biosynthetic genes were selected for characterization. Figure 6A shows that
the mRNAs encoding the enzymes anthranilate synthase (AS$_{\alpha}$1 and AS$_{\beta}$2),
phosphoribosylanthranilate transferase (PAT), indole-3-glycerolphosphate synthase
(IGPS), and tryptophan synthase (TS$_{\alpha}$) were induced upon senescence. The induction
patterns differed. The mRNAs encoding the AS$_{\alpha}$1, AS$_{\beta}$2, and PAT enzymes were
induced after 4 d upon senescence treatment whereas the mRNA transcripts for IGPS
and TS$_{\alpha}$ were shown to be induced at 6 d. In addition, glutamine synthetase (GS) 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 tryptophan 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-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 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 upon senescence (Fig. 7A). Serotonin concentrations in leaves were 16 µg/g fw in the wild type, whereas the transgenic lines (lines 10 and 14) produced more than 250 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, tryptophan levels were lower in the transgenic lines (10 and 14) than in the wild type because the tryptophan was 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 (Zea mays) ubiquitin promoter, and 20 independent transgenic lines were generated through Agrobacterium-mediated transformation (Fig. 8). Among them, two lines (RNAi-11 and RNAi-16) of T1 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 μg g⁻¹ fw serotonin 6 d after senescence, whereas the RNAi lines (T₀), such as RNAi-11 and RNAi-16, only produced 125 and 207 μg 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, tryptophan levels did not dramatically change in the RNAi lines, although the RNAi lines had lower levels of tryptophan 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 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 tryptophan 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 two-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 differences 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 two-fold higher radical scavenging activity (RSA) 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. 10C, 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. 10D, 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 tryptophan 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
While most genes are inactivated during senescence, particular sets of genes, referred to as senescence-related genes (SAGs), are activated and participate in catabolic activities. The SAGs 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 upregulated (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 tryptophan-derived secondary metabolite, was found to be employed in rice as either a substrate to synthesize serotonin derivatives such as feruloylserotonin and 4-coumaroylserotonin or incorporated directly into the cell wall upon pathogen attack (Jang et al., 2004; Ishihara et al., 2008). In this study, we found for the first time 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 Tryptophan 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 glutamine and asparagines, which serve as long-distance transport forms
of organic nitrogen (Hayashi and Chino, 1990). In addition to these amino acids, other amino acids, including tryptophan, were reported to increase in concentration during senescence in tobacco flowers and in the detached leaves of oats and *Arabidopsis*, and were presumably subjected to remobilization for developing vegetative and reproductive tissues (Soudry et al., 2005). In plants, free tryptophan levels are tightly regulated via allosteric inhibition of anthranilate synthase (AS) by micromolar tryptophan levels, which cause the plant cells to maintain a low level of free tryptophan (Radwanski and Last, 1995). An increase in free tryptophan 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 tryptophan, independent of the induction of tryptophan biosynthetic enzymes (Dubouzet et al., 2007). In addition, we found that free tryptophan levels are also heightened during senescence in rice leaves and that the induced synthesis of tryptophan occurs via the coordinated upregulation of tryptophan biosynthetic genes encoding AS, phosphoribosyl anthranilate transferase (PAT), and tryptophan synthase (TS) enzymes. The induction of tryptophan biosynthetic enzymes was also observed in pathogen-infected rice and *Arabidopsis* leaves, where serotonin and camalexin accumulated in parallel with coordinated induction of tryptophan biosynthetic transcripts (Zhao and Last, 1996; Ishihara et al., 2008). In pathogen-infected rice leaves, the elevated synthesis of tryptophan occurred upon induction of the *AS* genes of which *ASα2*, *ASβ1*, and *ASβ2* were upregulated upon pathogen treatment and *ASα1* was downregulated (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 tryptophan synthesis was followed by the induction of ASα1 as well as ASβ2, suggesting that ASα1 plays a pivotal role in the enhanced synthesis of tryptophan as well as serotonin in a senescence-specific manner. As for serotonin synthesis, the key enzyme is TDC, which catalyzes the conversion of tryptophan into tryptamine. TDC of rice exhibits a high (0.75 mM) $K_m$ toward tryptophan, and thus functions efficiently in the presence of high levels of tryptophan—termed a tryptophan feast (Kang et al., 2008). As expected, the induction of TDC took place at the same time as the tryptophan feast upon senescence. In addition to the induction of tryptophan biosynthetic genes, glutamine synthetase was also induced upon senescence, but its induction occurred at a later stage of senescence compared to that of other tryptophan 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 feruloylserotonin and 4-coumaroylserotonin were also shown to accumulate 8 d after senescence.

**Role of Serotonin in Senescence**

The report on tryptophan-overproducing transgenic rice demonstrated that tryptophan 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 tryptophan levels were significantly induced upon senescence and that the increased tryptophan was readily converted into serotonin by the induction of TDC. Thus, it seems reasonable to think that serotonin, rather than tryptophan, 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 tryptophan and chlorogenic acid. The antioxidant activity of serotonin far exceeds that of tryptophan, 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 being the first report of 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. tryptophan-derived oxidation products or serotonin-derived metabolites). Also, we cannot rule out the possible involvement of tryptophan in indoleacetic acid (IAA) biosynthesis upon senescence, as IAA 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 tryptophan that was induced upon senescence, although tryptophan 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 glutamine synthetase enzyme, which catalyzes the conversion of glutamate to glutamine, 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 tryptophan 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 were surface-sterilized and sown in half-strength Murashige and Skoog (MS) 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 were 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 range was carried out to find the significant differences at $P < 0.05.$
In Vitro Senescence of Leaf Segments

Seeds of wild-type and transgenic rice were immersed in tap water for 2 d to induce germination, and then potted in a tray filled with greenhouse compost (Boonong Soil, Seoul, Korea). Seedlings were grown in a plant growth room at 28°C and 70% humidity in a 16-h light/8-h dark cycle at 150 μmol m⁻² s⁻¹ photosynthetic photon flux density (PPFD) for 4 weeks. The apical 15 cm of the third leaf was used in all experiments. A group of ten segments was transferred into a 50-mL polypropylene conical tube containing 10 mL of water without nutrients and incubated under the same growth conditions mentioned above for specified time periods.

Measurements of Chlorophyll, ROS, and MDA

Chlorophyll content was spectrophotometrically determined according to the method of Lichtenthaler (1987). Relative ROS levels were measured using 2',7'-dihydrodichlorofluorescein diacetate (DCF-DA) (Sigma, St. Louis, MO, USA). Five 5-mm leaf squares were incubated in 50 mM MES buffer (pH 6.2) containing 10 μM DCF-DA for 30 min at 37°C (Rosenvasser et al., 2006). The fluorescence was measured with excitation (485 nm) and emission (530 nm) wavelengths using a Spectra MAX GEMINI (Molecular Devices, Sunnyvale, CA, USA). For the measurements of oxidative stress in leaves, malonyldialdehyde (MDA) was measured using the thiobarbituric acid (TBA) test. Leaves (0.1 g) were homogenized in a 2-mL solution of 0.5% TBA in 20% trichloroacetic acid. The supernatants after 20,000g centrifugation for 15 min were subjected to spectrometric analysis as described previously (Lee et al.,
RNA Gel Blot Analysis

Total RNA (10 µg) was isolated from leaves of transgenic or wild-type rice plants using TRI reagent (Sigma). 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.rgrc.dna.affrc.go.jp/). These cDNAs were anthranilate synthase α1 (AK072053), anthranilate synthase β1 (AK105178), phosphoribosyl anthranilate transferase (AK064915), indole-3-glycerolphosphate synthase (AK059358), tryptophan synthase α (AK066734), and tryptophan synthase β (AK072921), which are all part of the tryptophan biosynthetic pathway. Other cDNAs used in this study include glutamine 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, La Jolla, CA, USA) and used for hybridization during Northern blot analysis.

Construction of the TDC1 RNAi Binary Vector and Transgenic Rice Plants

Agrobacterium-mediated transformation was used to generate TDC1 RNAi transgenic rice plants. The rice TDC1 gene was amplified with the following primers: 5´-CTGGGTACC ACTAGTATGACGCACTGGGCGAGC-3´ (KpnI and SpeI sites underlined) and 5´-GGGATCC GAGCTCCCTGCATCGCCTCCAGCA-3´ (BamHI and SacI sites underlined). The PCR product of 519 bp was digested with either SacI 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 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 tumefaciens* LBA4404. Rice transformation was performed as previously described (Lee et al., 2000).

**Immunoblotting**

A polyclonal mouse antiserum raised against the purified rice **TDC1** protein was employed for immunoblot analysis (Peptron, Daejeon, Korea). 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 metabisulfate, 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 13,500g, the supernatant extracts were used as total soluble proteins. Proteins were separated by 11% SDS-PAGE and electroblotted onto PVDF membranes. Immunodetection was performed according to standard procedures (Boehringer Mannheim, Mannheim, Germany).

**Measurements of Tryptophan, 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 (Millex-LG; Millipore, Billerica, MA, USA). 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, Milford, MA, USA) that was equilibrated with 80% MeOH. The cartridge was washed with 500 µl of 80% MeOH and the effluent concentrated in a vacuum centrifuge. The resulting residue was dissolved in 40 µl of 50% MeOH. This solution was analyzed by reversed-phase HPLC (Shimadzu, Kyoto, Japan) for quantification of serotonin and tryptophan. 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 C18 cartridge (Waters) and further fractionated with 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

Anthranilate synthase (AS) activity was measured as described previously (Bücker et al., 1995; Matsukawa et al., 2002). In brief, tissue extracts containing 20 mM glutamine were incubated with 1 µM chorismate in 0.1 M citrate/phosphate buffer (pH7.5) 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 0.1% (v/v) phosphoric acid at a flow rate of 0.8 mL/min. The anthranilate was determined via fluorescence detection (λ_ex 340 nm/λ_em 400 nm). Tryptamine 5-hydroxylase (T5H) and TDC activities were measured as described previously (Kang et al., 2007a, b).

**Radical Scavenging Activity Using the DPPH Method**

The radical scavenging activity of a series of compounds including tryptophan 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: % 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 antisera against rice TDC at a dilution of 1:7,000 or serotonin (Alpha Diagnostic, San Antonio, TX, USA) at a dilution of 1:100, respectively. According to the manufacturer’s instructions, the primary
antibodies were detected with the Dako LSAB®2 System (Dako North America, Carpinteria, CA, USA) and colorized using 3-amino-9-ethylcarbazole (AEC) for TDC or enhanced diamino benzidine (DAB) for serotonin, respectively. Control experiments using preimmune sera were unreactive (data not shown).

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. Eight-day-old rice seedlings were transferred into conical tubes containing water supplemented with either 100 μM or 500 μM serotonin and incubated as described in Figure 1. Rice leaves were harvested at the indicated time points and subjected to analysis. Data represent the means ± S.D. of two replicate samples. *Different letters indicate significant differences according to Duncan’s multiple range test (P < 0.05).

Supplemental Figure S2. Effects of serotonin on Osl2 gene expression and TDC enzyme activity in attached leaves during senescence of rice seedlings. Experimental conditions were the same as in Figure S1.

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We thank Dr. Kang Chong (Institute of Botany, Chinese Academy of Sciences, Beijing, China) for the pTCK303 RNAi binary vector and the National Institute of Agrobiological Sciences (http://www.rgrc.dna.affrc.go.jp/) for providing the tryptophan biosynthetic cDNAs.
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FIGURE LEGENDS

**Figure 1.** Biosynthetic pathway of serotonin and its induced synthesis during senescence in the attached leaves of rice seedlings. A, Biosynthesis of serotonin. Serotonin is produced from tryptophan by the activities of two enzymes, tryptophan decarboxylase (TDC) and tryptamine 5-hydroxylase (T5H). B, Phenotype of rice seedlings upon senescence. C, Induced synthesis of serotonin upon senescence. Eight-day-old rice seedlings were transferred into conical tubes containing water without nutrients and incubated for the indicated time periods at 28°C with a 16-h light/8-h dark cycle. The data are the means ± S.D. of two replicates.

**Figure 2.** Biochemical analysis of changes upon senescence of attached leaves of rice seedling. A, Chlorophyll levels. B, Changes in reactive oxygen species (ROS). C, Malondialdehyde (MDA) production. D, Expression of senescence-related marker genes. Rice tissues from the attached leaves of rice seedlings were harvested and subjected to analyses at the indicated time periods. ROS levels were determined by 2’,7’-dihydrodichlorofluorescin diacetate (H\textsubscript{2}DCF-DA) using leaf disks. *Osl2* (AK102306) and *Osl139* (AK073816) were employed as senescence-related marker genes. The presented data (A–C) are the means ± S.D. of two replicates.

**Figure 3.** Expression of *TDC* mRNAs and enzyme activity measurements of TDC and T5H during the senescence of attached leaves of rice seedlings. A, Expression patterns of *TDC1* mRNA. B, Expression patterns of *TDC2* mRNA. C, TDC and T5H enzyme activities. TDC and T5H enzyme activities were measured using rice leaf extracts and are expressed as pkat mg\textsuperscript{-1} protein. Data represent the means ± S.D. of two replicate
samples.

**Figure 4.** Immunoblotting of TDC expression and levels of serotonin and tryptophan in different parts of a senescing leaf. A, Immunoblot analysis of TDC. B, Levels of serotonin and tryptophan. Leaves used were from the attached leaves of rice seedlings 16 d after the onset of senescence treatment as described in Figure 1. The expression of tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) indicates the equal loading of protein samples. Data represent the means ± S.D. of two replicate samples.

**Figure 5.** Effects of plant hormones on the induced synthesis of serotonin, tryptophan, 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 tryptophan levels. C-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 ten 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 ± S.D. of two replicate samples.

**Figure 6.** Regulation of the tryptophan biosynthetic pathway upon senescence of detached rice leaves. A, Expression of tryptophan biosynthetic mRNAs. B, Measurement of anthranilate synthase (AS) enzyme activity. Experimental conditions were the same as described in Figure 5 except for the hormonal treatments. ASα1, anthranilate synthase α1 (AK072053); ASβ1, anthranilate synthase β1 (AK105178);
**Figure 7.** Biochemical and physiological analyses of TDC-overexpressing transgenic lines on the senescence of detached rice leaves. A, Immunoblot analysis of the TDC protein. B, Analysis of serotonin contents. C, Analysis of tryptophan contents. D, Phenotype of detached rice leaves after 8 d of senescence. E, MDA production. F, Chlorophyll levels. The detached rice leaves were subjected to senescence as described in Figure 5. WT, wild type; 10–18, TDC-overexpressing transgenic lines. Data represent the means ± S.D. of three replicate samples. *Different letters indicate significant differences according to Duncan’s multiple range test (P < 0.05).

**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 tryptophan levels. D-E, Chlorophyll levels (D) and phenotype 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 ± S.D. of five replicate samples. *Different letters indicate significant differences according to Duncan’s multiple range test (P < 0.05).
Figure 9. Biochemical and physiological analyses of the SHT-overexpressing transgenic line and measurements of the radical scavenging activity of tryptophan-derived compounds. A, Serotonin contents. B, Tryptophan contents. C, Levels of serotonin derivatives. D, Phenotype of detached rice leaves after 8 d of senescence. E, Radical scavenging activity assessed by 1,1-diphenyl-2-picrylhydrazyl (DPPH). Detached rice leaves were subjected to senescence as described in Figure 5. WT, wild type; TDC-10, TDC-overexpressing transgenic line (T₄); SHT-13, SHT-overexpressing transgenic line (T₄); FS, feruloylserotonin; CS, 4-coumaroylserotonin; FT, feruloyltyramine; CT, 4-coumaroyltyramine. Data represent the means ± S.D. of three replicate samples. Data (chlorophyll and DPPH) from duplicates of the same sample were identical. *Different letters indicate significant differences according to Duncan’s multiple range test ($P < 0.05$).

Figure 10. Immunohistochemical localization of the TDC protein and serotonin in rice leaves. A and C, A transverse section of rice leaves incubated with TDC polyclonal antiserum at day 0 and day 7 after the senescence of detached rice leaves, respectively. B and D, A transverse section of rice leaves exposed to serotonin monoclonal antiserum at day 0 and day 7 after the senescence of detached rice leaves, respectively. E, Typical structure of the small vein of the leaf sheath stained using the periodic acid Shiff’s reaction on day 7. F, A magnification of the vascular bundle in C. G, A magnification of the vascular bundle in D. A’-D’, pre-immune controls of A-D; BS, bundle sheath; CC, companion cells; MC, mesophyll cells; MX, metaxylem; S, sieve cells; VP, vascular parenchyma cells; XP, xylem parenchyma cells. The bar represents 10 μm.
Figure 1. Biosynthetic pathway of serotonin and its induced synthesis during senescence in rice and phenotype of rice seedlings. A, Biosynthesis of serotonin. Serotonin is produced from tryptophan by the activities of TDC and T5H. B, Phenotype of rice seedlings upon senescence. C, Induced synthesis of serotonin upon senescence. Eight-day-old rice seedlings were transferred into conical tubes containing water without nutrients and incubated for the indicated time periods at 28°C with a 16-h light/8-h dark cycle. The data are the means ± S.D. of two replicates.
Figure 2.
Figure 3.
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Figure 6.
Figure 7. Biochemical and physiological analyses of TDC-overexpressing transgenic lines on the senescence of detached rice leaves.
Figure 8. Biochemical and physiological analyses of TDC RNAi transgenic lines on the senescence of detached rice leaves.
Figure 9. Biochemical and physiological analyses of the SHT-overexpressing transgenic line and measurements of the radical scavenging activity of tryptophan-derived compounds.
Figure 10. Immunocytochemical localization of the TDC protein and serotonin in rice leaves.

A and C, transverse section of rice leaves incubated with TDC antibodies at day 0 and day 7 after the senescence of detached rice leaves, respectively. B and D, transverse section of rice leaves exposed to serotonin polyclonal antibodies at day 0 and day 7 after the senescence of detached rice leaves.

A-D, pre-immune controls of A-D; BS, bundle sheath; E, companion cells; MC, mesophyll cells; MIX, metaxylem; S, sieve cells; V, vascular parenchyma cells; XP, xylem parenchyma cells. The bar represents 10 μm.