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Serotonin accumulation in transgenic rice by over-expressing tryptophan decarboxlyase results in a dark brown phenotype and stunted growth

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Abstract A mutant M47286 with a stunted growth, low fertility and dark-brown phenotype was identified from a T-DNA-tagged rice mutant library. This mutant contained a copy of the T-DNA tag inserted at the location where the expression of two putative tryptophan decarboxlyase genes, *TDC-1* and *TDC-3*, were activated. Enzymatic assays of both recombinant proteins showed tryptophan decarboxlyase activities that converted tryptophan to tryptamine, which could be converted to serotonin by a constitutively expressed tryptamine 5′ hydroxylase (T5H) in rice plants. Over-expression of TDC-1 and TDC-3 in transgenic rice recapitulated the stunted growth, dark-brown phenotype and resulted in a low fertility similar to M47286. The degree of stunted growth and dark-brown color was proportional to the expression levels of TDC-1 and TDC-3. The levels of tryptamine and serotonin accumulation in these transgenic rice lines were also directly correlated with the expression levels of TDC-1 and TDC-3. A mass spectrometry assay demonstrated that the dark-brown leaves and hulls in the TDC-overexpressing transgenic rice were caused by the accumulation of serotonin dimer and that the stunted growth and low fertility were also caused by the accumulation of serotonin and serotonin dimer, but not tryptamine. These results represent the first evidence that over-expression of TDC results in stunted growth, low fertility and the accumulation of serotonin, which when converted to serotonin dimer, leads to a dark brown plant color.

Keywords Tryptophan decarboxylase · Tryptophan · Tryptamine · Serotonin dimer · Brown pigmentation · T-DNA tagging · Transgenic rice

Abbreviations

DAI	Days after imbibition
GUS	β-Glucuronidase
LC/MS/MS	Liquid chromatography tandem mass spectrometry
NMR	Nuclear magnetic resonance
RT-PCR	Reverse transcription-PCR
TDC	Tryptophan decarboxylase
TYDC	Tyrosine decarboxylase
TT	Homozygous genotype
TW	Heterozygous genotype
WW	Wild type genotype

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Introduction

Serotonin (5-hydroxytryptamine) is a well-known aromatic amine neurotransmitter that controls several important physiological functions such as mood, sleep and anxiety in animals and humans (Veenstra-VanderWeele et al. 2000). Evolutionarily, serotonin existed in plants even before the appearance of animals (Azmitia 2001), with the first report coming 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 suggested to be involved in several physiological functions in plants, such as growth regulation (Csaba and Pal 1982), seed germination (Roshchina 2001), flowering, ion permeability, adaptation to environmental changes (Okjakova and Hadjiivanova 1997) and morphogenesis (Murch et al. 2001). Recently, serotonin has been reported to accumulate in rice leaves to delay senescence (Kang et al. 2009) and at sites of infection as part of the defensive response (Ishihara et al. 2008, 2011). Serotonin is also incorporated into the cell wall at disease lesions infected with *Bipolaris oryzae* and *Magnaporthe grisea*, suggesting that serotonin played an important role in controlling the strength of cell walls as the mechanical barrier against pathogens (Ishihara et al. 2008, 2011). Although multiple physiological functions of serotonin are currently known in plants, the effects of serotonin accumulation on plant growth and pigmentation have not been reported.

In plants, serotonin can be synthesized by L-tryptophan decarboxylases (TDCs), which catalyze the decarboxylation of tryptophan to produce tryptamine, followed by the catalysis of tryptamine 5-hydroxylase (T5H) to form serotonin (Schroder et al. 1999). The study of TDC has been extended to the staple food crop rice for their involvements in the serotonin biosynthesis (Kang et al. 2007a) and for their functions in pathogenic defense (Ishihara et al. 2008, 2011) and delaying the senescence of rice leaves (Kang et al. 2009). In rice, TDC is expressed at very low or negligible levels, while T5H is constitutively expressed (Kang et al. 2007b). Therefore, TDC serves as a bottleneck point regulating serotonin biosynthesis. Accordingly, the accumulation of serotonin can be achieved by over-expressing TDC in transgenic rice plants. For example, the ectopic over-expression of the TDC AK53 and AK31 genes in rice plants resulted in 7- and 25-fold increases in serotonin accumulation, respectively, in the leaves of 14-day-old seedlings (Kang et al. 2007a). Therefore, the key enzyme TDC can be used to genetically modify the metabolic pathways for the production of the pharmaceutically important compounds serotonin and monoterpenoid indole alkaloids in an approach similar to the over-expression of TYDC to produce dopamine or octopamine and to serve as a starting point for benzylisoquinoline alkaloid biosynthesis in several plant species (Facchini et al. 2000; Lee et al. 2009).

Seven putative *TDC* and *TYDC*-like genes have been identified in the rice genome (Supplementary Table S1). Among these genes, one *TYDC* (accession number AK065830, or AK30) and two *TDCs* including AK31 (accession number AK069031, also named *TDC-1*) and AK53 (accession number AK103253, also named *TDC-2*) have been characterized (Kang et al. 2007a, b; Lee et al. 2009). In vitro enzymatic assays demonstrated that both AK31 and AK53 have tryptophan decarboxylase activity and revealed high substrate specificity to tryptophan (Kang et al. 2007a; Park et al. 2011). Over-expression of *TDC* and *TYDC* genes ectopically in transgenic rice plants have been previously reported, and the production of important compounds in these transgenic plants and their effects on plant growth have also been recorded (Kang et al. 2007a; Lee et al. 2009). The ectopic over-expression of the *TYDC* gene (*AK30*) in rice plants resulted in an 80-fold increase in tyramine synthesis, and all of these transgenic rice plants showed stunted growth (Kang et al. 2007a). A further study revealed that the accumulation of tyramine reduced cell division that caused the dwarf phenotype of these *TYDC*-overexpression rice plants (Kim et al. 2011). Whereas the transgenic rice plants that over-expressed *TDC* genes accumulated 7- to 11-fold more tryptamine in 8-week-old leaves and up to 25-fold additional increase of serotonin in seedlings and both grew normally as the phenotype difference or detrimental growth effects were not observed (Kang et al. 2007a).

In the present study, we isolated a T-DNA insertion mutant M47286, which contained a copy of the T-DNA activation-tag inserted at the location where the expression of two *TDC* genes, *TDC-1* and *TDC-3* (accession number BAD11583, named *TDC-3* in this study), were activated and over-expressed. Accordingly, we detected an elevated level of serotonin accumulation in this mutant and in the transgenic rice plants over-expressing either TDC-1 or TDC-3. Uniformly, all the TDC-overexpressing transgenic and mutant rice plants showed stunted growth and had dark-brown leaves, panicles and grains. However, this relationship between the accumulation of serotonin and the phenotypes of dark-brown color and stunted growth has not been previously reported in rice plants. These observations led to the proposition that the phenotypes that appeared in mutant M47286 and in the TDC-over-expressing transgenic rice plants resulted from the accumulation of serotonin and/or serotonin dimer, with the effects of serotonin or its dimer resulting in stunted growth and the accumulation of the serotonin dimer resulting in brown pigmentation.

Materials and methods

Plant materials and growth conditions

Wild-type rice (*Oryza sativa* cv Tainung 67) and M47286 mutant seeds (from Taiwan Rice Insertional Mutants library http://trim.sinica.edu.tw/) were surface sterilized and germinated on MS medium (Murashige and Skoog Basal Medium; Sigma-Aldrich) in a 28°C temperature-controlled growth chamber with a 16 h light/8 h dark cycle for approximately 15–20 days and then transferred to a green house or an isolated paddy field. Arabidopsis seeds (*Arabidopsis thaliana*, Columbia ecotype) were surface sterilized and germinated on GAMBORG B-5 Basal Medium (Duchefa Biochemie) containing 2% sucrose (pH 5.7 with KOH) and 0.8% agar. For transgenic screening, 20 mg/ml of hygromycin was added to the GAMBORG B-5 medium. The germinated Arabidopsis seedlings were transferred to the pot containing soil:vermiculite (3:1) and raised in a 24°C temperature-controlled growth chamber under a 10 h light/14 h dark cycle.

DNA extraction and southern hybridization

Genomic DNA was extracted with CTAB extraction buffer (Doyle and Doyle 1987). Rice genomic DNA (15 μg) was digested with *Not* I or *Xba* I and fractionated on a 1% agarose gel, transferred to a nylon Hybond^{TM}-N+ membrane (Amersham Biosciences) and hybridized with ^{32}P-labeled probes containing the GUS DNA fragment. After washing, the membrane was visualized by a phosphoimager (Typhoon 9200, Amersham Biosciences). The GUS DNA fragment was PCR-amplified with the primer sets listed in the Supplementary Table S2.

Genotyping of the progenies of mutant M47286

Genotyping for the segregated progenies of T-DNA mutant M47286 was performed by the two sets of PCR primers shown in the Supplementary Table S2. One is the gene-specific primer set, and the other is a primer set with a gene-specific primer and a T-DNA specific right border (RB) primer. The PCR was conducted and its product was fractionated on a 1.5% agarose gel in TAE buffer and visualized by ethidium bromide staining.

RNA extraction and RT-PCR

Total RNA was isolated from various stages of rice tissues and Arabidopsis leaves using Trizol reagent (Invitrogen) and then treated with RNase-free DNase I (Promega) to eliminate genomic DNA contamination. The concentration and quality of total RNA were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). First-strand cDNA was synthesized from 4.5 μg of total RNA using the Superscript III reverse transcriptase synthesis system (Invitrogen). RT-PCR analysis was performed using GoTaq DNA polymerase (Promega) using the primers listed in the Supplementary Table S2. RT-PCR products were fractionated on a 1.5% agarose gel in TAE buffer and visualized by ethidium bromide staining.

Western-blot analysis

The rice leaves were milled to a fine power in liquid nitrogen using a mortar and pestle and then extracted with extraction buffer (0.125 M Tris–HCl, pH 8.0, 1 mM EDTA-Na_2, 0.1% SDS). SDS-PAGE-resolved proteins were transferred to Hybond^{TM}-P PVDF membranes (Amersham Biosciences) for western blotting. The membrane was blocked with 5% non-fat powdered milk in TBS (20 mM Tris–HCl pH 7.5, 0.5 M NaCl) for 30 min and then incubated with rabbit immune serum containing specific polyclonal antibodies against the TDC-1 or TDC-3 proteins for 2 h. The membrane was washed 3 times with TBST (TBS containing 0.1% (v/v) Tween 20) and incubated with horseradish peroxidase-conjugated secondary antibodies (GeneTex, Inc.) for 1 h. The antibody recognized protein signals were visualized using the ECL Detection Kit (Millipore) and photographed using a chemiluminescence-sensitive camera device (Fuji-Film LAS-4000, Fuji-Film Life Science).

Construction of TDC overexpression vectors and plant transformation

Full-length cDNAs of *TDC-1* (1,545 bp) and *TDC-3* (1,572 bp) were isolated by PCR from rice and fused downstream of the maize ubiquitin (Ubi) promoter, generating Ubi:*TDC-1* and Ubi:*TDC-3* constructs for rice transformation. The primer pairs are shown in the Supplementary Table S2. The PCR products were ligated into the pGEM-T Easy cloning vector (Promega), and their sequences were confirmed by DNA sequencing. The TDC-1 and TDC-3 DNA fragments were digested with *BamH*I, gel-purified and ligated into the same restriction sites between the Ubi promoter and the nopaline synthase (Nos) terminator in plasmid pAHC18 (Bruce et al. 1989). Plasmids containing Ubi:*TDC-1* and Ubi:*TDC-3* were linearized with *Hind*III and inserted into the same site in pCAMBIA1301 (Hajdukiewicz et al. 1994). The resulting binary vectors were transferred into *Agrobacterium tumefaciens* strain EHA105 for rice transformation (Hiei et al. 1997) as well as for Arabidopsis transformation using the floral dip method (Clough and Bent 1998).

Enzyme activity and an assay for the substrate-specificity of tryptophan decarboxylase

The same full-length cDNAs of *TDC-1* and *TDC-3* were inserted into the pET30a vector (Novagen) for protein expression in *Escherichia coli* BL21 (DE3) (Novagen). Protein expression was induced by adding 0.1 mM IPTG at 28°C for 6 h. His-tagged TDC proteins were purified using His-Bind resin (Novagen). The protein concentration was measured using the Bradford method with bovine serum albumin as the standard. The purified proteins were subjected to enzymatic assay and used for polyclonal antibody induction in rabbits.

The substrate specificity and enzymatic activity assays for the purified TDC proteins were performed according to the method described in Kang et al. (2007a) with minor modifications. In brief, 6 μg of purified TDC protein was incubated in the 50 mM Tris–HCl buffer (pH 7.0) with 10 mM L-tryptophan, L-tyrosine or L-dopa and 1 mM pyridoxal-5-phosphate at 30°C for various reaction times, and then the reaction was stopped by heating at 95°C for 5 min. The resulting solutions were subjected to HPLC for product analysis using a C-18 column (Inertsil 10 ODS, 3.2 × 250 mm, VERCOPAK) with an isocratic elution of 10% (v/v) methanol in water containing 0.3% trifluoroacetic acid at a flow rate of 1.0 ml/min. Elution of the compounds was detected at 280 nm using a Waters 2996 Photodiode Array Detector (Waters, Milford, MA).

Measurement of tryptamine and serotonin

Tryptamine and serotonin were analyzed using the method described in Kang et al. (2007a) with minor adjustments. In brief, the rice leaves (0.1 g) were ground with liquid nitrogen into fine powders and extracted with 2 ml of methanol. The sample was centrifuged at 3,500×*g* for 5 min, and the supernatant was filtered through a 0.22 μM PVDF membrane filter (Chrom Tech, Germany), and 250 μl of DIH_2O was added to a 1 ml aliquot of the filtrate. The mixture was then filtered through an Oasis® HLB cartridge (Waters, Milford, MA) that was equilibrated with 80% methanol. The filtered sample was evaporated to dryness under vacuum and dissolved in 200 μl of 50% methanol. The tryptamine and serotonin contents of the filtered samples were detected under the same HLPC conditions mentioned above.

UV-catalyzed photochemical reaction and identification of the serotonin dimer structure

Serotonin (5 mM) dissolved in 50% methanol was exposed to a continuous UV light using one 10 watt 254 nm lamp (Sankyo-Denki G10T8, Japan) at 25°C for 10 days. The control serotonin was kept in the dark for the same period of time. These samples were subjected to LC/MS/MS as described in the next section below for product analysis.

^{1}H NMR spectroscopy (400 MHz, Me2SO-d6, 298 K) was used to identify the structure of the serotonin dimer. For the photo dimerization reaction, the serotonin compound was kept in a half-open UV-transmitted quartz cuvette and placed under a Rayonet RPR-200 photoreactor (Southern New England Ultraviolet, Hamden, CT) containing eight 35 watt 254 nm lamps for 2.5 and 5 h. All NMR data were collected on a Varian Mercury Plus-400 spectrometer in Me_2SO-d_6.

Pigment extraction, liquid chromatography and mass spectrometry

Rice materials such as hulls and leaves (0.1 g) were extracted with 2 ml of methanol and then centrifuged, and the supernatant was filtered through a 0.22 μM PVDF membrane filter as mentioned above. The filtrate was evaporated to dryness and stored at −20°C until use.

The solutions extracted from rice hulls, leaves and serotonin standards (Sigma, St. Louis, MO) were dissolved in 50% methanol and infused into the mass spectrometer via an electrospray source at a flow rate of 4 μl/min. All mass spectrometric analyses were performed on a Thermo Fisher Scientific LTQ^{XL} (San Jose, CA) linear ion trap mass spectrometer. For MS/MS analysis with collision-induced dissociation (CID), the precursor ion width was set at 2 Da, and the normalized collision energy was 35% eV. Samples for LC–MS/MS analyses were introduced into the mass spectrometer via high-performance liquid chromatography (HPLC) using an Agilent 1200 series binary HPLC pump and standard autosamplers (Agilent, Palo Alto, CA). Samples were separated using an XBridge Shield RP-18 column (2.5 μm, 2.1 × 50 mm, Waters, Milford, MA). Mobile phase A consisted of 2 mM ammonium acetate (Sigma, St. Louis, MO) and mobile phase B consisted of 2 mM ammonium acetate in 75% acetonitrile (Merck, Darmstadt, Germany). The flow rate for HPLC was 0.15 ml/min with linear gradients of: (1) isocratic at 0% B for 3 min; (2) 0–100% B for 10 min; and (3) isocratic at 100% B for 10 min. Data were collected and analyzed using the vendor-supplied Xcalibur data system.

For quantitative experiments with selective reaction monitoring (SRM) mode, precursor ion width was set at 2 Da and the automatic gain control (AGC) of the precursor ions for MS/MS scan was set at 1×10^5. For serotonin dimer, the SRM transition was m/z 351 → 334. The area under the curve was calculated for serotonin dimer using the vendor-supplied Xcalibur data system.

Analysis of the growth effects of tryptamine, serotonin or serotonin dimer treatments

Seeds of wild type Arabidopsis (*A. thaliana,* Columbia ecotype) were sterilized and grown on GAMBORG B-5 media (Duchefa Biochemie) as described above for either 10 days (for experiment I) or 24 days (for experiment II) before treatment. The Arabidopsis plants were then transferred to a Magenta box (Glassco, India) containing GAMBORG B-5 media supplemented with one of the following compounds: (1) 1 mM tryptamine, (2) 1 mM serotonin (Sigma, St. Louis, MO) or (3) serotonin dimer derived from UV-treated 1 mM serotonin (exposed to 254 nm UV at 25°C for 10 days) for the time indicated in Fig. 11. Plants grown in the Magenta box were kept in the growth chamber at 24°C under a 10 h light/14 h dark cycle. All compounds with concentration indicated above were dissolved in water and added into the media.

Pollen viability assay

The pollen viability was evaluated using the same method described in Chhun et al. (Chhun et al. 2007). Anthers before flowering were collected and crushed into a fine powder on a glass slide and then stained with 20 µl of 1% (v/v) of I_2 in 3% (v/v) KI. A 5 µl portion of the pollen sample was examined using a light microscope. Grains stained black were recognized as viable pollen, and those stained yellow or light red were recognized as non-viable or sterile pollen.

Results

Characterization of mutant M47286

The T-DNA insertion mutant M47286 isolated from the Taiwan Rice Insertional Mutants (TRIM) T-DNA-tagged rice mutant library was found to have stunted growth, dark-brown panicles and grains and dark green leaves (Fig. 1). The T-DNA tag (Hsing et al. 2007) used to generate the TRIM library contained multiple *CaMV35S* enhancers adjacent to the left border, which activated promoters located near the T-DNA insertion sites. This mutant M47286 contained a copy of the T-DNA tag (Fig. 2a) inserted at the location where the expression of two putative tryptophan decarboxlyase genes, *TDC-1* and *TDC-3*, were activated (Fig. 3a, b).

The progenies by selfing of mutant M47286 with heterozygous genotype (TW) were separated into three different phenotypes (Fig. 1a), including (1) the wild type (WW) line, which showed the same phenotype as the wild type; (2) the heterozygous (TW) line, which showed a slight growth retardation and dark-brown-colored panicles; and 3) the homozygous (TT) line, which showed stunted growth with dark-brown colored panicles and leaves (Fig. 1a). The different genotype derived from the

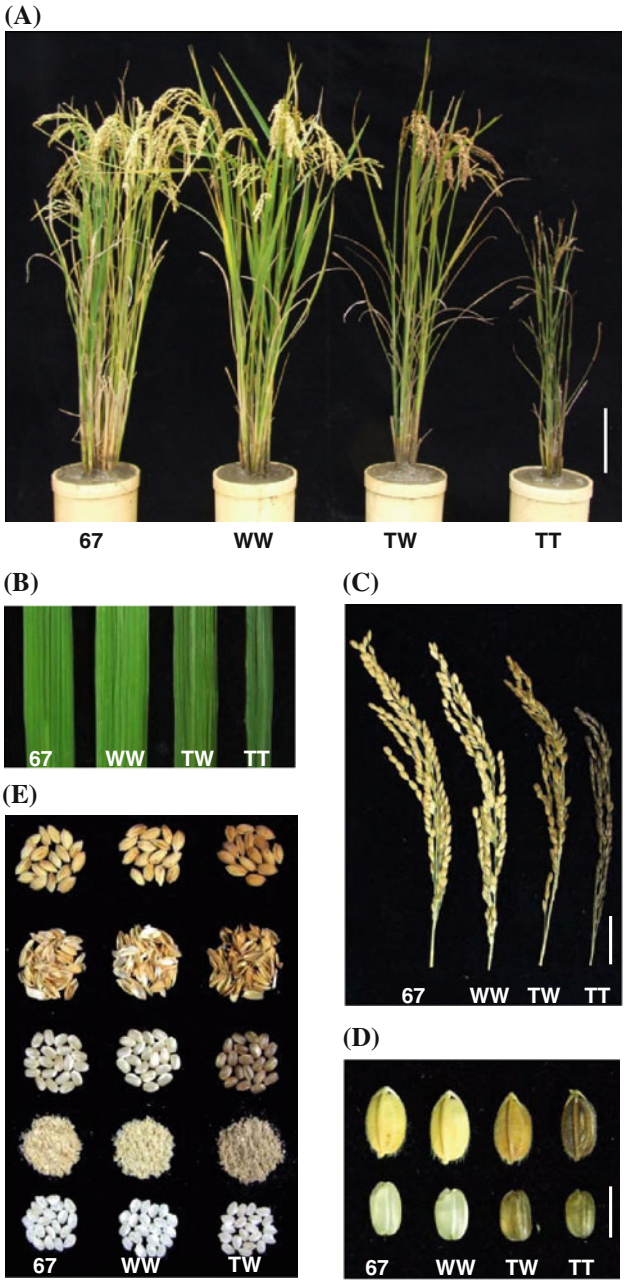

Fig. 1 Morphological characteristics of the T-DNA insertion mutant M47286. **a** Comparison of a wild type plant (TNG67) and progenies of mutant M47286 (T4 plant) with wild type-like (WW), heterozygous (TW) and homozygous (TT) genotypes at the mature stage. *Bar* = 15 cm. **b–d** Comparison of the appearances of the mature leaves (**b**), panicles (**c**) and whole grains and brown rice (**d**) between wild type and various genotypes of mutant M47286. The scale bar is 3 cm in (**c**) and 0.5 cm in (**d**). **e** Color comparison among different parts of rice grains. The materials from the top to bottom are whole grains, hulls, brown rice, rice bran and milled rice

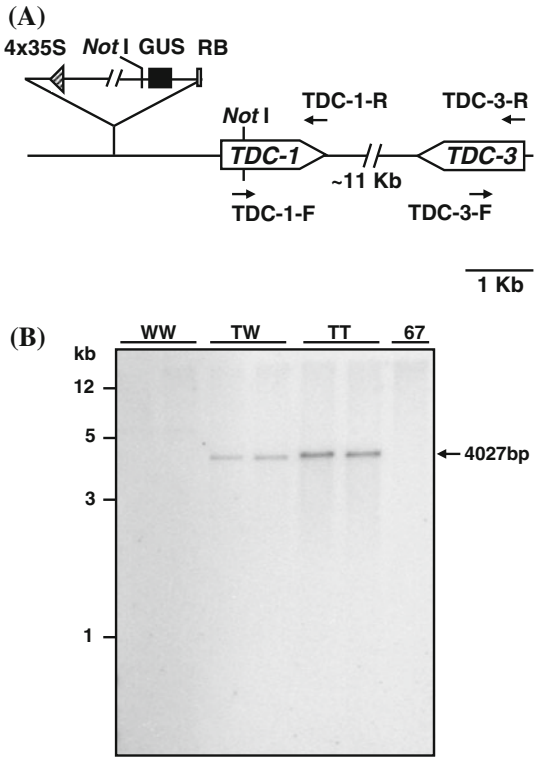

Fig. 2 T-DNA insertion site and its flanking genes in mutant M47286. **a** Schematic diagram showing the genomic organization of tryptophan decarboxylase genes *TDC-1* and *TDC-3* and the location of the T-DNA insertion site. The 4 copies of 35S enhancers (*triangle*) at the *left border* are indicated along with the location of the *right border* (RB) and the GUS gene (*filled box*) in the T-DNA. Gene-specific primer sets for *TDC-1* and *TDC-3* used in RT-PCR are indicated with *arrows*. *Bar* = 1 kb. **b** Southern blot analysis to verify the location and copy numbers of T-DNA insertion in mutant M47286. Genomic DNA (15 μg) from wild type and various genotypes of M47286 was digested with *Not*I and hybridized with a GUS DNA probe. Molecular size markers in kb are shown on the *left* of the membrane

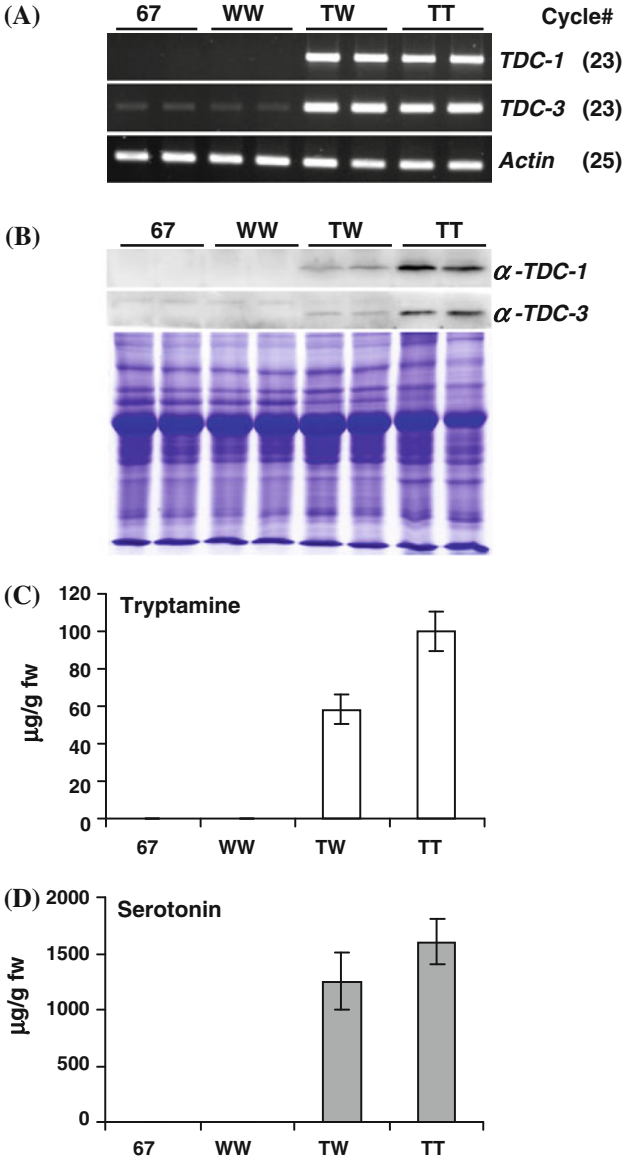

Fig. 3 Expression of *TDC-1* and *TDC-3* genes and the levels of accumulation of tryptamine and serotonin in various genotypes of M47286. **a** Enhanced RNA expression of *TDC-1* and *TDC-3* in M47286. Total RNA isolated from the 70-day-old leaves of wild type (67) and various genotypes of mutant M47286 were used to detect the expression of *TDC-1* and *TDC-3* genes by RT-PCR assay. The expression of the actin gene was used as an RNA quality control. *TDC-1* and *TDC-3* were amplified with 23 PCR cycles. **b** Western blot analysis of TDC-1 and TDC-3. Proteins (60 μg) extracted from 100 DAI (days after imbibition) leaves were subjected to SDS-PAGE, visualized by *Coomassie Blue* (*lower panel*) or detected by antibodies generated against TDC-1 and TDC-3, respectively (*upper two panels*). **c** and **d** The levels of accumulation of tryptamine (**c**) and serotonin (**d**) in 100-day-old leaves of wild type and various genotypes of M47286 were analyzed by HPLC. The data represent the mean values of three independent assays

heterozygous line of M47286 was confirmed by genotyping and Southern blot assay (Fig. 2b). Figure 1 shows the appearance of the leaves (Fig. 1b), the mature panicles (Fig. 1c), the whole grains and brown rice (Fig. 1d) and different parts of the rice grains (Fig. 1e, not enough seeds to show the homozygous line) of various genotypes of M47286. The milled rice of the TW line showed the same white color as that of the wild type (Fig. 1e, bottom panel), suggesting that the brown color mainly existed on the grain surface (Fig. 1e, the 2nd panel from the bottom). All of the appearances of the homozygous (TT) line including leaves, panicles and grains were darker than those of the heterozygous (TW) lines, suggesting that the dark-brown appearance of the mutant lines correlate with the number of T-DNA insertions in the chromosomes.

The inheritance of the dark-brown phenotype was tracked for more than 5 generations, and all progenies from the cross of TT × WW showed heterozygous genotypes (Supplementary Fig. S1B, by PCR genotyping) and had the same phenotype as the TW lines. The progenies from

Table 1 Characterization of T-DNA mutant M47286 rice plants with various genotypes

Traits	WT67[a]	WW[a]	TW[a]	TT[a]
Plant height (cm) at 130 DAI	94.2 ± 3.3[b] (100)[c]	91.6 ± 3.9 (97)	87.3 ± 3.8 (93)	57.3 ± 3.7 (61)
Total tiller number	18.6 ± 2.9 (100)	17.5 ± 1.9 (94)	8.0 ± 1.3 (43)	6.5 ± 1.1 (35)
Heading day (DAI)	88.1 ± 2.0	88.3 ± 2.4	91.4 ± 2.4	101.2 ± 3.8
Panicle length (cm)	21.5 ± 1.0(100)	21.6 ± 1.2(101)	20.4 ± 1.2(95)	15.0 ± 1.3(70)
Panicle weight (g)	3.6 ± 0.4 (100)	3.6 ± 0.5 (98)	2.4 ± 0.2 (66)	0.3 ± 1.0 (8)
Florets per panicle (count)	154.6 ± 10.2 (100)	155.3 ± 13.6 (100)	133.1 ± 10.7 (86)	53.8 ± 10.8 (35)
Fertility (%)	96.2 ± 0.9(100)	93.7 ± 2.7 (97)	75.5 ± 3.0(78)	8.4 ± 7.0(9)
Weight of a thousand seeds (g)	24.7 ± 0.7 (100)	24.9 ± 1.0 (101)	20.9 ± 0.9 (85)	16.0 ± 2.4 (65)

The various genotypes used in this study were the progenies segregated from the T3 plant of M47286

DAI days after imbibition

[a] WT67, wild type host TNG67; WW, the wild type-like lines; TW, the heterozygous lines; TT, the homozygous lines

[b] Standard errors were calculated from sample sizes of n = 10 for all traits, except for the weight of a thousand seeds of TT, where n = 4

[c] Values in parentheses indicate % of the WT67

selfing of TW genotype plants exhibited three different appearances, with genotypes of WW:TW:TT at a ratio of approximately 1:2:1. Table 1 shows the characteristics of various genotypes of mutant M47286 compared to TNG67. In addition to the dark-brown appearance where the homozygous (TT) lines exhibited stunted growth, late heading (101 days compared to 88 days for the TNG67), low fertility (9% of the TNG67), low seed production (35% of the TNG67) and smaller seeds (65% of the size of TNG67 seeds).

Activation of TDC gene expression in mutant M47286

The flanking sequence and Southern blot analysis (Fig. 2) demonstrated that a copy of T-DNA was inserted in chromosome #8 of mutant M47286 at a position 1.6 kb upstream of the translation start codon of TDC-1 and about 15.9 kb upstream of the translation start codon of TDC-3 (Fig. 2a). The expression levels of mRNAs (Fig. 3a) and proteins (Fig. 3b) of TDC-1 and TDC-3 genes were significantly enhanced in both heterozygous (TW) and homozygous (TT) lines. While the accumulation levels of TDC-1 and TDC-3 mRNAs were similar in both the TW and TT lines using semi-quantitative RT-PCR, the real time quantitative PCR results showed that the TT lines were approximately 1.6 fold higher than those in TW lines (Supplementary Fig. S2). Similarly, the TT lines accumulated more TDC proteins than the TW lines (Fig. 3b, due to the antibodies of TDC-1 and TDC-3 cross-reacting with both TDC-1 and TDC-3 antigens, the signals detected in the mutant represent the sum of both TDC-1 and TDC-3 proteins). In addition to *TDC-1* and *TDC-3*, five other TDC-related genes (Supplementary Table S1) were also analyzed by RT-PCR. Results showed either no expression or the same low level expression in *TDC-2* which was observed across different mutant genotypes and TNG67 (Supplementary Fig S3). This observation indicated that expressions of these five TDC-related genes were not altered by the activation of *TDC-1* and *TDC-3* genes in this T-DNA mutant.

The levels of tryptamine, an immediate product of tryptophan catalyzed by TDCs, were also significantly enhanced in TW and TT lines ranging from 60 to 100 μg per gram fresh weight of the 100 DAI (days after imbibition) leaves, while no tryptamine was detected in the leaves of WW and TNG67 specimens (Fig. 3c). Serotonin, converted from tryptamine by tryptamine 5-hydroxylase (T5H), accumulated to approximately 1,200 μg in the TW lines and approximately 1,600 μg in the TT lines, while no serotonin was detected in the WW lines and TNG67 (Fig. 3d).

TDC-3 has tryptophan decarboxylase activity

There are seven tryptophan decarboxylase (TDC)- and tyrosine decarboxylase (TYDC)-like genes in the rice genome (Supplementary Table S1). The evolutionary relationships of these TDC- and TYDC-like genes were described by an unrooted tree constructed using the ClustalW program (Supplementary Fig. S4A). The phylogenetic analysis revealed that the *TDC-3* gene with the accession number BAD11583 was closely related to *TDC-1*, with an amino acid sequence identity of 87% (Supplementary Table S3). To determine the enzymatic activities of TDC-3, full-length recombinant TDC-3 and TDC-1 (used as control) proteins expressed in *E. coli* were purified and subjected to enzymatic assay using various substrates. Results showed that he TDC-3 recombinant

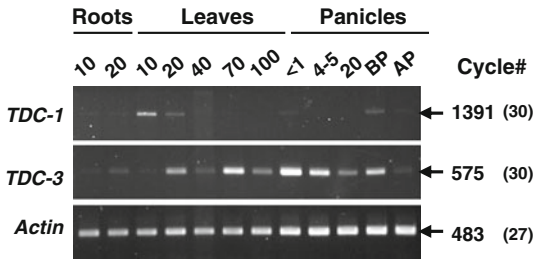

Fig. 4 Expression of *TDC-1* and *TDC-3* in rice. Total RNA isolated from various tissues of wild type plants (TNG67) at various developmental stages were analyzed by RT-PCR using *TDC*-1 and *TDC*-3 gene-specific primers. Vegetative tissues were analyzed, including roots and leaves from young seedlings (10 and 20 DAI) and leaves from the active tillering stage (40 DAI) and maturing stages (70 and 100 DAI) as well as reproductive tissues, including panicles in the early developmental stages with sizes of less than 1 cm, between 4 and 5 cm, and 20 cm in the booting stages and mature panicles after heading but before pollination (BP) or after pollination (AP). *TDC-1* and *TDC-3* were amplified with 30 PCR cycles, and the actin gene was used as an internal control

protein exhibited the same tryptophan decarboxylase activity of converting L-tryptophan, but not L-tyrosine or L-dopa, into tryptamine as TDC-1 (Supplementary Fig. S4C).

Expression profiles of the *TDC-1* and *TDC-3* genes in rice

In Fig. 3, the mRNA expression of *TDC-1* and *TDC-3* were analyzed by RT-PCR using 70-day-old leaves with primers specific for *TDC-1* and *TDC-3*, and the results showed significant expression levels of *TDC-1* and *TDC-3* in the TW and TT lines of mutant M47286, while no *TDC-1* and barely detectable *TDC-3* signals were observed in the WW line using 23 PCR cycles (Fig. 3a). The low expression levels of *TDC* in the wild type led us to pursue further analysis of the expression profiles of *TDC-1* and *TDC-3* in various rice tissues and their possible effects at different growth stages.

This assay used vegetative tissues including the roots and leaves of young seedlings, leaves from the active tillering and maturing stages, reproductive tissues represented by panicles in the early development stages with sizes ranging from less than 1 to 20 cm in the booting stages, and mature panicles before pollination (BP) or after pollination (AP). The mRNA in all these tissues were not clearly detected using the same 23 PCR cycles as previously performed (Fig. 3a), thus 30 PCR cycles reactions were used. The results showed no detectable *TDC-1* mRNA signal in most tissues, except for low levels in the young leaves and very low signals in the mature panicles before pollination (Fig. 4). However, *TDC-3* mRNA was detected in most panicles and leaves, with stronger signals in young panicles and 70 day-old leaves, and both *TDC-1* and *TDC-3* expression levels were higher in the panicles before pollination (Fig. 4). The relatively low expression levels of *TDC-1* and *TDC-3* suggested that only low levels of TDC may be needed for plant growth. On the other hand, that the activation of TDCs in the M47286 mutants may have triggered de novo phenotypic changes such as stunted growth and dark-brown color. To investigate this hypothesis, the *TDC-1* and *TDC-3* genes were overexpressed in TNG67 host plants to analyze their effects on plant growth.

Over-expressing *TDCs* in transgenic rice recapitulate the mutant phenotype

To verify the cause of stunted growth and dark-brown phenotypes in mutant M47286, full-length cDNAs of TDC-1 (1,545 bp) and TDC-3 (1,572 bp) were isolated from rice and fused downstream of the maize ubiquitin (Ubi) (Sun and Gubler 2004) promoter, generating Ubi:*TDC-1* and Ubi:*TDC-3* constructs for rice transformation. More than 20 independent transgenic rice lines were obtained for each construct and showed various degrees of stunted growth and dark-brown colored phenotypes. Five independent transgenic lines confirmed by Southern blot assay (Supplementary Fig. S4) from each construct were selected for further investigations. The morphological characteristics of these transgenic lines were compared to those of transgenic rice carrying the pCAMBIA 1301 vector only (WT-V) (Fig. 5), and their molecular profile were compared, including the levels of mRNA and protein expression for TDC-1 and TDC-3 genes and the accumulations of their metabolic products, tryptamine and serotonin (Fig. 6).

All transgenic lines generated from the Ubi:*TDC-1* and Ubi:*TDC-3* constructs resulted in various degrees of stunted growth (Fig. 5a, b) and showed dark-green leaves (Fig. 5c) and dark-brown panicles and grains (Fig. 5d, e). These morphological observations indicated that overexpression of either TDC-1 or TDC-3 in transgenic rice lines was able to recapitulate partly, if not completely, the stunted growth and dark-brown colored phenotype of mutant M47286. The degrees of recapitulation were slightly different in the Ubi:*TDC-1* and Ubi:*TDC-3* transgenic lines. Most of the Ubi:*TDC-3* transgenic lines (except line #9) have a darker brown color in the leaves, panicles and grains than the Ubi:*TDC-1* transgenic lines (Fig. 5c–e).

In the Ubi:*TDC-1* transgenic rice, four lines (lines # 1, 5, 6 and 10) had significantly increased expression of *TDC-1* mRNA. Among them, line #1 had the highest mRNA level (Fig. 6a) and also the highest protein level as determined by western blot (Fig. 6b) and the highest accumulations of tryptamine and serotonin (Fig. 6c, d). In contrast, in line #8, low TDC-1 mRNA expression (Fig. 6a), no protein expression (Fig. 6b) and no detectable tryptamine were

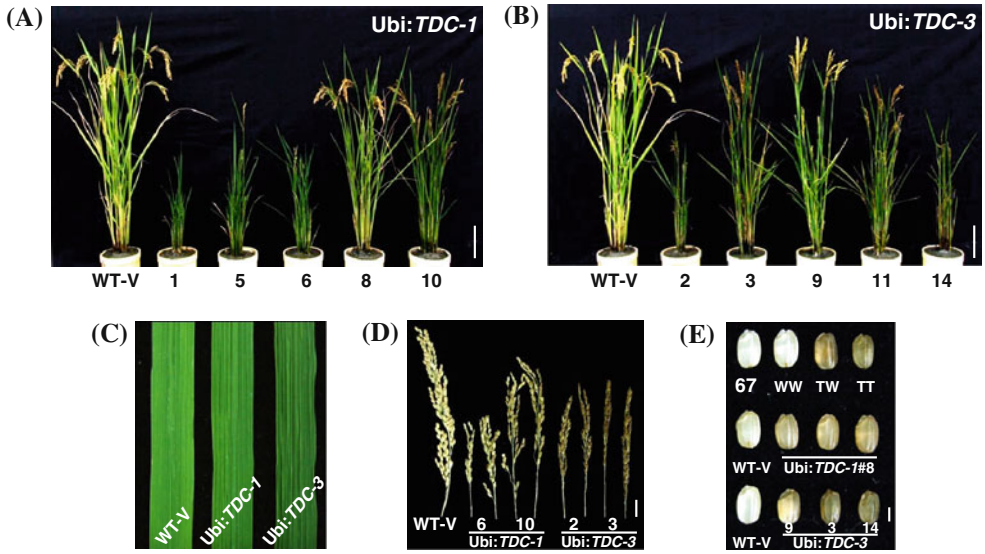

Fig. 5 Morphological characteristics of TDC-1 and TDC-3 overexpressing transgenic rice. **a** and **b** Comparison of a wild type-like vector only (WT-V) transgenic plant and 5 representative T_0 Ubi:*TDC-1* (**a**) and Ubi:*TDC-3* (**b**) transgenic lines at the mature stage. *Bars* = 15 cm. **c** The leaves of WT-V and *Ubi-TDC* transgenic lines after heading. **d** Comparison of the mature panicles of WT-V and *Ubi-TDC* transgenic lines. *Bar* = 2 cm. **e** Comparison of brown rice grains from various genotypes of M47286 (*top*) and transgenic lines of Ubi:*TDC-1* (*middle*) and Ubi:*TDC-3* (*bottom*). *Bar* = 0.2 cm

observed (Fig. 6c), and it grew similar to the WT-V plants and produced fertile seeds (Fig. 5). These data confirm the correlation between molecular characteristics and stunted growth seen in Fig. 5.

Similar relationships were also observed in the Ubi:*TDC-3* transgenic lines. Line #9 showed lower levels of TDC-3 mRNA and protein and low accumulation of tryptamine and serotonin (Fig. 6e–h), and thus grow better than all other lines with higher expression levels of TDC-3 (Figs. 5, 6). Taken together, the studies of transgenic lines suggest that the higher the expression levels of TDCs, the larger the accumulation of tryptamine and serotonin, resulting in more stunted growth.

Polymerization of serotonin dimer caused the dark-brown colored phenotype in rice plants

The correlation of the dark-brown phenotype to the expression levels of TDC genes and the accumulation of tryptamine and serotonin in the T-DNA insertion mutant M47286 (Figs. 1, 3) and in the Ubi:*TDC* transgenic lines (Figs. 5, 6) suggested that serotonin is responsible for the brown color in these rice plants. However, serotonin is a colorless compound, and the accumulation of serotonin itself does not contribute to the dark-brown color in these rice plants. Therefore, we subsequently investigated the cause of the dark-brown colored phenotypes in the TDC-overexpressing transgenic and mutant rice plants.

At first, a 5 mM serotonin solution in clear glass tubes was exposed to UV light with a wavelength of 254 nm for 10 days to stimulate the photochemical reaction and to observe whether the color changed. A brown color developed two days after UV treatment and turned darker after 10 days of UV exposure; the control kept in the dark remained clear with no color development (Fig. 7a). These reaction samples were subjected to ESI–MS analysis with infusion to verify the compound produced after UV treatment. For the dark-treated control serotonin sample, in addition to the major serotonin monomer $[M + H]^+$ (m/z 177, Fig. 7b1 data not included), another product ion detected at m/z 353 (Fig. 7b1) was suggested to be a non-covalent dimer $[2M + H]^+$ of serotonin. Further analysis by MS^2 showed that the product ions detected at m/z 177 and m/z 160 represented a serotonin monomer of $[M + H]^+$ and the fragment ion that lost NH_3 (data not shown). This MS^2 result confirmed the non-covalent serotonin dimer structure inferred by the m/z 353 shown in Fig. 7b1. For the UV-treated serotonin sample, in addition to the serotonin m/z 353 peak, a strong peak at m/z 351 was apparent (Fig. 7b2). The MS^2 assay of m/z 351 produces m/z 334 (Fig. 7b3), which could be further degenerated to m/z 317 and m/z 305 by MS^3 analysis (Fig. 7b4). These results indicated that the product ion at m/z 351 correlated with the brown color was a covalent serotonin dimer, which was generated after exposing serotonin to UV treatment.

To demonstrate that the dark-brown color phenotype in rice plants resulted from the accumulation of the covalent serotonin dimer, the TDC-overexpressing rice plants, including the various genotypes of mutant M47286, were

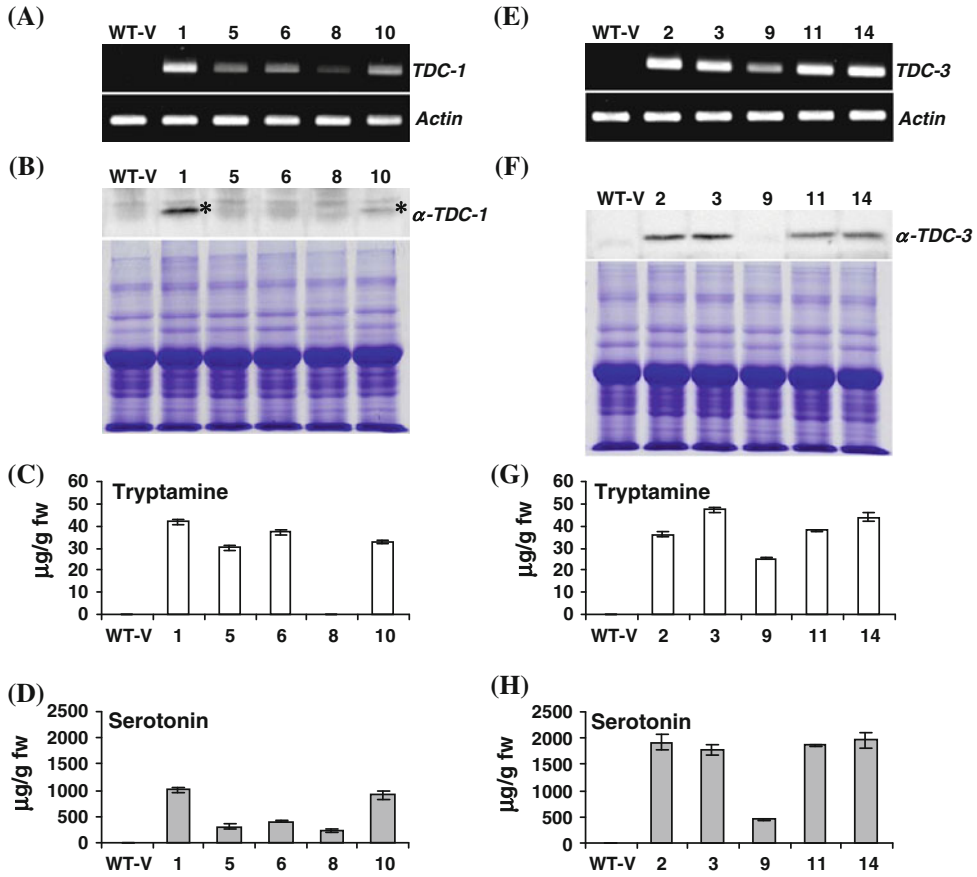

Fig. 6 Expression of *TDC* genes and serotonin accumulation in TDC-1 and TDC-3 overexpressing transgenic rice plants. **a** and **e** RT-PCR results of the expression of *TDC-1* and *TDC-3* genes using total RNAs extracted from the leaves of 5 representative Ubi:*TDC-1* (**a**) and Ubi:*TDC-3* (**e**) transgenic rice lines at 80 days after transplantation to pots housed in the greenhouse. Twenty-three amplification cycles were used for *TDC-1,* and 19 cycles were used for *TDC-3*. The actin gene was used as an internal control. **b** and **f** Western blot analysis of TDC-1 and TDC-3 in TDC-overexpressing transgenic rice. Leaf proteins (60 µg) extracted from each of the same Ubi:*TDC-1* (**b**) and Ubi:*TDC-3* (**f**) transgenic lines as shown in (**a**) and (**b**) were subjected to SDS-PAGE, visualized by *Coomassie Blue* (*lower panel*) or detected by antibodies against TDC-1 or TDC-3, respectively (*upper panels*). **c** and **d** The levels of accumulation of tryptamine (**c**) and serotonin (**d**) in mature leaves of Ubi:*TDC-1* transgenic lines analyzed by HPLC. The data represent the mean values of three independent assays. **g** and **h** The levels of accumulation of tryptamine (**g**) and serotonin (**h**) in mature leaves of Ubi:*TDC-3* transgenic lines analyzed by HPLC. The data represent the mean values of three independent assays

analyzed by LC/MS/MS assay to detect the relative amounts of the covalent serotonin dimer. The results showed that the leaves of homozygous TT lines accumulated more of the covalent serotonin dimer than those of the heterozygous TW lines (Fig. 7c, upper panel), similar to the proportion of serotonin accumulation in the TT and TW lines (Fig. 3d). Three of each Ubi:*TDC-1* and Ubi:*TDC-3* transgenic lines with various degrees of darkness in the leaves were also analyzed, and the results showed a positive correlation between the darkness of color and the amount of the covalent serotonin dimer (Fig. 7c, bottom panel). The covalent serotonin dimer was also collected from the mature rice hulls of the same rice plants and analyzed, and the results showed a similar correlation between the darkness of the color and the amount of dimer (Fig. 7d, e). These results demonstrated that the dark-brown phenotype of TDC-overexpressing transgenic rice plants was closely associated with the accumulation of the covalent serotonin dimer. In addition, a similar brown material deposit in the infection site caused by rice blast pathogen was examined and contained the covalent serotonin dimer as well (Supplementary Fig. S6).

To identify the precise structure of the covalent serotonin dimer produced after UV treatment, ^{1}H NMR spectroscopy analysis was applied. The identities and relative levels of the serotonin products after various UV exposure times was determined by 400 MHz NMR spectroscopy. The UV-treated serotonin products were identified as the covalent serotonin dimer based on the results of ^{1}H NMR spectroscopy (Fig. 8c). The protons with different chemical shifts of the serotonin monomer (precursor) and dimer (products of UV irritation) were marked in ^{1}H NMR

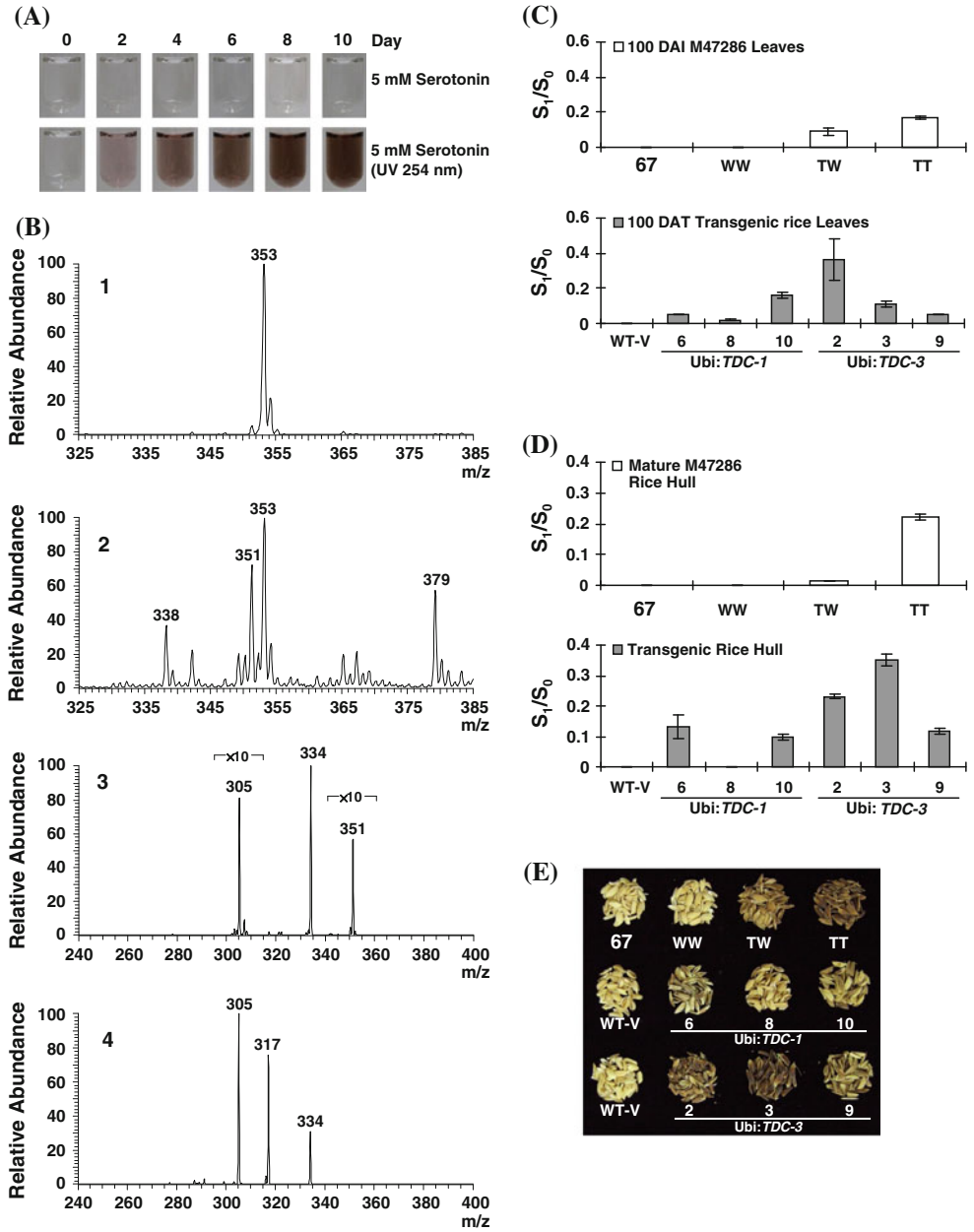

Fig. 7 Identification of serotonin dimer and detection of the levels of accumulation of serotonin dimer in plants. **a** The color development of serotonin exposed to UV light. A 5 mM solution of serotonin was kept in the dark (*top*) or exposed to UV light (*bottom*), and the color development of each treatment was recorded at 2 day intervals for 10 days. **b** Mass spectrum of the serotonin sample kept in the dark for 10 days (*b1*). Mass spectrum of serotonin sample exposed to UV light for 10 days (*b2*). MS^2 spectrum of the precursor ion m/z 351 observed in *b2* (*b3*). MS^3 spectrum of the precursor ion m/z 334 observed in *b3* (*b4*). **c** The relative amount of serotonin dimer in 100-day-old leaves of various genotypes of M47286 (*top*) and Ubi:*TDC-1* and Ubi:*TDC-3* transgenic rice lines (*bottom*) determined by mass spectrometric analysis. **d** The relative amount of serotonin dimer in rice hulls of various genotypes of M47286 (*top*) and Ubi:*TDC-1* and Ubi:*TDC-3* transgenic rice lines (*bottom*) by mass spectrometric analysis. **e** The appearances of rice hulls of various genotypes of M47286 and Ubi:*TDC-1* and Ubi:*TDC-3* transgenic rice lines. The relative amounts of serotonin in rice leaves (**c**) and hulls (**d**) were calculated based on the proportion of the serotonin dimer detected (S_1) to the serotonin dimer detected in the 50 μg sample of serotonin treated with UV light for 10 days (S_0)

spectra for the structure confirmation (Fig. 8c). The identified structures of the serotonin monomer and dimer are shown in Fig. 8a, b, respectively. The ^{1}H NMR spectra obtained from 0, 2.5, and 5 h periods of UV (254 nm) irradiation are shown in Fig. 8d. This figure shows the time course of changes in the serotonin monomer (blue dots) and dimer (red dots) concentrations through the course of the experiment. UV irradiation consumed the serotonin

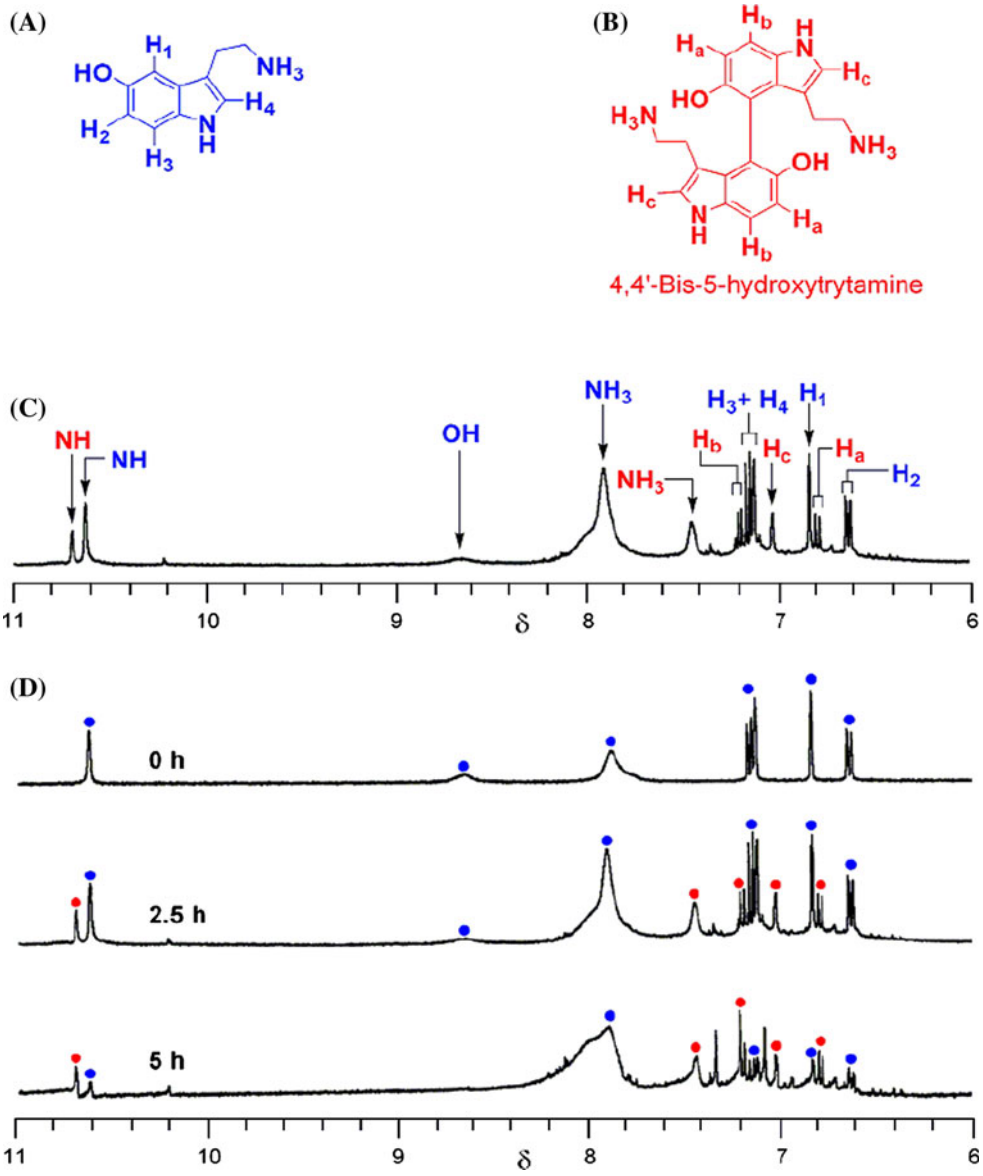

Fig. 8 Partial ^{1}H NMR spectra and the structure of serotonin compounds before and after a period of UV irradiation. Serotonin compound (5 mM) dissolved in methanol was exposed to UV (254 nm) for the time indicated and then subjected to ^{1}H NMR spectroscopy (400 MHz, Me2SO-d6, 298 K) for structure confirmation. **a** and **b** Structure of the serotonin monomer (**a**) and dimer (**b**). **c** ^{1}H NMR spectra of serotonin compounds after a 2.5 h period of UV (254 nm) irradiation. **d** ^{1}H NMR spectra of serotonin compounds before and after a 2.5, 5 or 7 h period of UV irradiation. The proton with a different chemical shift between the serotonin monomer (*blue dot*) and dimer (*red dot*) was marked

monomers to produce serotonin dimer, which turned out to be the compound responsible for the brown color (Fig. 8d).

Over-expressing *TDCs* reduced pollen viability and affected fertilization in female gametophyte

In addition to stunted growth, all of the TDC-over-expressing transgenic and mutant lines showed low fertility (Table 2). The morphology of anthers in the spikelet right before pollination was examined to determine the cause of low fertility. The anthers of the TT line of M47286 were pale yellow, while TNG67, WW line and WT-V plants had a healthy bright yellow color, and the TW line was in between the two. Two representative lines from each TDC-1 and TDC-3 transgenic rice also showed a pale yellow color (Fig. 9a). The pollen viability measured by I_2-KI staining showed less viable pollen in the pale yellow anther samples (Fig. 9b), indicating that less active pollen was available for fertilization.

To verify the fertilization ability of female gametophyte, various female genotypes were crossed with WW line that produced normal pollens. The WW female parent produced more fertile seeds (53%) than the female parents of TW (42%) or TT (21%), suggesting that the female gametophyte in the TW and TT lines were partially defective. Though, the female organ of the TT line was much more severely affected than that of the TW line; however, the TT line remained partially functional as demonstrated by the result that approximately 21% of fertile seeds could be produced (Table 3). The cross of WWxTW (with the TW line as the pollen donor and the WW line as the female parent) produced a similar ratio (57%) of fertile seeds as the WW × WW cross

Table 2 Pollen viability and seed fertility in T-DNA mutant M47286 and TDC transgenic rice

	M47286				
	WT67	WW	TW	TT	
Viable pollen (%)[a]	80.2 ± 5.7 (1,673)[c]	78.6 ± 1.7 (1,680)	68.0 ± 5.9 (1,717)	13.9 ± 9.1 (1,769)	
Fertility (%)[b]	96.2 ± 0.9	93.7 ± 2.7	75.5 ± 3.0	8.4 ± 7.0	
	Transgenic rice				
	WT-V	TDC1-6	TDC1-10	TDC3-2	TDC3-3
Viable pollen (%)[a]	94.8 ± 1.5 (2,228)[c]	15.2 ± 8.8 (1,833)	55.4 ± 19.7 (1,964)	14.1 ± 2.0 (2,019)	78.8 ± 10.3 (2,390)
Fertility (%)[b]	95.3 ± 1.6 (706)[d]	0.0 (112)	0.3 (618)	0.0 (186)	0.2 (491)

[a] The percentage of viable pollen was calculated relative to the total pollen counted. The data represent the mean value ± SD from 3 individual spikelets

[b] Fertility represents the percentage of spikelets that set fertile seeds. The data represent the mean value ± SD from 3 to 10 available individual panicles

[c] Values in parentheses indicate total pollen grains counted

[d] Values in parentheses indicate total seeds counted

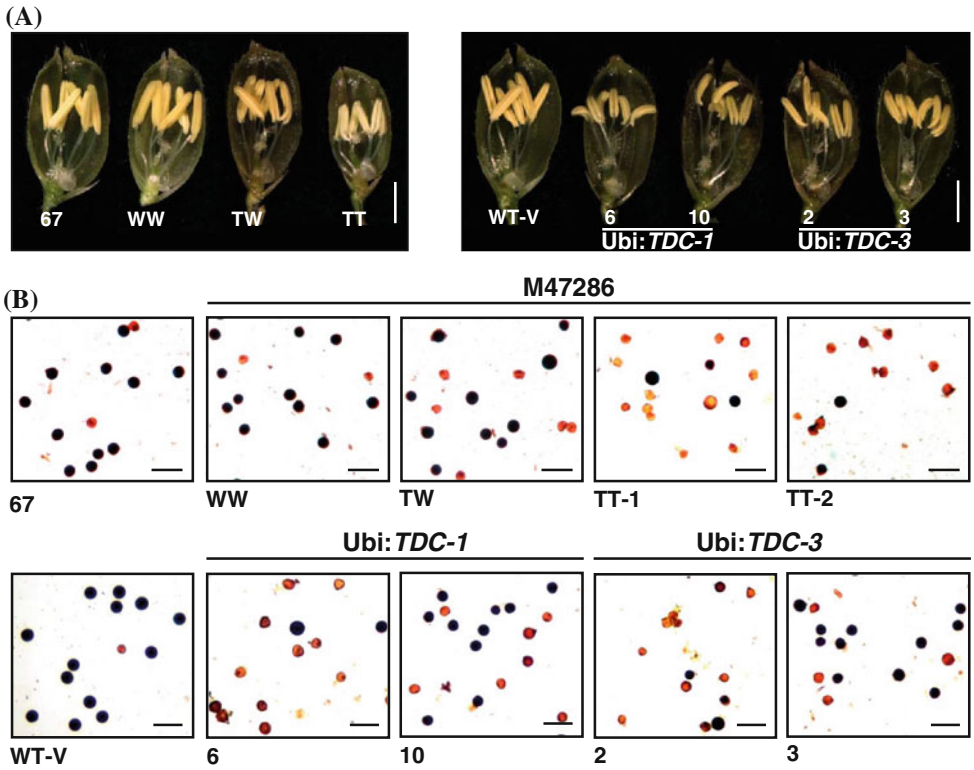

Fig. 9 Pollen viability in various genotypes of M47286 and TDC over-expressing transgenic lines. **a** The anthers in the spikelet of wild type (67) plants, various genotypes (WW, TW and TT) of M47286 (*left panel*) and two of each Ubi:*TDC-1* and Ubi:*TDC-3* transgenic rice lines (*right panel*). *Bars* = 2 mm. **b** The I_2-KI stained pollen grains of the wild type, various genotypes of M47286 (*top panel*) and Ubi:*TDC-1* and Ubi:*TDC-3* transgenic rice lines (*bottom panel*). *Bars* = 100 μm

indicating the number of pollens collected from the TW line was enough for fertilization (Table 3).

Ectopic expression of rice *TDC* genes in Arabidopsis affected growth and produced brown and sterile flower buds

Both Ubi:*TDC-1* and Ubi:*TDC-3* constructs were used for Arabidopsis transformation to examine whether rice TDCs were functional in dicots, i.e., could reduce plant growth and enhance the dark-brown phenotype. The T1 transgenic Arabidopsis showed the same retardation of plant growth as the transgenic rice plants, but to different extents. Two transgenic Arabidopsis lines representing different degrees of retardation of plant growth were shown (Fig. 10f, g), and the severely retarded line #1 died without flower development. For those that developed into flowers, such as line #2, the flower buds were smaller (Fig. 10a) and

Table 3 Fertile seed production in various cross combinations between wild type and different genotypes of M47286

Crosses	Fertile[a]	Sterile[b]	Total
WW × WW	309 (53)[c]	273 (47)	582 (100)
TW × WW	601 (42)	828 (58)	1,429 (100)
TT × WW	71 (21)	266 (79)	337 (100)
WW × TW	548 (57)	417 (43)	965 (100)

[a] Flowers that set seeds were judged as fertile

[b] Flowers that failed to set seeds were judged as sterile

[c] Values in parentheses indicate the % of total seeds

exhibited defective flower organ development (Fig. 10b, c). Most of these flower buds stopped growing, did not open and eventually became brown colored (Fig. 10d) with no formation of siliques and seeds (Fig. 10e).

The expression levels of *TDC-1* and *TDC-3* in the representative transgenic lines were analyzed by RT-PCR, and the results showed that lines #1 in both constructs exhibited much higher levels of *TDC-1* or *TDC-3* expression than lines #2 (Fig. 10h). Lines #1 also revealed stronger growth inhibition effects than lines #2, suggesting that the higher the TDC expression, the greater the detrimental effect on plant growth and flower development. In addition, we identified high levels of tryptamine and serotonin accumulations in the transgenic Arabidopsis (Supplementary Table S4). Although no ortholog of T5H gene has been identified, the serotonin accumulation clearly suggests the existence of T5H activity in Arabidopsis.

Exogenous serotonin and serotonin dimer affect plant growth and flower development

The T-DNA activation-tagged mutant M47286 and TDC-overexpressing transgenic rice and Arabidopsis lines all revealed stunted growth, sterility and transformation to dark-brown appearances. These effects were related to the level of expression of TDC, the enzyme that converts tryptophan to tryptamine. However, the accumulation of tryptamine in addition to serotonin and serotonin dimer were detected in the TDC over-expressing plants (Figs. 6, 7, 8), which led to the investigation of which compounds play the major role in affecting plant growth and flower/seed development.

Experiments were performed with the exogenous addition of compounds including tryptamine, serotonin and serotonin dimer to the medium of both the growing seedlings (10-DAI seedlings, experiment I) and the bolting stage of Arabidopsis plants (24-DAI plants, experiment II). In the seedling treatment (experiment I), the seedlings fed with serotonin or serotonin dimer showed stunted growth 14 days after treatment (Fig. 11a) and then turned brown and withered at 28 days after treatment, although a tiny bolt grew out. The tryptamine treatment showed no difference from the control (Fig. 11b). Similarly, the bolting Arabidopsis plants (experiment II) revealed retarded growth and delayed flower development after treatment with serotonin or serotonin dimer, while the tryptamine treatment showed no difference from the control (Fig. 11c), indicating that the mature Arabidopsis plants at the reproductive stage could still be affected by treatments with either serotonin or serotonin dimer (Fig. 11c).

Discussion

Serotonin accumulation in TDC-overexpressing transgenic rice affects plant growth and reduces fertility

In the present study, we observed high levels of tryptamine and serotonin accumulation in a T-DNA insertion mutant M47286 and in TDC-overexpressing transgenic rice plants. These tryptamine and serotonin-accumulated rice plants all revealed stunted growth, low fertility and a dark-brown phenotype. In addition, we also noticed a much larger amount of serotonin accumulation than tryptamine in these rice plants (Figs. 3c, d, 6c, d, g, h). This raises the suspicion of which compound accumulation causes the stunted growth and low fertility.

The study of Back's laboratory showed no morphological changes in the TDC-overexpressing transgenic rice plants that accumulated tryptamine and serotonin (Kang et al. 2007a). In contrast, TYDC-overexpressing transgenic rice that accumulated high levels of tyramine showed stunted growth and no seed production (Kang et al. 2007a; Lee et al. 2009). Tyramine accumulation could reduce rice cell division (Kim et al. 2011) and has been shown to be toxic toward the calli of many plant species, including soybean, tobacco, sunflower and corn (Christou and Barton 1989). The toxic effect was further confirmed by exogenously added tyramine which resulted in the inhibition of seedling growth (Lee et al. 2007). While tyramine is toxic to plants, tryptamine does not exhibit the same cytotoxicity to transgenic species that over-produce tryptamine (Gill and Ellis 2006; Kang et al. 2007a; Songstad et al. 1990; Thomas et al. 1995; Thomas et al. 1999). Similarly, we showed that exogenous addition of tryptamine to the medium did not inhibit the vegetative growth and flower development in Arabidopsis (Fig. 11).

Judging from the different degrees of phenotype and the different levels of accumulation of tryptamine (ranging from 60 to 100 μg/g fw) and serotonin (ranging from 1200 to 1,600 μg/g fw) in various genotypes of mutant M47286 (Figs. 1, 3), and that certain levels of the tryptamine concentration does not inhibits plant growth, we therefore

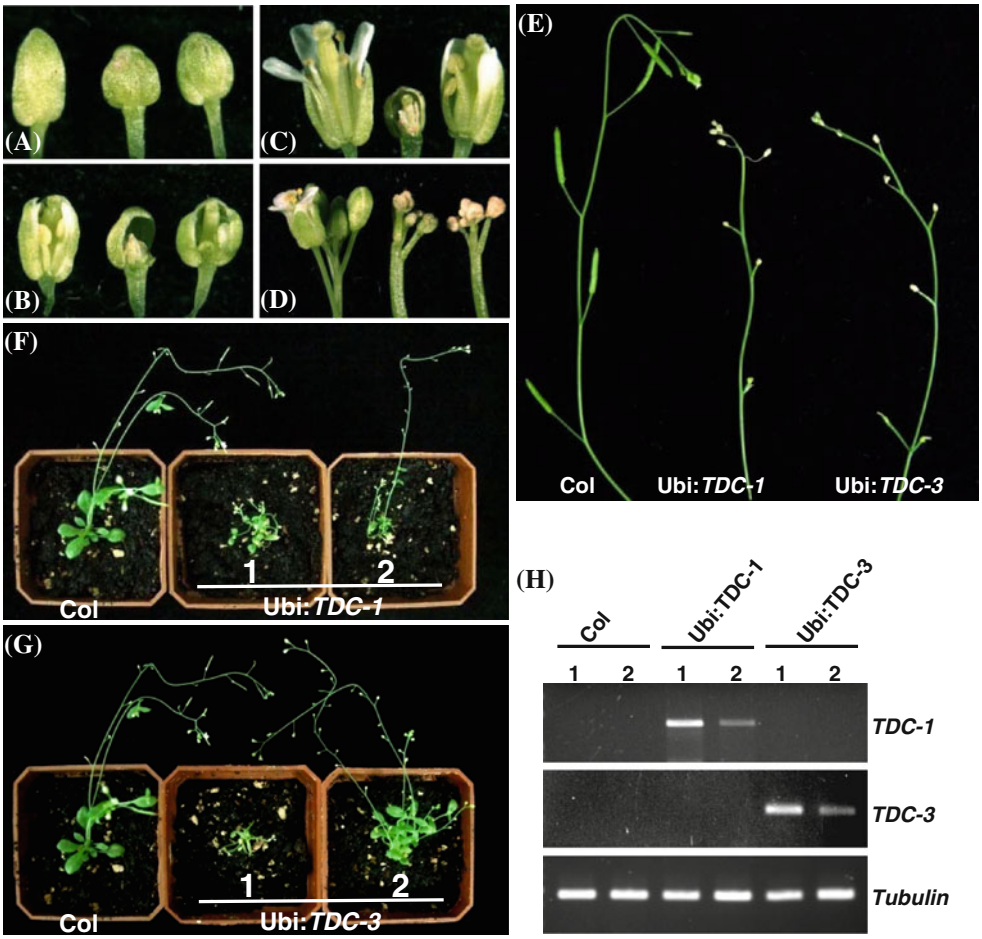

Fig. 10 Morphological and molecular characteristics of TDC overexpressing transgenic Arabidopsis. **a**–**d** Comparison of the young flower buds (**a**) with their sepals removed (**b**) and mature flowers (**c**) at the stage where the wild type flower is completely open, and older flower panicles (**d**) from the wild type (*left*), Ubi:*TDC-1* (*middle*) and Ubi:*TDC-3* (*right*) transgenic Arabidopsis. **e** Normal siliques developed in wild type Arabidopsis (Col) while Ubi:*TDC-1,* Ubi:*TDC-3* transgenic Arabidopsis failed to produce normal siliques. This photo was taken at 65 days after germination. **f** and **g** Comparison of a wild type (Col) and 2 representative lines of each Ubi:*TDC-1* and Ubi:*TDC-3* transgenic Arabidopsis grown in the pot for 6 weeks. **h** RT-PCR results of *TDC-1* and *TDC-3* genes using total RNA extracted from whole plants of the wild type and the same 2 transgenic lines from Ubi:*TDC-1* (**f**) and Ubi:*TDC-3* (**g**). The *TDC-1* gene was amplified using 23 PCR cycles, and 21 cycles were used to amplify the *TDC-3 gene*. The tubulin gene was used as a control

postulated that the different levels of stunted growth might not be caused by the accumulation of tryptamine, but instead, by the different accumulation levels of serotonin. This notion can be further supported by the TDC-overexpressing transgenic rice studies (Figs. 5, 6) and that exogenously addition of serotonin inhibited the growth of Arabidopsis (Fig. 11). Although we demonstrated that the accumulation of a large amount of serotonin would affect plant growth, we did not rule out the possibility that there are other metabolic products produced in the TDC-overexpressing transgenic and mutant lines which exert different functions. To demonstrate the functional roles of these metabolic products, further detection of these compounds and more complete analysis of the affected phenotype need to be performed.

In addition to stunted growth, we observed low fertility in all of the TDC-overexpressing transgenic and mutant rice lines (Table 2). The results of the pollen viability assays partially explain the low fertility in TT line of mutant M47286, line #6 of Ubi:*TDC-1* and line # 2 of Ubi:*TDC-3*. However, in line #10 of Ubi:*TDC-1* and line #3 of Ubi:*TDC-3*, 55 and 79% of the pollens were viable, respectively, but still no fertile seeds were produced. Although the morphological abnormal pistils were not observed in florets of these lines, the results of the crossing experiments with various genotypes of mutant lines (Table 3) explained that the female gametophytes were partially defective and may have been affected by overexpressing TDCs in these transgenic rice lines. Taken together, our results suggest that the low fertility of these

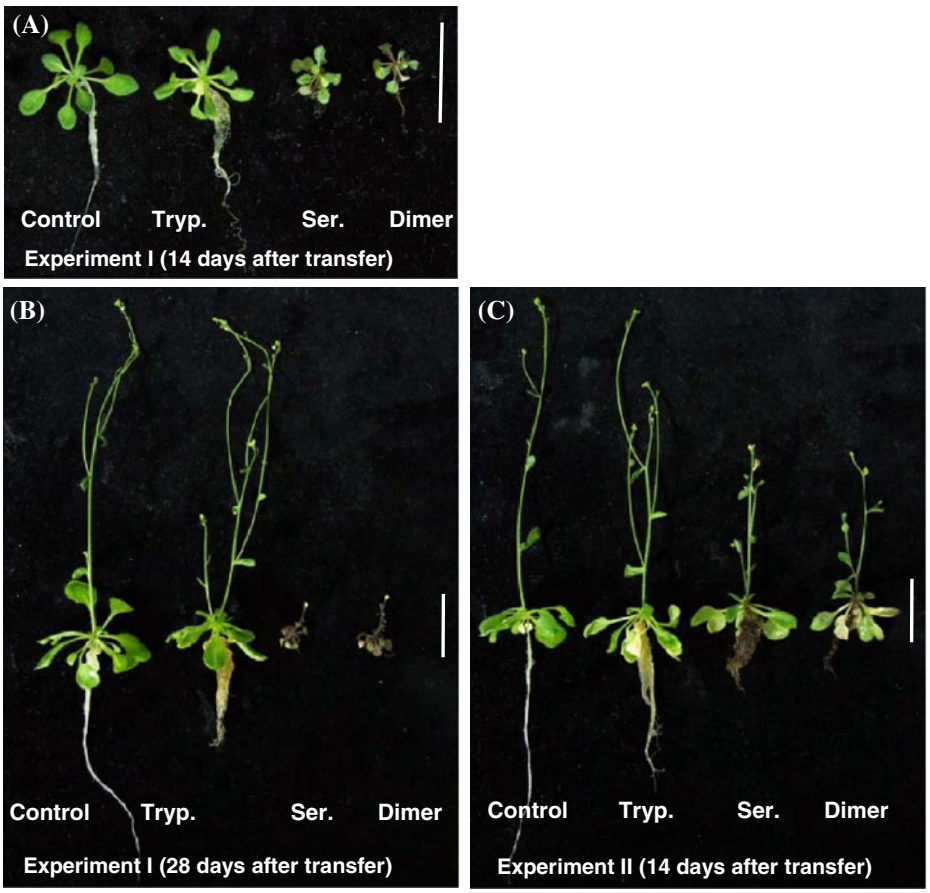

Fig. 11 The effects of tryptamine, serotonin and serotonin dimer on the growth of Arabidopsis. **a** and **b** Comparison of Arabidopsis plants after 14 days (**a**) or 28 days (**b**) of growth in the B-5 medium (control) or medium supplemented with 1 mM tryptamine (Tryp), 1 mM serotonin (Ser) or serotonin dimer (Dimer). Experiment I used 10-day-old Arabidopsis plants. **c** Comparison of Arabidopsis plants 14 days after the same media treatments as in (**a**) and (**b**), except that the starting Arabidopsis plants (experiment II) were 24-day-old and at the bolting stage. *Bars* = 2 cm

TDC-overexpressing transgenic and mutant rice lines could result from both the reduced pollen viability and the defective female gametophyte, which may have been caused by the excessive accumulation of TDC metabolic products, such as serotonin.

The ectopically over-expressing of rice TDC genes in Arabidopsis caused a detrimental effect on plant growth and flower development (Fig. 10). In addition to the RNA expression of TDC genes in transgenic Arabidopsis, we detected high levels of tryptamine and serotonin (Supplementary Table S4). Although no functional ortholog T5H has been reported in Arabidopsis, our transgenic study suggested that Arabidopsis containing T5H activity which would produce serotonin which affected plant growth. Overall, these results demonstrated that rice TDC-1 and TDC-3 genes have similar functions in monocots and dicots and that their overexpression resulting in accumulation of serotonin would affect plant growth and flower development.

High levels of serotonin accumulation in rice plants are necessary but not sufficient for the development of the dark-brown phenotype

In addition to the stunted growth revealed in the TDC-overexpressing transgenic and mutant rice lines, dark-green leaves and brown panicles and grains were also observed (Figs. 1, 5). The degree of the dark-brown phenotype was proportional to the expression levels of TDCs (Figs. 5, 6). Moreover, while the maximum levels of tryptamine were similar between these two types of transgenic lines (Fig. 6c, g), the serotonin contents in the Ubi:*TDC-3* transgenic lines were higher than those in the Ubi:*TDC-1* transgenic lines (2,000 vs. 1,000 μg/g fw, Fig. 6d, e), and the Ubi:*TDC-3* transgenic lines showed a darker phenotype (Fig. 5c–e). Therefore, it implied that the accumulation of serotonin was the cause of the darker brown phenotypes in these TDC-overexpressing transgenic and mutant rice plants.

However, various transgenic plants over-expressing TDC ectopically did not exhibit the same phenomena as those observed in rice in this study. For example, transgenic tobacco (Guillet and de Luca 2005; Guillet et al. 2000; Songstad et al. 1990; Thomas et al. 1995), canola (Chavadej et al. 1994), *Peganum harmala* (Berlin et al. 1993), potato (Yao et al. 1995) and petunia (Thomas et al. 1999) over-expressing *TDC* genes from *Catharanthus roseus* (periwinkle) all showed normal growth phenotypes. The same normal phenotype was also observed in transgenic poplar over-expressing the *Camptotheca acuminata TDC1* gene (Gill and Ellis 2006). The lack of a dark brown phenotype in these studies could be explained by the heterogeneous system used in these transgenic lines, or it could be due to the fact that the downstream enzyme was lacking in the host plants. For example, tobacco has no endogenous T5H activity, resulting in the accumulation of high levels of tryptamine but not serotonin (Di Fiore et al. 2002).

Furthermore, a recent study on a transgenic rice system over-expressing the rice AK31 (the same as the TDC-1 gene in our study) or AK53 (TDC-2) genes in a homogenous transgenic rice system in contrast to the heterogeneous system mentioned above increased the amount of TDC enzymes, resulting in an 11- to 25-fold increase of serotonin accumulation (Kang et al. 2007a). However, transgenic rice plants retained their normal phenotypes. Although the amount of serotonin in the AK31 and AK53 transgenic rice plants were about 11- to 25-fold higher than those in the control plants, the highest absolute amount of serotonin accumulated in the seeds or leaves of the transgenic rice plants were only approximately 2 or 5 μg/g fw, respectively (Kang et al. 2007a). This amount of serotonin was about 200- to 400-fold less than what we measured in our TDC-overexpressing transgenic rice plants, which ranged from 1,000 to 2,000 μg/g fw. Therefore, we postulated that the drastic difference in the levels of serotonin accumulation between Kang's study and the current study was the major cause of the different observed phenotypes.

Recent study from Back's laboratory indicated that the TDC-overexpressing transgenic rice accumulated serotonin up to 2,700 μg/g fw in the detached leaves after 8 days of senescence, showing delayed senescence compared to the wild type which accumulated serotonin up to 1,700 μg/g fw (Kang et al. 2009). After 8 days of detachment, the leaves from serotonin-overproducing transgenic rice were greener than the wild type control (Kang et al. 2009). Although the serotonin contents of the detached leaves were similar to those of the attached leaves (1,000–2,000 μg/g fw) of our TDC-overexpressing transgenic rice, the leaves in these two studies appeared different. The detached leaves remained green, while the attached leaves of our study became dark green and brownish. The difference in the leaf appearances between Back's study and our study could be attributed to the different sources of rice materials and treatments.

The rice materials used in the senescence study were either young seedlings depleted of nutrients or detached leaves from 4-week-old seedlings (Kang et al. 2009), and the increase in TDC expression and serotonin accumulation resulted from nutrient depletion or detachment. The rice materials used in our study were mature leaves, panicles and grains (Figs. 1, 5), and the increase in TDC expression and serotonin accumulation resulted from TDC-activation and ectopic TDC gene over-expression. Although the detached leaves in Back's study did reach very high levels of serotonin, this outcome caused delayed senescence but no brown pigmentation. These results suggest that not only high levels of serotonin accumulation but also additional factors such as the oxidation/polymerization of serotonin were required for the development of the dark-brown phenotype.

Serotonin dimer involved in brown pigmentation in TDC-overexpressing transgenic and mutant rice and at pathogen infection sites

In the present study, we demonstrated that the dark-brown pigmentation in the mature leaves and seed-hulls of TDC-transgenic rice was closely linked to the levels of serotonin dimer (Fig. 7d, e). This dimer formation required a serotonin oxidation reaction that could be catalyzed by peroxidase (Napolitano et al. 1988; Siraki and O'Brien 2002) or by various environmental conditions (Eriksen et al. 1960; Napolitano et al. 1988), such as exposure to UV light (Fig. 7; Eriksen et al. 1960) or copper (Jones et al. 2007). Without direct stimulation under natural field-grown environments, the serotonin-accumulated plants may require a longer exposure time to form the dimer. Therefore, in addition to the serotonin accumulation, time and environmental factors are also involved in the development of serotonin dimer in rice plants.

The present study showed that a serotonin solution developed brown pigmentation when exposed to UV light (Fig. 7a) and that this color was correlated with the production of the covalent serotonin dimmer identified by LC/MS/MS and NMR analysis (Figs. 7b3, b4, 8). These results suggest that the covalent serotonin dimer with its conjugated molecular ring systems can give rise to the brown color. Using the same LC/MS/MS assay method, we demonstrated that the samples extracted from the brown leaves and hulls of the TDC-overexpressing transgenic rice plants contained a compound with the same retention time and fragment ions as the covalent serotonin dimer shown in Fig. 7b. In addition, the darkness of the rice materials was tightly linked to the amount of the covalent serotonin dimer

(m/z 351, Fig. 7c–e). Therefore, we concluded that the dark-brown leaves and hulls in the TDC-overexpressing transgenic and mutant rice were caused by the accumulation of the covalent serotonin dimer.

A recent study showed that the expression of TDC was significantly up-regulated by inoculation with *B. oryzae*, which caused a marked increase in serotonin production in the defense responses of rice against pathogenic infection (Ishihara et al. 2008). These serotonins have been demonstrated to be incorporated into the cell walls of lesion tissue where the deposition of brown material was apparent, resulting in the restriction of the pathogen hyphal growth and the achievement of plant protection (Ishihara et al. 2008, 2011). Based on their results, these authors strongly suggested that oxidative polymerization and cross-linking of serotonin caused the formation of brown deposits at the infection site (Ishihara et al. 2008, 2011). Similar to the experiment of Ishihara et al. (2008), the infection sites caused by rice blast pathogen showed the deposition of brown material, and high levels of serotonin and the covalent serotonin dimer were detected at these infection sites (Supplementary Fig. S5). Based on the observations that the brown pigmentation in the mature leaves and seed-hulls of the TDC-overexpressing transgenic and mutant rice were correlated with the relative level of the covalent serotonin dimer (Fig. 7c–e), we therefore suggest that the covalent serotonin dimer is responsible for the brown material deposition in the infection sites.

In summary, our results provide strong evidence that the over-expression of rice TDC-1 and TDC-3 increased the accumulation of serotonin, which affected plant growth. In addition, extended exposure of plants to sunlight (UV) triggered the formation of covalent serotonin dimer in the plants, resulting in brown phenotypes. Moreover, the deposition of brown material at pathogen infection sites could also resulted from the accumulation of the covalent serotonin dimer.

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