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Isolation and Characterization of Triacontanol-Regulated Genes in Rice (*Oryza sativa* L.): Possible Role of Triacontanol as a Plant Growth Stimulator

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Triacontanol (TRIA) is a saturated long-chain alcohol that is known to have a growth promoting activity when exogenously supplied to a number of plants. In this study, dry weight, protein and chlorophyll contents of rice seedlings were increased by foliar application of TRIA. Leaf net photosynthesis rate (Pn) was increased very quickly and persistently at a given photon flux density (PFD). The TRIA-regulated genes in rice were isolated from cDNA library by differential screening with probes generated from the forward- and reverse-suppression subtractive hybridization (SSH) populations and confirmed by Northern blot. Sequence analysis revealed that most of the up-regulated genes encoded the photosynthetic and photorespiratory proteins. Two down-regulated genes were identified as those encoding an ABA- and stress-related protein and a wounding-related protein. These results suggested that TRIA up-regulated the photosynthesis process and suppressed stresses in rice plants. Time-course profiles of expression of *rbc*S isogenes suggested the complex mechanisms involved in the regulation of photosynthesis promoted by TRIA.

Keywords: cDNA — Differential screening — Photosynthesis — Stress — Suppression subtractive hybridization (SSH).

Abbreviations: PFD, photon flux density; Pn, net photosynthesis rate; SSH, suppression subtractive hybridization; TRIA, triacontanol.

Introduction

Triacontanol (TRIA), a saturated primary alcohol (*n*-$C_{30}H_{61}OH$), is a natural component of plant epicuticular waxes with a plant growth enhancing property (Ries et al. 1977). Recent researches on a variety of plant species have provided convincing evidence for this property (Nagoshi and Kawashima 1996, Muthuchelian et al. 1997, Borowski et al. 2000, Kumaravelu et al. 2000). In ornamental plants, it can be used to activate the micropropagation (Kissimon et al. 1999, Tantos et al. 1999, Tantos et al. 2001). Past decades have witnessed much success in increasing the yields of food crops and vegetables with TRIA (Eriksen et al. 1981, Ries 1985, Nagoshi and Kawashima 1996, Asane et al. 1998, Borowski et al. 2000).

Efforts have been made to elucidate the mechanisms of TRIA action. Assumption of a cascade effect (Ries and Houtz 1983) lead to the identification of 9-β-L(+)-adenosine as a second messenger of TRIA (Ries 1991). Exogenous application of TRIA to barley roots results in a rapid stimulation of membrane-associated Ca^{2+}/Mg^{2+}-dependent ATPase activity (Lesniak et al. 1986) in a calmodulin-dependent manner (Lesniak et al. 1989). Besides that, activity of NADH oxidase of the plasma membrane is potentiated by TRIA application (Morré et al. 1991). Dynamic membrane studies reveal an increase in the fluidity of membranes in vitro (Ivanov and Angelov 1997, Shripathi et al. 1997). All these seem to suggest a hormonal mode of action. However, how plants sense TRIA is not understood nor is it known how the signal is transduced to elicit an appropriate response.

Plants respond very rapidly to TRIA application. Whole rice and maize plants respond to TRIA application within 10 min (Ries and Houtz 1983). The response of whole plants has been characterized by increases in dry weight, leaf area, and level of reducing sugars, amino acids, soluble proteins, and total nitrogen (Ries 1985). An increase in dry weight has been assumed as the result of the increased photosynthetic activity and the accumulation of photosynthates (Haugstad et al. 1983). However, little is known about the molecular mechanism for those responses. Isolation and characterization of TRIA-regulated genes is a first step towards understanding the TRIA action since it can give clues to the biochemical pathways and physiological processes that TRIA regulates, and reveal the components involved in TRIA signaling.

Many methods have been developed to study differentially expressed genes. Notable among these is suppression subtractive hybridization (SSH; Diatchenko et al. 1996). It is an improved method based on representational difference analysis (RDA; Lisitsyn et al. 1993) and it has provided new insights into many old stories (Hufton et al. 1999, Sävenstrand et al. 2000, Voiblet et al. 2001).

As one of the most important crops worldwide, rice has also become a model system in the genomic project. Here, we confirmed the stimulating effects of TRIA on rice growth. TRIA increased photosynthesis activity at a wide range of pho-

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Table 1 Effects of TRIA on the growth of 15-day-old rice seedlings

	Dry wt (mg/20 shoots)	Protein content (mg (g FW)$^{-1}$)	Chlorophyll content (mg (g FW)$^{-1}$)
Control	61.73 ± 1.51	32.97 ± 0.36	1.91 ± 0.09
TRIA	62.43 ± 1.48	38.25 ± 0.28	2.39 ± 0.12
Increase rate (%)	1.13	16.01	25.01

Growth and biochemical parameters were investigated in 15-day-old rice seedlings 4 h after TRIA or control treatment (16:00 in the day). Each test was conducted with 20 seedlings. Data presented were means of four independent tests (±SE).

tosynthetic photon flux densities. We isolated and characterized TRIA-regulated genes by a combination of SSH and differential screening of a cDNA library in this organism. A large number of the TRIA-responsive genes were photosynthesis-associated ones. The photosynthesis-associated genes were up-regulated, and the stress-related genes were down-regulated by TRIA. In addition to providing a number of molecular markers for TRIA action, this work is a step towards a better understanding of the physiological processes that TRIA regulates at the molecular level in plants.

Results

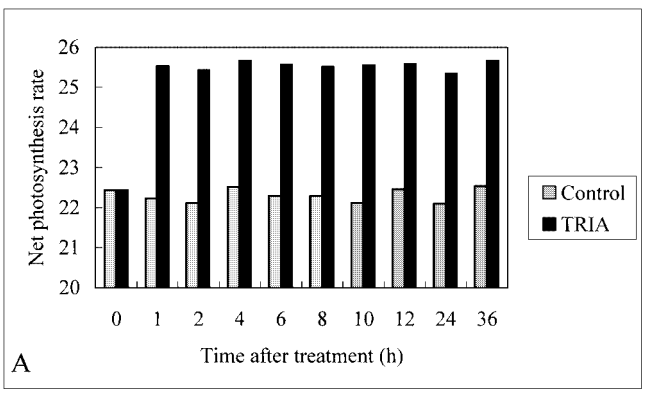
Effect of TRIA on the growth of rice seedlings

The growth of 15-day-old rice seedlings was stimulated by foliar application of TRIA at a very low concentration (10 μg liter^{-1}). A higher level of dry weight was observed in TRIA-treated plants as compared to the controls 4 h after TRIA treatment (Table 1). The protein and chlorophyll contents increased significantly by 16% and 25% respectively in TRIA-treated plants. Results obtained here were consistent with previous reports (Bittenbender et al. 1978, Ries and Wert 1988), where a growth response of rice seedlings was noticeable as early as 3 h after TRIA treatment.

Leaf net photosynthesis rate and light saturation point

Leaf net photosynthesis rate (Pn) was monitored at 800 μmol m^{-2}s^{-1} photon flux density (PFD) at different time courses after TRIA treatment on a CI-310 photosynthesis system (CID, Inc.). Leaf Pn was higher in TRIA-treated plants as compared to the controls (Fig. 1A). Leaf Pn did not exhibit a notable time-course difference for both groups, and it remained a higher level among time courses in TRIA-treated plants as compared to the controls. These data suggested that TRIA increased photosynthesis persistently.

In order to construct leaf Pn curves in TRIA-treated and the control plants, leaf Pn was recorded at different photosynthetic PFDs (Fig. 1B). It increased rapidly with the increasing PFD until a plateau was reached regardless of treatment. However, it exhibited a higher level in TRIA-treated plants at any irradiance investigated. The increased level of leaf Pn by TRIA ranged from 14.1% to 18.6% as compared to the controls. Leaf



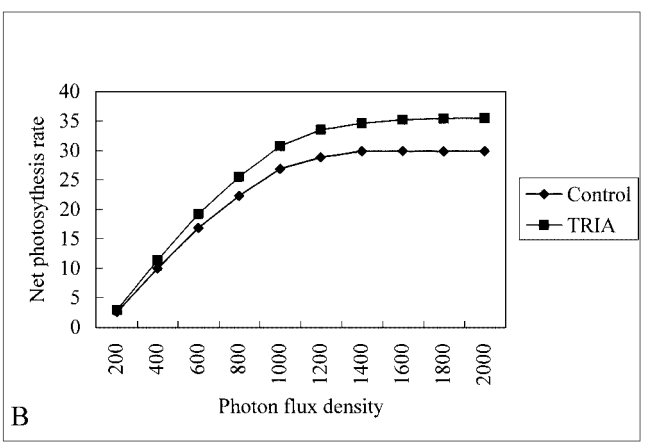


Fig. 1 Leaf Pn (μmol m^{-2}s^{-1}) and light response curves of Pn (μmol m^{-2}s^{-1}) in the control and TRIA-treated rice seedlings. TRIA treatment was conducted parallel to the control assay at 12:00. The leaf temperature was controlled at 28°C by CI-310CS temperature control module. Measurements were carried out automatically five times each from the two top leaves of five plants. Data were presented as the means of 25 measurements. (A) Leaf Pn at 800 μmol m^{-2}s^{-1} PFD during different time-courses. The photosynthetic photon flux density was controlled by CI-310LA light attachment. (B) Light response curves of Pn. The photosynthetic PFD was controlled by CI-310LA light attachment. Measurements were carried out between 13:00 (1 h after treatment) and 24:00 (12 h after treatment).

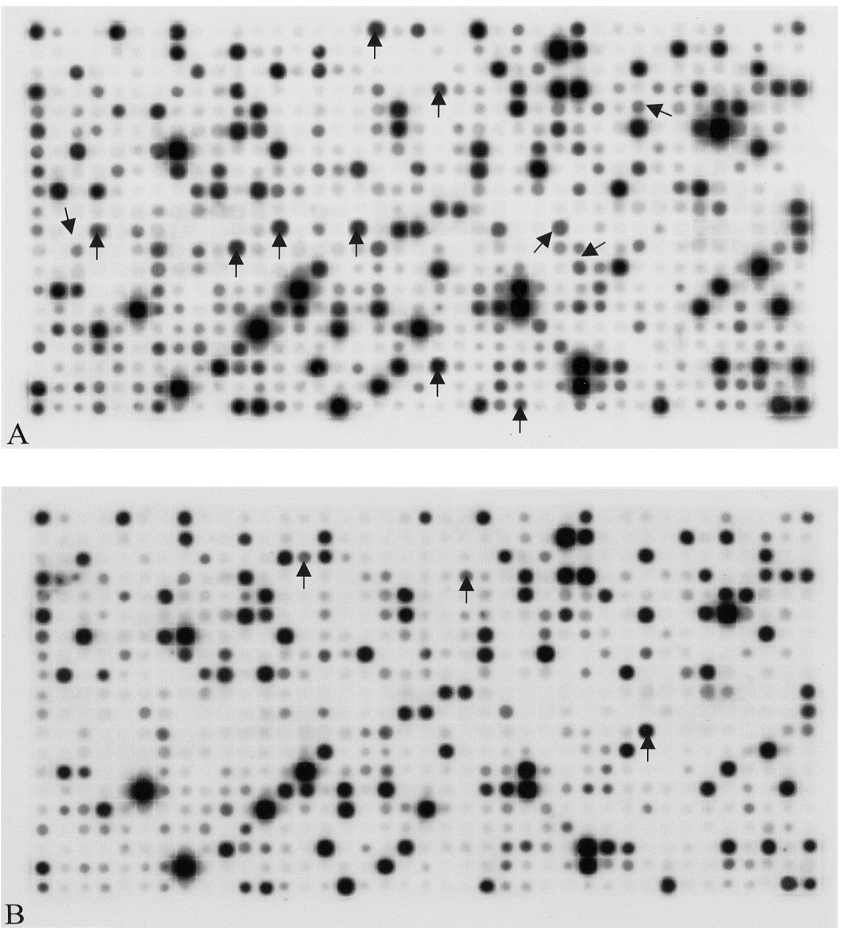


Fig. 2 An example for differential screening with probes made from forward- and reverse-SSH populations. Plasmid cDNAs isolated randomly from a cDNA library prepared from TRIA-treated plants were arrayed onto two sets of membranes. The ubiquitin cDNA was arrayed on the up-left. Two sets of filters were hybridized with probes made from forward- and reverse-SSH populations respectively. After autoradiography, the hybridization profiles were compared, the signal of an given clone was then standardized relative to that of the ubiquitin cDNA. Clones that hybridized stronger with probes made from forward-SSH population were taken as putative up-regulated ones, and those hybridized stronger with probes made from reverse-SSH population taken as down-regulated ones. (A) The filter was hybridized with probes from forward-SSH population. Up-regulated clones are indicated by arrows. (B) The filter was hybridized with probes from reverse-SSH population. Down-regulated clones are indicated by arrows.

Pn reached to its highest level at about 1,400 μmol $m^{-2}s^{-1}$ in control plants, while it reached to its highest level at about 1,600 μmol $m^{-2}s^{-1}$ in TRIA-treated plants. These observations demonstrated that TRIA increased leaf Pn and light saturation point of rice plants.

Differential screening of TRIA-regulated genes

Sixteen-hundred clones were picked up at random in the cDNA library constructed from TRIA-treated plants and arrayed onto membranes. The genes were differentially screened with probes made from the forward- and the reverse-SSH populations respectively. An ubiquitin cDNA was first confirmed by Northern blot not to be regulated by TRIA and was then used to normalize the signal differences on the membranes. An example of the screening was shown in Fig 2. Clones detected with a stronger signal with the forward-SSH probe were considered as putative TRIA up-regulated ones; those detected with a stronger signal with the reverse-SSH probe were considered as putative down-regulated ones. A total of thirty clones were found to be up-regulated and three were down-regulated. Many fewer down-regulated clones were obtained relative to the up-regulated ones, possibly because their abundance in the TRIA-treated cDNA library was low.

Sequencing and identification of TRIA-regulated genes

The putative clones were sequenced automatically with a sequencer. Sequences were determined from the putative 5′ end of the cDNA in order to enhance the probability of obtaining a coding sequence. Database comparison characterized 15 different genes (Table 2).

Table 2 Identification of TRIA regulated genes in rice (*Oryza sativa* L.)

Clone	Number of clones	Putative identity	Related accession number [a]	% homology	Overlap	Regulated pattern
Rtr1	5	Rubisco SSU	AF017364	95	409/512	Up
Rtr2	4	Rubisco SSU	L22155	95	615/642	Up
Rtr3	3	Rubisco SSU	D00644	92	450/488	Up
Rtr4	4	Rubisco SSU	AF017363	82	314/382	Up
Rtr5	4	Rubisco SSU	D00643	93	387/414	Up
Rtr6	2	oxygen evolving protein	X57408	82	312/380	Up
Rtr7	2	glycine decarboxylase P subunit	AF024589	85	281/328	Up
Rtr8	2	serine hydroxymethyltransferase	Z5863	85	301/353	Up
Rtr9	1	ribosomal protein S9	AB022675	94	302/320	Up
Rtr10	1	Dof zinc protein	AB028132	98	488/497	Up
Rtr11	1	ABA and stress-induced protein	AF039573	99	443/446	Down
Rtr12	1	wound induced protein	X71396	88	169/192	Down
Rtr13	1	Rice cDNA clone	gi4880975 AU065274	91	482/528	Not
Rtr14	1	Rice shoot cDNA clone	gi3769233 AU033260	96	327/339	Up
Rtr15	1	No significant homologue				Up

Identified clones were termed as Rice TRIA Regulated (Rtr).
[a] Database accession number of related sequences.

Northern blot analysis was employed to verify whether those candidate genes were really regulated by TRIA. Twelve were confirmed in Northern blot as up-regulated and two were confirmed as down-regulated (Fig. 3). The 12 up-regulated genes included ten known genes that encode eight photosynthetic and photorespiratory proteins, a ribosomal protein S9, a Dof zinc finger protein, a rice cDNA with unknown function and a novel gene. The two down-regulated genes were identified as those encoding an ABA- and stress-related protein and a wounding related protein (Table 2).

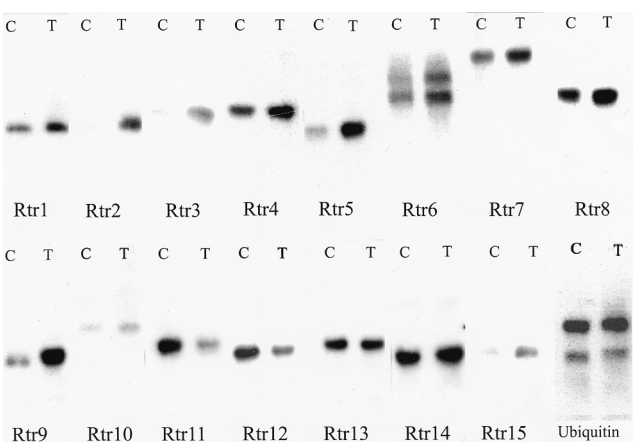


Fig. 3 Northern verification of TRIA regulated genes. For TRIA-treated or control group, RNAs from each time-course were equally mixed to constitute their corresponding RNA pool. Twenty μg of RNA from each pool were separated aside in the agarose-formaldehyde gel and blotted onto Hybond N membrane. Northern blot was used to hybridize with the corresponding cDNA and ubiquitin cDNA. After sequential hybridizing to the given cDNA and ubiquitin cDNA, the signal was then standardized. "C" indicates the control RNA pool, and "T" indicates the TRIA-treated RNA pool. The corresponding gene is indicated by the clone name.

Quantitative analysis of relative expression levels of photosynthesis-associated genes

Dot blot analysis was performed to reveal the quantitative level to which the photosynthesis-associated genes were regulated by TRIA. Such presentation permits many replicates in a single experiment (Fig. 4). The regulated levels of genes for photosynthetic carbon fixation and PSII proteins were higher than those of genes for photorespiratory proteins. The regulated levels varied among different members of *rbc*S. For example, transcripts for Rtr1, Rtr2, Rtr3, Rtr4, and Rtr5 were

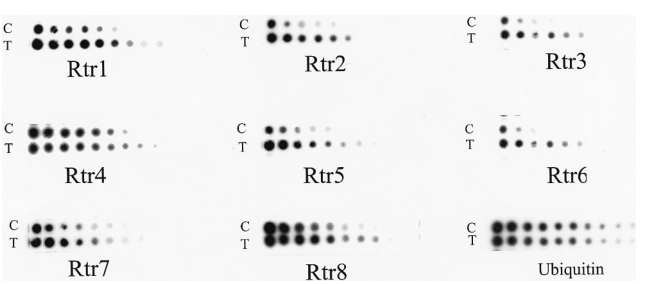


Fig. 4 Quantitative analysis of the relative expression level for photosynthesis-associated genes. RNAs were pooled as described in Northern blot. They were arrayed onto the membrane with a gradient of concentration in dot blots. After sequential hybridizing to a given cDNA and ubiquitin cDNA, the signal was then standardized, and the relative expression level was determined between pools. "C" indicates control RNA pool, and "T" indicates TRIA-treated RNA pool.

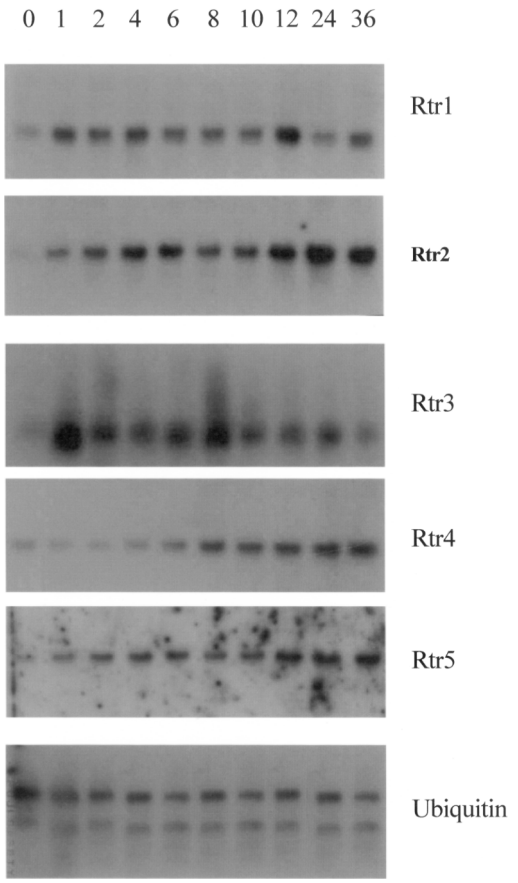


Fig. 5 Time-course expression of different *rbc*S isogenes. Twenty μg of RNA, for each time-course, were separated along in the gel. Northern blot was conducted as above. Time courses are indicated as numbers. *rbc*S isogenes are indicated as clone names.

up-regulated by 3.8, 4.4, 3.4, 2.2 and 5.0 folds respectively. *psb*O transcripts were up-regulated by 3.2 folds. The transcripts of GDC P protein gene were up-regulated by 2.5 folds, and those of *SHMT* gene were up-regulated by 2.2 folds.

Time-course expression of rbcS

Time-course expression of five isoforms of *rbc*S was conducted to see how soon these genes respond to TRIA treatment (Fig. 5). Interestingly, the expression profile varied among the *rbc*S isogenes. Except for Rtr4, other isogenes were rapidly induced within 1 h by TRIA. Only Rtr3 fluctuated with two peaks. Except for Rtr3, others expressed at a high level 36 h after treatment.

Discussion

TRIA favored the photosynthetic process

The present study represented the first report on isolation and characterization of TRIA-regulated genes in plants. As an important plant growth regulator, TRIA has attracted much attention in characterizing the physiological effects in a number of plants. However, the molecular mechanisms for TRIA action remain to be elucidated. Isolation and characterization of TRIA-responsive genes would serve as an initial step towards understanding these mechanisms.

Data collected here supported its effects on the photosynthetic process. Here, total protein and chlorophyll contents were both increased significantly, and dry weight was also increased by TRIA. Straightforward was seen leaf Pn. At a given PFD, TRIA increased leaf Pn very quickly and persistently (Fig. 1A). From the light-response curves of leaf Pn, TRIA increased leaf Pn at a wide range of PFDs (Fig. 1B). Combining SSH and differential screening of a cDNA library, we isolated many TRIA-responsive genes. Most of these were photosynthesis-associated ones. Five were *rbc*S isogenes (Matsuoka et al. 1988), which encodes the small subunits of Rubisco. Another was a *psb*O gene (Meadows et al. 1991), which is one of the three extrinsic components of oxygen evolving complex of PSII. Another was a gene for the P protein of glycine decarboxylase (GDC), and a SHMT gene (Kopriva and Bauwe 1995). Although not always, the *rbc*S transcript levels frequently correlate well with the Rubisco CO_2 fixation activity, the *rbc*S has been thus widely used as an indicator or marker for photosynthesis activity (Roitsch et al. 2000). For example, a depression in *rbc*S transcription is associated with suppressed photosynthesis in methyl jasmonate treatment (Reinbothe et al. 1993). Increases in *rbc*S transcripts are also associated with increases in photosynthesis during the acclimation to low CO_2 level (Gesch et al. 2000). Here, higher *rbc*S levels were associated with improved photosynthetic activity in TRIA-treated plants. Most of *rbc*S isogenes were induced rapidly (Fig. 5), and so was increased leaf Pn. Higher *psb*O transcript levels indicated an increase in photosystem efficiency in TRIA-treated plants. GDC and SHMT are involved in the glycine cleavage of the photorespiratory process. By acting in concert, these enzymes catalyze the interaction of two molecules of glycine to form a molecule of serine, CO_2, NH_3 and to reduce NAD^+ to NADH concomitantly (Douce et al. 2001). Increases in the transcript levels of the gene for the P protein of GDC and *SHMT* indicate a higher photorespiration activity in TRIA-treated plants.

Interestingly, the extent to which the transcripts regulated by TRIA differed among different members of *rbc*S (Fig. 4). In addition, time-course expression of *rbc*S isogenes revealed five different profiles (Fig. 5). Except for Rtr4, others were induced as quickly as 1 h after treatment, suggesting that *rbc*S isogenes respond to TRIA very quickly. Except for Rtr3, others expressed at a high level 36 h after treatment, indicating *rbc*S isogenes were induced persistently rather than transiently. *rbc*S belongs to a gene family that consists of many members. Previously, it has been shown that the expression pattern varies among different members (Galili et al. 1998). We did not know exactly the evolutionary significance for various regulated patterns of *rbc*S. However, this study demonstrated the complex

regulation mode for *rbc*S expression. Further studies on the regulation mechanisms are needed to deepen our understanding of photosynthetic assimilation.

Relationships between TRIA and stresses

In this study, one of the down-regulated clones was identified as a gene for the ABA-induced protein OsAsr1. The *OsAsr1* transcript has been reported to be up-regulated by exogenous ABA, salt stress and mannitol stress in the shoot (Vaidyanathan et al. 1999). The other down-regulated clone was a gene for wounding-inducible protein WIP1 (Rohrmeier and Lehle 1993), which shows significant homology to Bowman-Birk proteinase inhibitors. It has been reported that plant hormones regulate expression of proteinase inhibitors (Pena-Cortes et al. 1989). Finding of stress-related genes in this study not only revealed the cross-talk between TRIA signal and others, but also helped to understand the role that TRIA plays in stress responses. It has been previously found that TRIA extenuates the unfavorable effects of stress on proliferation of woody plants in vitro (Kissimon et al. 1999). Rajasekaran and Blake (1999) also find that TRIA can reverse the damaging effects of drought and enhance drought tolerance of pine seedlings. It has been found that TRIA inhibits lipid peroxidation (Ramanarayan et al. 2000). As we know, the breakdown products of lipid peroxidation have been implicated in triggering stress-related responses (Dionisio-Sese and Tobita 1998). All those data suggest that TRIA serves as an alleviant of stresses. In this study, an ABA- and stress-related gene was down-regulated by TRIA, and so was a wounding-related gene. These observations provided an excellent explanation at the molecular level for the findings obtained elsewhere and also deepened our understanding of the relationships between TRIA and stresses.

In conclusion, this study confirmed the stimulating effects of TRIA on plant growth. TRIA increased photosynthesis activity at a wide range of PFDs. Isolation and characterization of TRIA-regulated genes indicated the role of TRIA in plants at the molecular level. Among those TRIA-responsive genes, a large number were photosynthesis-associated ones. Photosynthesis-associated genes were up-regulated by TRIA, and stress-related genes were down-regulated. The regulation level was different among photosynthesis genes. Time-course expression of *rbc*S isogenes revealed five different profiles. This study would contribute to a better understanding of the molecular response of plants to TRIA.

Materials and Methods

Plant growth condition and TRIA treatment

Seeds of rice cultivar B5 (*Oryza sativa* L.; Wang et al. 2001) were sown (Ries and Wert 1988) and grown in a growth chamber at 28°C for 16 h light (6:00–22:00) and 8 h dark (22:00–6:00). High purity of TRIA (Sigma, U.S.A.) was dissolved in Tween 20 to 1.0 mg g^{-1} (w/w) at 90°C for 15 min. Prior to use, TRIA was diluted with distilled water to 10 μg $liter^{-1}$ (Ries 1985). Fifteen-day-old rice seedlings were subjected to TRIA treatment and mock treatment in parallel on 12:00 in the day. For the TRIA treatment assay, TRIA solution (10 μg $liter^{-1}$) was sprayed onto the foliage. For the control assay, an equal amount of 10 mg $liter^{-1}$ Tween 20 was sprayed. Seedlings were sampled at different time courses after treatment of 0, 1, 2, 4, 6, 8, 10, 12, 24 and 36 h respectively for each group, frozen immediately in liquid nitrogen and stored at –80°C.

Growth parameter investigations

Dry weight measurement was carried out by keeping the seedlings in a hot air oven at 80°C for uniform weight determination. Total protein was estimated following the method of Bradford (1976). The absorbance was recorded spectrophotometrically at 595 nm (UV-1601, Shimadzu) using bovine serum albumin as standard. The chlorophyll content of the leaves was determined after extraction in 80% acetone at –20°C (Arnon 1949). Absorbance was measured, using a spectrophotometer (UV-1601, Shimadzu, Japan), at 646 and 663 nm and chlorophyll content was calculated using the equation:

$$\text{Total chlorophyll content} = 7.18 \times A_{663} + 17.32 \times A_{646}$$

where A_{663} and A_{646} are light absorption values at 646 and 663 nm. Four replicates were made of each measurement.

Leaf Pn measurements

Leaf Pn of the two top leaves in each of five plants in a given group (TRIA-treated or control) were measured with a CI-310 photosynthesis system (CID, Inc.) in an open-air circulation system. The photosynthetic PFD was controlled by CI-310LA light attachment (CID, Inc.). The leaf temperature was controlled at 28°C by CI-310CS temperature control module (CID, Inc.). The airflow through the cuvette was set as 0.3 liter min^{-1}, and the interval time 20 s. Measurements were carried out automatically five times.

RNA isolation

RNA was isolated respectively from samples of each time point with a TRIZOL reagent (Gibco BRL, U.S.A.), dissolved in DEPC-treated water, and denatured for 10 min at 65°C immediately before 10-min chilling on ice. RNA was quantified with a PERKIN-ELMER LAMBDA BIO 20/1.0 nm UV/VIS spectrophotometer (Perkin-Elmer, U.S.A.). For either treated or control group, the RNA pool from each time course was used to extract mRNA with MESSAGEMAKER reagent assembly (Gibco BRL, U.S.A.).

Suppression subtractive hybridization

SSH was performed based on two RNA pools between groups with the PCR-Select cDNA subtraction kit (Clontech, U.S.A.). Two μg of mRNA were reverse-transcribed following the manufacturer's protocol. Forward-SSH was performed using cDNA synthesized from TRIA-treated plants as tester and that from the control plants as driver to enrich genes that are up-regulated by TRIA. Reverse-SSH was performed using cDNA from control plants as tester and that from TRIA-treated plants as driver aiming to enrich genes that are down-regulated by TRIA. Suppression PCR was performed with AdvanTAge PCR cloning kit (Clontech, U.S.A.) as suggested by the manual.

Construction of a cDNA library for TRIA-treated plants

Five μg of mRNA from TRIA-treated materials were used as starting materials to construct a cDNA library in the λ-ZAP vector (Stratagene, U.S.A.) according to the manufacturer's instructions. The first strand cDNA was synthesized with methylated cytidine to protect the integrity of cDNA from cutting by *Xho*I before cloning into the λ-ZAP vector. The cDNA inserts were oriented into the *Eco*RI and *Xho*I

cloning sites of the ZAP vector and packaged in Gigapack III Gold packaging extracts (Stratagene, U.S.A.). The primary library (before amplification) contained 3.9×10^6 dependent clones. One million clones were amplified once to a titer of 1.5×10^{10} pfu ml^{-1}. Before screening, ten million plaques were converted to pBluescript SK phagemid clones as suggested by the manual.

Differential screening of a cDNA library with probes made from SSH populations

Sixteen-hundred clones were randomly selected from the phagemid cDNA library prepared from TRIA-treated plants. The plasmid DNA was isolated in a 1.5 ml tube containing 0.8 ml of overnight-growth cultures. One hundred ng plasmid in 1 µl of water was arrayed for each set of membranes. One hundred ng of ubiquitin cDNA in 1 µl of water was arrayed onto the up-left of the membranes. Twenty-five ng of either forward- or reverse-SSH population devoid of adaptor was labeled with [α-^{32}P]dCTP using Prime-a-Gene labeling system (Promega, U.S.A.) to hybridize with the filters. After autoradiography, the expression profiles between the two populations were compared.

Sequencing

Plasmid DNA was used as template for automated sequencing with an ABI PRISM Dye terminator cycle sequencing kit and an ABI Model 377 DNA sequencer (Perklin-Elmer, U.S.A.). M13 reverse universal primer was used to sequence DNA templates. The sequences devoid of vector sequences were compared to all known DNA sequences in the National Center for Biotechnology Information (www. ncbi.nlm.nih.gov) database with a Blastn program.

Northern blot

Twenty µg of total RNA were separated on a 1.5% agarose-formaldehyde gel and blotted onto a Hybond N membrane (Amersham-Pharmacia, U.S.A.). Probe was made with Prime-a-Gene labeling system (Promega, U.S.A.) from 25 ng of PCR products of the corresponding clone. After hybridization, the filters were exposed to an X-ray film (Fuji, Japan) at –80°C for appropriate time. The filters were stripped with boiling 0.1% (w/v) SDS and re-hybridized with the ubiquitin gene.

Dot blot analysis

Samples of about 5.00, 3.00, 1.80, 1.08, 0.65, 0.39, 0.23, 0.14, 0.08 and 0.05 µg of total RNA in 1 µl of DEPC-treated water were arrayed onto membranes (Hybond-N, Amersham-Pharmacia, U.S.A.) previously wetted with 2× SSC. After 1–5 min, when RNA samples were absorbed, RNA was baked to the membrane by incubating at 80°C for 2 h. The membranes were kept dry until use. Probe labeling and hybridization were all as described in Northern blot. The relative hybridization signals were determined by scanning the autoradiograms using ArtixScan 2500 (Microtek, U.S.A.) connected to Photoshop 5.0 program. Relative mRNA levels were measured by densitometry from the scanned films with SxImage software (Shanghai, China) using the ubiquitin gene as a loading control.

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