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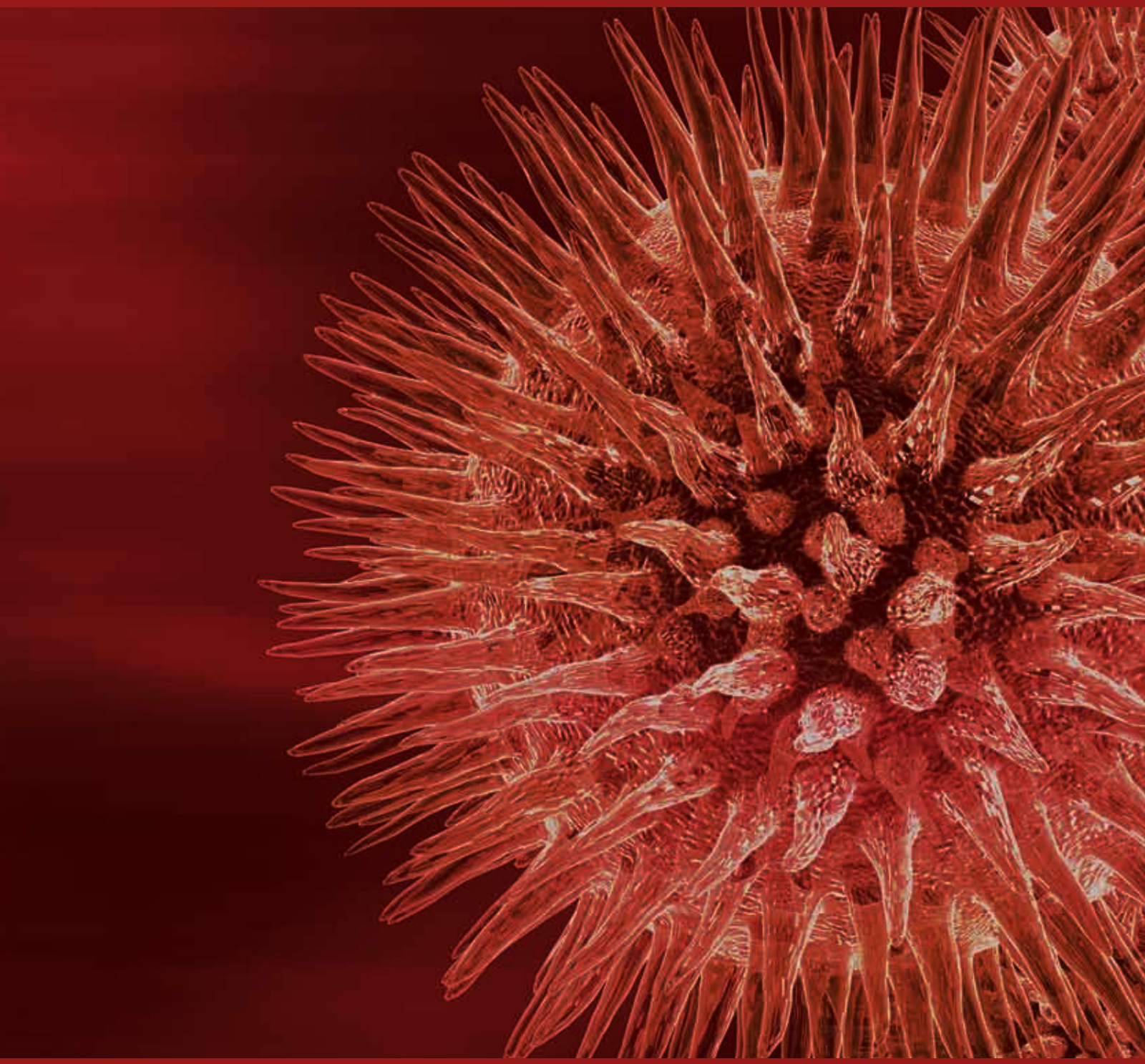
Plant Stress and Biotechnology

Guest Editors: Juan Francisco Jiménez Bremont, Margarita Rodríguez Kessler, Ji-Hong Liu, and Sarvajeet S. Gill



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Contents

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Editorial

Plant Stress and Biotechnology

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Nowadays plant biotechnology faces many important challenges, including the development of strategies to secure global food supply, the adequate acquisition and management of plant derived products for human subsistence (woods, pharmaceuticals, biofuels, etc.), as well as the development of plants that can cope with the constant effects of biotic and abiotic stress conditions in the era of changing climatic conditions. Being sessile, plants are able to adapt or acclimate after constant exposition to environmental stress conditions; nevertheless, in most cases growth rate and yield are reduced below optimum levels. Increases in global warming, climatic changes, soil degradation, and pollution, are affecting dramatically the plant kingdom.

Advances in plant molecular biology and biotechnology have changed our capabilities for gene discovery, the study of tissue-specific promoters, the functional characterization of genes, gene pyramiding, and the generation of efficient methods for plant genetic transformation and/or plant genome manipulation. All of these beneficial applications offer the possibility to achieve pest resistance, abiotic stress tolerance, crop yield improvement, and excel food nutritional quality.

In this regard, the understanding of plant molecular mechanisms underlying biotic and abiotic stress tolerance, the characterization of hormone mediated signaling pathways, and the molecular interaction and crosstalk among pathways will improve our knowledge in plant stress biology.

This special issue consists of six papers related to abiotic and biotic plant stress. Papers by M. Pérez-Clemente et al., L.-T. Yang et al., and A. S. Dubrovina et al. present interesting reviews of diverse topics such as plant response to stress, aluminium tolerance in higher plants, and pre-mRNA splicing under plant stress conditions, respectively. The following three are research papers focusing on identification of rice genes in response to heat stress (Y. Cao et al.), transcriptional profiling of canker-resistant transgenic sweet orange (X.-Z. Fu and J.–H. Liu), and characterization of the newly developed soybean cultivar (DT2008) under drought tolerance (C. V. Ha et al.).

M. Pérez-Clemente et al. outline the main biotechnological approaches used to study plant stress responses, such as the "omics" technologies (genomics, proteomics, and metabolomics), and transgenic-based approaches. As well, considerable advances in plant physiology, genetics, and molecular biology are included, which have greatly improved our understanding of plant responses to abiotic stress conditions.

A. S. Dubrovina et al. review recent data on alternative splicing in plant genes involved in stress signaling; in particular the authors focus on the occurrence, properties, and functional consequences of unconventional splicing and splicing-like events in plants. Precursor mRNAs with introns can undergo alternative splicing to produce multiple transcripts from the same gene by differential use of splice sites, thereby increasing the transcriptome and proteome complexity. In order to survive the stress conditions, plants actively employ pre-mRNA splicing as a mechanism to regulate expression of stress-responsive genes and reprogram intracellular regulatory networks. This review is attractive

since it provides data on transcript diversity generated in response to environmental stresses, an aspect that could be important to plant biotechnology in terms of developing new strategies for crop breeding and protection.

L.-T. Yang et al. describe the main mechanisms related to aluminium tolerance in plants facing acidic soils, where aluminium toxicity is an important factor that limits crop productivity. Importance is given to the secretion of organic acid ions from plant roots and the possible mechanisms that regulate this process, including ion channels or transporters, internal concentration of organic acid ions, root plasma membrane H-ATPase, and temperature, among others. As well, transgenic plants attempting to increase the secretion and biosynthesis of organic acid anions as an approach for the acquisition of aluminium tolerance are discussed as potential research area.

In the research article presented by Y. Cao et al., the importance of studying heat stress responses in plant crops, such as rice, is emphasized, in particular, because of global warming and its increasing impact on crop production at the present time. In rice, several papers involving heat shock proteins (HSPs) and heat shock transcription factors in the heat stress response have been published; nevertheless, little is known about other genes induced under this condition. Based on cDNA-AFLP analysis, the authors identified 49 differentially expressed genes, including genes related to carbohydrate metabolism, photosynthesis and energy production, and amino acid and polyamine metabolism and transport, among others, as heat stress-responsive genes in rice. The possible implication of these genes in heat stress response is discussed.

In the research article presented by X.-Z. Fu and J.-H. Liu, they analyzed the global transcriptional profiling of transgenic sweet orange line (TG9) using the Affymetrix Citrus GeneChip microarray. TG9 line overexpresses *Malus domestica* spermidine synthase (MdSPDS1); it has increased levels of the polyamines, spermidine and spermine, and confers canker resistance. Nowadays, the gene regulation network in polyamine which involved plant pathogen response is largely unclear, particularly in perennial plants like citrus. A profile of genes up- and downregulated under high polyamine levels in the TG9 line is presented. It is hypothesized that genes implicated in stimulus response and immune system process, cell wall and transcriptional regulation, and cellular and metabolism processes, such as starch and sucrose metabolism, glutathione metabolism, biosynthesis of phenylpropanoids, and plant hormones play major roles in the canker resistance of TG9.

In the research article presented by C. V. Ha et al., drought-tolerant phenotypes of DT2008 soybean variety and W82 soybean cultivar were compared by examining the dehydration-induced water loss and membrane stability of the shoot parts of young seedlings. DT2008 is an improved variety of soybean that shows enhanced drought tolerance and stable yield in field conditions. The authors propose that further comparisons between DT2008 and W82 cultivars using molecular approaches will enable the identification of mutations associated with drought tolerance in DT2008, which could be used to improve drought tolerance in soybean cultivars through genetic engineering.

In the research article presented by C. V. Ha et al., they compared the drought-tolerant phenotypes of DT2008 soybean variety and W82 soybean cultivar by examining the dehydration-induced water loss and membrane stability of the shoot parts of the young seedlings. DT2008 is an improved variety of soybean that showed enhanced drought tolerance and stable yield in the field conditions. Molecular analysis between these soybean cultivars will enable the identification of mutations, which could be candidates for genetic engineering to improve drought tolerance in soybean cultivars.

Acknowledgments

We thank the authors of the submitted papers for their contribution. The preparation of this special issue would not have been possible without the generous support and dedication of experts that evaluated the papers submitted.

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Research Article

Identification of Differential Expression Genes in Leaves of Rice (*Oryza sativa* L.) in Response to Heat Stress by cDNA-AFLP Analysis

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High temperature impedes the growth and productivity of various crop species. To date, rice (*Oryza sativa* L.) has not been exploited to understand the molecular basis of its abnormally high level of temperature tolerance. To identify transcripts induced by heat stress, twenty-day-old rice seedlings of different rice cultivars suffering from heat stress were treated at different times, and differential gene expression analyses in leaves were performed by cDNA-AFLP and further verified by real-time RT-PCR. In aggregate, more than three thousand different fragments were indentified, and 49 fragments were selected for the sequence and differential expressed genes were classified functionally into different groups. 6 of 49 fragments were measured by real-time RT-PCR. In addition, the variations of three different polyamine contents in response to heat stress through high-performance liquid chromatography (HPLC) analysis were also performed. The results and their direct and indirect relationships to heat stress tolerance mechanism were discussed.

1. Introduction

With the development of industrialization, the impact of ongoing climate change on the natural environment deterioration was more and more obviously shown. Due to an increase in mean global temperature, heat stress has become a major disastrous factor that severely affects the crop cultivation and productivity. Heat stress-induced decrease of the duration of developmental phases leading to fewer organs, smaller organs, reduced light perception over the shortened life cycle, and perturbation of the processes related to carbohydrate metabolism (transpiration, photosynthesis, and respiration) is most significant for losses in cereal yields [1].

Being a staple food and a principal calorie source for people living in Asia [2], rice (*Oryza sativa* L.) production and its factors has attracted more and more attention. However, rice is also a prime example of cereal crops whose growth and reproduction could be impaired by heat stress. It has been indicated that rice production will be severely affected by the aberrant change of temperature [3]. Comprehending the mechanisms of which rice response to heat stress would facilitate the development of heat-tolerant cultivars with enhanced productivity in a warmer future climate. The researches that underlie molecular responses both in genomics and proteomics level of plants to heat stress have, therefore, attracted a great deal of attention [4–6].

Previous proteomic analysis with the employment of various advanced techniques such as two-dimensional electrophoresis provides a cogent approach to investigate the molecular mechanisms of rice response to abiotic stresses [7, 8]. Relative proteomic studies about rice in response to abiotic stresses have been deep into subcellular proteome and posttranslational modifications [7]. However, it is well known that the expression of proteins is regulated by the gene transcription and translation processes, although proteomic

study about rice in response to heat stress is significant, the assessment of gene level regulation of such type of temperature variability on rice is also unneglectful. In addition, rice may confront with different high-temperature stresses during its lifespan, and different growth phases may have different responses to the stresses, which will be inevitably regulated by the variation of the expression of genes. Previous proteomic studies revealed that seedlings-phased rice could also be affected by the heat stress environment [9]. Although genomewide expression analysis showed that transcription level of heat shock proteins (Hsps) and heat shock factors (Hsfs) are induced under heat stress at young seedling stages [10, 11], further investigations to uncover the regulative mechanisms corresponding to heat tolerance at genomics level are still necessary.

Among techniques employed in gene identification, many techniques such as differential display reverse transcription-polymerase chain reaction (DDRT-PCR), representational difference analysis (RDA), serial analysis of gene expression (SAGE), suppression subtractive hybridization (SSH), and cDNA microarray provide effective approaches for transcription analysis [12]. As a high-efficient, less labor-intensive mRNA fingerprinting method for isolation of differentially expressed genes [13], cDNA-amplified fragment length polymorphism (cDNA-AFLP), which has been employed in the study, is a robust genomewide expression tool [12] for gene discovery without a prerequisite of the prior knowledge of the sequences [14]. In addition, due to its high detective sensitivity of cDNA-AFLP analysis, some rare transcripts could also be detected by this method [15]. This technique has been further ameliorated to avoid the possibility of several transcript-derived fragments (TDFs) from a single gene/cDNA [16].

Currently, several rice varieties defined by temperature thresholds [17] have been identified with genotype variation in spikelet sterility at high temperature in *indica* and *japonica* [18, 19]. In our study, forty-nine critical genes differentially expressed in rice seedling in response to heat stress using cDNA-AFLP technique have been identified. To validate their expression patterns, six gene fragments involving different physiological activities were analyzed through real-time PCR. In addition, due to direct interactions of polyamines with other metabolic pathways during the stress response [20] and their regulatory functions in plant abiotic stress tolerance [21], the variations of three different polyamine contents in response to heat stress through high-performance liquid chromatography (HPLC) analysis were also performed. These outputs may lay the fundamental basis of the rice variety breeding in the future.

2. Materials and Methods

2.1. Plant Material. Seeds of two *indica* rice cultivars (Shuanggui 1, heat sensitive; Huanghuazhan, heat tolerant) were provided by the Rice Research Institute of Guangdong Academy of Agricultural Sciences (Guangzhou, China). The seedlings were grown in Kimura B complete nutrient solution, under greenhouse conditions (28°C day/22°C night, relative humidity 60–80%, the light intensity 600–1000 μmol m^{-2} s^{-1}, and photoperiod of 14 h day/10 h night), with 50 seedlings per pot. Then, twenty-day old seedlings were heat-stressed (at 39°C day/30°C night) for two different treatment times: 24 h or 48 h, while control seedlings were grown under the conditions mentioned above. Each treatment had 3 pots as replications. Samples from rice seedling leaves were harvested and were immediately frozen in liquid nitrogen and stored at −80°C until use. Heights of rice seedlings of Shuanggui 1 at 28°C control condition or 39°C stress condition were determined with a ruler from the land surface to the top of the seedling leaves with means of three replications (50 plants per replication).

2.2. RNA Extraction. Total RNA was extracted using RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. The integrity of the RNA was checked by agarose gel electrophoresis and the concentration was determined at 260 nm by spectrophotometer.

2.3. cDNA-AFLP Analysis. An cDNA-AFLP method was adapted from Bachem et al. [13, 22] with a few modifications. Synthesis of double-stranded cDNA was performed with M-MLV RTase cDNA Synthesis kit (TaKaRa, China) and refined using phenolchloroform extraction. 5 μL was checked using agarose gel electrophoresis in order to observe an expected smear between 100 bp and 1000 bp. The rest of cDNA was digested using the restriction enzymes *Eco*R I (Fermentas; 2 h at 37°C) and *Mse* I (*Tru*1I, Fermentas; 2 h at 65°C). The digested products were ligated to adaptors (*Eco*R I, 5 μmol L^{-1}, forward primer: 5′-CTCGTAGACTGCGATCC-3′, reverse primer: 5′-AATTGGTACGCAGTCTAC-3′; *Mse* I, 50 μmol L^{-1}, forward primer 5′-GACGATGAGTCCTGAG-3′, reverse primer 5′-TACTCAGGACTCAT-3′) with T4-DNA ligase (Fermentas) for 13 h at 16°C. The products of ligation were amplified with the corresponding preamplification primers (*Eco*R I: 5′-GACTGCGTACCAATTC-3′; *Mse* I: 5′-GATGAGTCCTGAGTAA-3′). Preamplification was initiated at 94°C for 3 min and followed by 30 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, and terminated at 72°C for 5 min. The products of the preamplification were checked by agarose gel electrophoresis (expected smear between 100 bp and 1000 bp). From a 20-fold dilution of the pre-amplified samples, a 5 μL sample was used for the final selective amplifications using the primers 5′-GACTGCGTACCAATTCNN-3′ (*Eco*R I, NN represents AC, AG, GA, and GT) and 5′-GATGAGTCCTGAGTAAMM-3′ (*Mse* I, MM represents TC, TG, CA, and CT). After initial denaturation (94°C for 3 min), 12 cycles were performed with touchdown annealing (94°C for 30 s, 65°C to 56°C in 0.7°C steps for 30 s, 72°C for 1 min) followed by 24 cycles (94°C for 30 s, 56°C for 30 s, 72°C for 1 min) and final elongation (72°C for 5 min). Altogether 64 primer combinations of two base extensions (denoted as NN and MM) were used.

2.4. Polyacrylamide Gel Electrophoresis. The selective amplification products were separated on a sequencing polyacrylamide gel (6% polyacrylamide, 8 mol L^{-1} urea, 1×TBE) at a

constant current mode in a vertical slab gel electrophoresis apparatus (Hoefer Pharmacia Biotech Inc., CA, USA). The glass plates were treated by Repel-Silane and Binding-Silane, respectively (Dingguo, China), following the manufacturer's instructions. The system of electrophoresis was prerun in 1×TBE buffer about 30 minutes for 40°C–45°C of the gel surface temperature at 1500 v. Then the samples were separated about 4 h of migration until the bromophenol blue reached the bottom of the gel. The cDNA bands were visualized by silver staining according to Bassam et al. [23].

2.5. Isolation, Cloning, and Sequencing of Differential Fragments. Fragments of interest were eluted from silver-stained gels using the procedure of Frost and Guggenheim [24] with modifications. The band was eluted in 50 μL of sterile double-distilled water initially at 95°C for 15 min and then hydrated overnight at 4°C. Amplifications were performed with appropriate primers with 5 μL of this solution. PCR reactions were initiated at 94°C for 3 min followed by 35 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, and terminated at 72°C for 10 min. The PCR products were resolved in a 2% 1×TBE agarose gel, and each single band was isolated and eluted using the DNA Fragment Quick Purification Kit (Dingguo, China). Cloning was performed from fresh PCR products with the pGEM-T Easy vector (Promega) according to the manufacturer's instructions and using chemical transformation of one shot *E. coli* (DH5α) competent cells using Ampicillin as the selecting agent. After plasmid purification using a small-scale plasmid DNA purification kit (QIAGEN), the insert size was checked by PCR amplification using the corresponding selective amplification primer and the clones were sequenced by Invitrogen biotechnology service company (Shanghai, China).

2.6. Real-Time PCR Analysis. RNA extraction was performed according to the above mention. Total RNA was then purified with DNase I Kit (Invitrogen, China) according to the manufacturer's instructions. First-stranded cDNAs were synthesized using PrimeScript RT Master Mix Perfect Real Time Kit (TaKaRa, China). These cDNAs were used for PCR experiments using gene-specific primers designed with DNAMAN software. Forward and reverse primers were used for producing a single amplification for the following genes: *Os04g0429600* (5′-AGGTGCCGCAGAGTTTCTAC-3′ and 5′-TACAGTGGAAGCAACCCGTTC-3′); *Os06g0124900* (5′-CCCTCTTCGTCGTCTCCAAT-3′ and 5′-TAAGCCTCTCACCTTCACGG-3′), *SRP14* (5′-CGTTTCTGAGCGAGTTGACG-3′ and 5′-GTTCTTCTTGCCATCGGTGG-3′); *Os02g0611200* (5′-TCGGCTACAGCATTGAGGAC-3′ and 5′-GGAAGAAAGGAAGCAGGACTGA-3′); *Os02g0788800* (5′-TCGGAGTTTAGAGGAGTTGCG-3′ and 5′-TAAGAGCCATCACGAGACCG-3′). qRT-PCR experiments were performed in ABI 7500 real-time PCR system and 7500 Software v 2.0.4. All PCR reactions were mixed as follows: 2 μL of diluted cDNA, 10 μL of 2×SYBR Premix Ex Taq II (TaKaRa, China), 0.4 μL 50× ROX Reference Dye II, and 400 nmol/L of primers in a final volume of 20 μL PCRs. All reactions were repeated four times. As an internal standard, a fragment of rice *actin* gene (5′-CTTCATAGGAATGGAAGCTGCGGGTA-3′ and 5′-CGACCACCTTGATCTTCATGCTGCTA-3′) was used. All the PCRs were performed under the following program: 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 34 s at 60°C in 96-well optical reaction plates (Applied Biosystems, USA).

2.7. High-Performance Liquid Chromatography (HPLC) Analysis of Free Polyamines. Rice leaves (1.0000 g) were weighed and crushed to homogenate using precooled marble pestle and mortar with 4 mL 5% perchloric acid. The homogenate was then suspended at 4°C for 1 h and centrifuged at 14000 g for 30 min. Supernatant was collected for the following HPLC separation and identification. All standards and polyamines extracts of spermidine, spermine, and putrescine were analyzed on an Agilent 1200 HPLC system (Agilent Corp, USA). Chromatographic separation and collection of extracts were achieved using an Eclipse Plus C18 reversed-phase column (250 mm × 4.6 mm, 5 mm; Agilent). The condition for HPLC separation was determined to be a mobile phase composed of methanol and water (75 : 25, v/v) and a flow-rate of 1 mL per min. The volume of sample injected was 10 μL for the qualitative evaluation, the detection wavelength and the column temperature were set at 254 nm and 30°C, respectively. Quantification of spermidine, spermine, and putrescine in the product was determined by comparison with external standards.

2.8. Analysis of Sequences and Data Analysis. The sequences were analyzed for their homology against the publicly available nonredundant genes/ESTs/Transcripts in the database (http://www.ncbi.nlm.nih.gov/BLAST) using the BLASTN and BLASTX algorithms [25]. The data was analyzed for variance using the SAS/STAT statistical analysis package (version 6.12, SAS Institute, USA). Means were tested by Duncan's test at the $P_{0.05}$ level.

3. Results

3.1. Morphological Responses of Rice Seedlings to High-Temperature Treatment. 20-day-old seedlings were heat-stressed at 39°C day/30°C night for two days. After 48 h, the morphologies of the leaves were compared among these seedlings treated at 39°C with those treated at 28°C. As shown in Figure 1, at 28°C leaves developed normally and showed deep green (Figure 1(a)). However, conspicuous symptom has been appeared under high temperature: low-growing leaves with yellow color characters have been appeared in the heat stress treatment (Figure 1(b)). The average height of plant with 28°C treatment was 21.8 ± 1.25 cm, while 39°C heat-stressed plants showed 18.2 ± 0.95 cm high, which was much lower than those of 28°C-treated rice leaves (Figure 1(c)).

3.2. Identification of High-Temperature-Regulated Transcripts. In order to further understand the response of rice seedlings to heat stress, cDNA-AFLP gels of gene bands from rice leaves after treatment for 48 h at 39°C treatment were compared

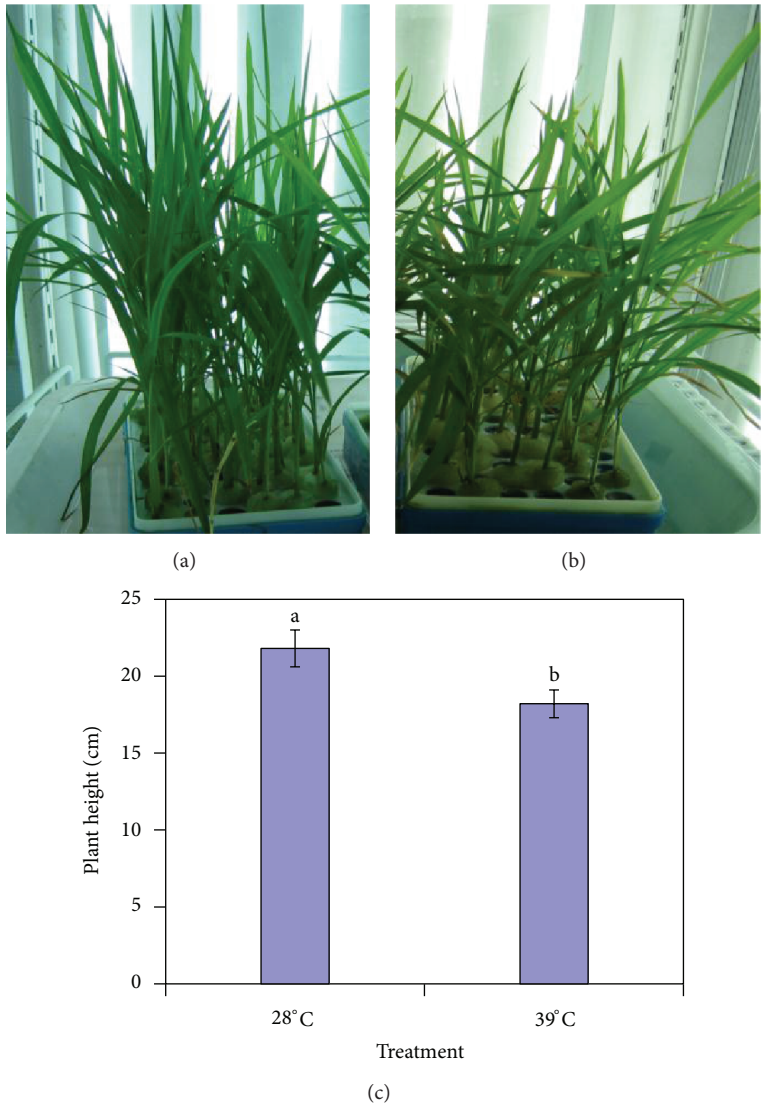

Figure 1: Behavior of rice seedlings (Shuanggui 1). (a) 28°C day/22°C night treatment; (b) 39°C day/30°C night treatment; (c) The comparison of plant height after 48 h at 39°C. Data from plant heights are means of three replications (50 plants per replication) ± SE. Dissimilar letters above bars differ significantly at $P \leq 0.05$.

with those reduce extra space at 28°C treatment. Accurate gene expression profiles were determined by visual observation and analysis of band intensities, and subtle differences in transcriptional activity were revealed. After cDNA-AFLP, average 72.4 TDFs per pair of primers could be reproducibly detected mainly at 28°C while a total of average 50.5 TDFs per pair of primers changed in abundance in response to 39°C (Table 1). Therefore, on average, 61 bands (TDFs) were produced with each primer combination, which yielded more than seven thousand TDFs from seedling leaves both under control (28°C) and heat stress (39°C) treatments. A total of 145 TDFs were isolated from the silver-stained cDNA-AFLP gels based on their presence/absence (qualitative variants) or difference in the levels of expression (quantitative variants) (see Figure S1 in Supplementary Material available online at doi: http://dx.doi.org/10.1155/2013/576189).

Table 1: Summary of PAGE showing bands after 64 pairs of primers amplification (per pair of primes).

Treatment	Average number of amplified gene bands	Average number of upregulated and nascent fragments
28°C	72.4	37.3
39°C	50.5	15.9

28°C : 28°C (day)/22°C (night); 39°C : 39°C (day)/30°C (night).

Compared with the genomics profile at 28°C, average 15.9 TDFs per pair of primers were upregulated under 39°C treatment. However, compared with the genomics profile at 39°C, an average of 37.3 TDFs per pair of primers were upregulated under 28°C condition (Table 1, Figure S1), and

more than three thousand differential expressing fragments were indentified. Thus, the quantity of both upregulated or downregulated cDNA fragments increased with increased temperature. This implied that the higher the temperature, the more rice life processes are affected.

3.3. Changes of Rice Leaf Genomics Profile Under High-Temperature Stress. With the 64 primer combinations tested, 65 reproducible (i.e., same expression profile in 2009 and 2010 seasons) TDFs were identified, and 49 of these were cloned and sequenced. 49 differential expressed gene sequences were performed according to BLASTN (score ≥ 50) and BLASTX ($E \leq 10^{-5}$) analyses in NCBI and Gene Ontology databases. According to MIPS (http://mips.helmholtz-muenchen.de/proj/funcatDB/search_main_frame.html) database, differential expressed genes in leaves suffered from heat stress were classified functionally as metabolism (including carbohydrate metabolism, protein metabolism, polyamine metabolism, amino acid metabolism, ribonucleotide metabolism, and cellulose synthesis), material transport, stress response, cell cycle and fate, signal transduction, and unclear functional protein. The sequence comparison against the database revealed that most of them could be sorted into the following ten functional groups (Table 2).

The most abundant groups were related to carbohydrate metabolism. Fourteen TDFs response to the heat stress were identified as related to carbohydrate metabolism. Among them, four TDFs (H51-4, H53-1, -2, and H57-3, Figure S1 and Table 2) corresponding to photosynthesis II-related protein, NADH dehydrogenase subunit 2, chloroplast ATP synthase a chain precursor, and chloroplast ATP synthase a chain precursor, respectively, were upregulated by heat stress treatment (Figure S1 and Table 2), while two TDFs (N21-1, N58-3), one of them related to photorespiration proteins (NAD-dependent epimerase/dehydratase family protein) and the other corresponding to phospho-2-dehydro-3-deoxyheptonate aldolase 1, were downregulated (Table 2). Two TDFs (H1-5, H51-5) corresponding to ubiquitin family protein and 60S ribosomal protein, which were sorted into protein metabolism group, were also upregulated by the heat stress (Table 2). One identified TDF (H57-5) corresponding to S-adenosylmethionine decarboxylase, which has been classified into polyamine metabolism, was also affected by heat stress treatment. To further validate the function of S-adenosylmethionine decarboxylase in rice seedling leaves, we used HPLC to investigate the effect of heat stress on the concentration of spermidine, spermine, and putrescine in rice seedling leaves. As shown in Figure 3, spermidine, spermine, and putrescine contents were increased dramatically at 39°C compared with those at 28°C. In addition, one TDF (H51-1) corresponding to glutamate decarboxylase, which could be sorted into amino acid metabolism, has also shown increasing trend under heat stress treatment. Two upregulated heat stress-induced differential fragments (H3-2-8, H46-3), which are corresponding to CTP synthase and cellulose synthesis family protein, were classified into ribonucleotide metabolism and cellulose metabolism, respectively. There are five heat-stressed induced genes identified and classified into material transport group: 2 (H51-3, H59-4) of 3 corresponding to phophate translocator and amino acid/polyamine transporter II were upregulated at 39°C (Table 2). Other two TDFs (H53-8, N60-4) corresponding to thioredoxin h isoform 1 and heat shock protein (HSP) DnaJ, respectively, could be sorted into stress response group. One upregulated heat stress-induced differential fragment (H4-1) and the other downregulated fragment (N6-1), which are corresponding to S-phase-specific ribosomal protein and putative senescence-associated protein, were classified into cell cycle and fate group (Table 2). Other gene fragments regulated with 39°C treatment included sixteen gene bands corresponding to hypothetical proteins (H3-2-1, H51-2, N60-5, etc.), whose functions were unknown or unclear (Table 2 and Figure S1).

3.4. Differential Response of Gene Fragments to Heat Stress. To validate the results of cDNA-AFLP experiment and quantitatively assess the relative abundance of the transcripts in rice seedling leaves under the heat stress, five upregulated TDFs (H46-3, *Os04g0429600*; H51-2, *Os06g0124900*; H51-6, *SRP14*;H57-5, *Os02g0611200*, and H59-4, *Os02g0788800*) and one downregulated TDF (N60-4, *Os07g0620200*) were selected for RNA expression analysis. Initially, a semiquantitative RT-PCR was performed to analyze the changes in transcripts of all six TDFs in response to the heat stress (data not shown). Obtained RT-PCR results were substantiated with quantitative real-time PCR (qRT-PCR) (Figure 2). Rice *actin* gene was selected as internal control for normalization.

The transcript of all six genes fragments (*Os04g0429600*, *Os06g0124900*, *SRP14*, *Os02g0611200*, *Os02g0788800*, and *Os07g0620200*) responded to heat stress (Figure 2). The most pronounced effect was observed in the case of *Os06g0124900* gene which increased more than twice after 48 h at 39°C treatment both in heat-sensitive "Shuanggui 1" and heat-tolerant "Huanghuazhan" and more increasing trend could be observed in heat-tolerant cultivar, though there was no obvious change after 24 h with 28°C treatment. Fast transcript accumulation was observed in case of *SRP14* and *Os07g0620200* with heat stress. *SRP14* showed significant increase in transcript accumulation only after 24 h at 39°C compared with that with same time at 28°C and relative expression of this gene presented more increasing trend in heat-tolerant cultivar compared to that in heat-sensitive cultivar. Fast downregulated trend has been observed in *Os07g0620200* gene, which has also happened only after 24 h heat stress treatment in heat-sensitive cultivar and considerable decreasing presented has been shown after 48 h under heat stress condition. However, fast effect of heat stress did not take place in heat-tolerant cultivar with no considerable variation after 24 h treatment both at 28°C or 39°C while obvious transcript increase could be observed after 48 h of heat stress treatment. The transcripts of *Os04g0429600*, *Os02g0611200*, and *Os02g0788800* also showed up-regulation with no more than twofold due to heat stress with no significant change after 24 h and gradual increase after 48 h. In general, five TDFs (*Os04g0429600*,

Table 2: The nucleotide-homology of the transcript-derived fragments (TDFs) with known gene sequences in the database using the BLASTN and BLASTX algorithms along with their expression patterns.

TDF no.	TDF size (bp)	GenBank/gene	Corresponding or related protein	Score (bits)	Identities	*E* value
			Carbohydrate metabolism			
N21-1	130	*ABF96013.1*	NAD-dependent epimerase/dehydratase family protein	73.2 (178)	32/33 (97%)	$2e-14^{*}$
H50-1	195	*PsaB*	PSI P700 apoprotein A2	200 (108)	108/108 (100%)	$3e-48$
H51-4	198	*YP_654233*	PS II protein H	57.8 (138)	26/27 (96%)	$2e-09^{*}$
H51-8	138	*ABI34757.1*	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*Pentameris aspera*)	80.5 (197)	34/35 (97%)	$3e-17^{*}$
H53-1	495	*NP_039432.1*	NADH dehydrogenase subunit 2	205 (522)	139/171 (81%)	$1e-60^{*}$
H53-2	389	*Os10g0527100*	Chloroplast ATP synthase a chain precursor	217 (117)	119/120 (99%)	$6e-53$
H53-3	299	*Os08g0472600*	Alpha-1, 3-fucosyltransferase	152 (82)	87/89 (98%)	$1e-33$
H53-6	261	*Os10g0527100*	Chloroplast ATP synthase a chain precursor	433 (234)	236/237 (99%)	$4e-118$
H53-7	220	*Os07g0662900*	4-Alpha-glucanotransferase	76.8 (41)	46/48 (96%)	$6e-11$
H56-3	229	*AAA84588.1*	atpB gene product	145 (366)	71/76 (93%)	$1e-39^{*}$
H57-3	294	*Os10g0527100*	Chloroplast ATP synthase a chain precursor	224 (121)	123/124 (99%)	$3e-55$
H57-4	290	*Os10g0527100*	Chloroplast ATP synthase a chain precursor	224 (121)	123/124 (99%)	$3e-55$
H57-6	195	*Os01g0881600*	Photosystem II reaction center J protein	110 (59)	61/62 (98%)	$5e-21$
N58-3	270	*Os08g0484500*	Phospho-2-dehydro-3-deoxyheptonate aldolase 1, chloroplast precursor	207 (112)	116/118 (98%)	$3e-50$
			Protein metabolism			
H1-5	227	*Os03g0131300*	Ubiquitin domain containing protein	283 (153)	159/162 (98%)	$3e-73$
H51-5	185	*AF093630*	60S ribosomal protein L21 (RPL21)	298 (161)	168/171 (98%)	$1e-77$
N53-6	294	*Os07g0555200*	Eukaryotic translation initiation factor 4G	147 (79)	82/83 (99%)	$6e-32$
			Polyamine metabolism			
H57-5	260	*Os02g0611200*	S-adenosylmethionine decarboxylase proenzyme (AdoMetDC) (SamDC)	169 (91)	96/98 (98%)	$1e-38$
			Amino acid metabolism			
H51-10	118	*GAD*	Glutamate decarboxylase	172 (93)	103/107 (96%)	$3e-40$
			Ribonucleotide metabolism			
H3-2-8	102	*Os12g0556600*	CTP synthase family protein	143 (77)	79/80 (99%)	$2e-31$
			Cellulose synthesis			
H46-3	193	*Os04g0429600*	Cellulose synthase-like protein H1	124 (67)	100/115 (87%)	$2e-25$
			Material transport			
N51-17	181	*Os12g0166000*	Peptidase S59, nucleoporin family protein	189 (102)	111/115 (97%)	$6e-45$

Table 2: Continued.

TDF no.	TDF size (bp)	GenBank/gene	Corresponding or related protein	Score (bits)	Identities	*E* value
H51-3	238	*Os01g0239200*	Phophate translocator	399 (216)	218/219 (99%)	3*e* − 108
N53-1	372	*Os08g0517200*	Ca^{2+}-ATPase isoform 9	580 (314)	316/317 (99%)	2*e* − 162
H57-2	304	*Os02g0176700*	Potential calcium-transporting ATPase 9, plasma membrane type	505 (273)	282/286 (99%)	9*e* − 140
H59-4	276	*Os02g0788800*	Amino acid/polyamine transporter II family protein	466 (252),	252/252 (100%)	4*e* − 128
			Stress response			
H53-8	191	*Os07g0186000*	Thioredoxin h isoform 1	189 (102)	104/105 (99%)	6*e* − 45
H53-9	186	*Os07g0186000*	Thioredoxin h isoform 1	187 (101)	104/105 (99%)	2*e* − 44
N60-4	307	*Os07g0620200*	Heat shock protein DnaJ, N-terminal domain containing protein	403 (218)	218/218 (100%)	3*e* − 109
			Signal transduction			
H51-6	175	*SRP14*	Signal recognition particle Subunit 14	279 (151)	156/158 (99%)	3*e* − 72
H51-7	144	*SRP14*	Signal recognition particle Subunit 14	209 (113)	122/126 (97%)	3*e* − 51
			Cell cycle and fate			
H4-1	357	*RSPSP94*	S-phase-specific ribosomal protein	604 (327)	329/330 (99%)	1*e* − 169
N6-1	296	*AY436773.1*	Putative senescence-associated protein (*Pyrus communis*)	503 (272)	277/279 (99%)	3*e* − 139
			Unclear functional proteins			
H3-2-1	200	*Os05g0156800*	Hypothetical protein	263 (142)	144/145 (99%)	4*e* − 67
N19-2	369	*BAH80065.1*	Putative retrotransposon protein	173 (438)	97/100 (97%)	4*e* − 48*
N51-2	436	*Os12g0597400*	Hypothetical protein	758 (410)	414/416 (99%)	0.0
N51-3	411	*Os12g0444500*	Hypothetical protein	479 (259)	270/275 (98%)	8*e* − 132
N51-4	399	*BAD68598.1*	Hypothetical protein	46.6 (109)	29/59 (49%)	5*e* − 04*
H51-2	250	*Os06g0124900*	Hypothetical protein	265 (143)	179/194 (92%)	1*e* − 67
H51-9	128	*Os01g0750800*	Hypothetical protein	174 (94)	102/106 (96%)	1*e* − 40
N53-5	298	*AAQ56570.1*	Polyprotein	145 (367)	79/125 (63%)	1*e* − 41*
H53-11	143	*Os04g0599650*	Tetratricopeptide-like helical domain containing protein	230 (124)	139/145 (96%)	3*e* − 57
H53-12	140	*Os04g0599650*	Tetratricopeptide-like helical domain containing protein	228 (123)	133/137 (97%)	9*e* − 57
H55-2	169	*Os01g0795000*	Hypothetical protein	274 (148)	148/148 (100%)	1*e* − 70
H56-4	212	*Os03g0685500*	Hypothetical protein	172 (93)	93/93 (100%)	7*e* − 40
H56-5	151	*Os12g0590400*	Hypothetical protein	147 (79)	91/96 (95%)	3*e* − 32
H56-8	173	*Os03g0395600*	Hypothetical protein	204 (110)	110/110 (100%)	2*e* − 49
N60-5	303	*EEC84589.1*	Hypothetical protein OsI_31400	90.9 (224)	45/48 (94%)	5*e* − 19*
N62-8	305	*AAT44171.1*	Hypothetical protein	73.2 (178)	35/36 (97%)	1*e* − 15*

The "H" before the numbers of the TDFs represents upregulated expression in high-temperature treated plants, while "N" represents upregulated expression in normal temperature treated plants (downregulated gene under high temperature). All are BLASTN scores except for those marked with "*", which are BLASTX scores.

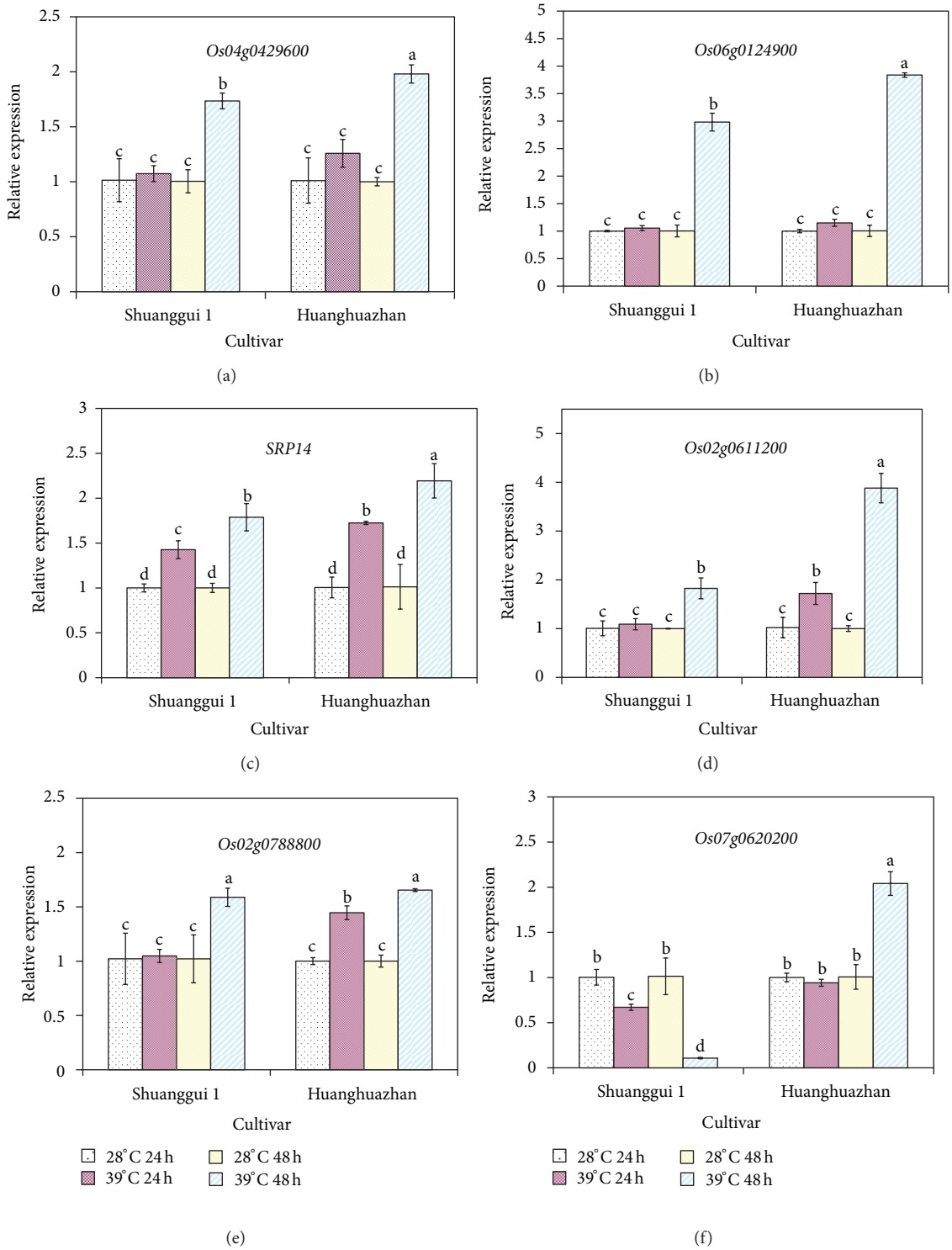

Figure 2: Relative expression of *Os04g0429600*, *Os06g0124900*, *SRP14*, *Os02g0611200*, *Os02g0788800*, and *Os07g0620200* compared to that of *actin* detected by real-time quantitative RT-PCR in plants at 28/22°C or plants at 39/30°C for 24 hours and 48 hours in heat-sensitive Shuanggui 1 and heat-tolerant Huanghuazhan. Values indicate relative expression levels against those of the same genes at 28/22°C in three biological replications from cDNA prepared from leaves. *Os04g0429600*, cellulose synthase-like protein H1; *Os06g0124900*, hypothetical protein; *SRP14*, signal recognition particle subunit 14; *Os02g0611200*, S-adenosylmethionine decarboxylase proenzyme (AdoMetDC) (SamDC); *Os02g0788800*, amino acid/polyamine transporter II; *Os07g0620200*, heat shock protein DnaJ, N-terminal domain containing protein.

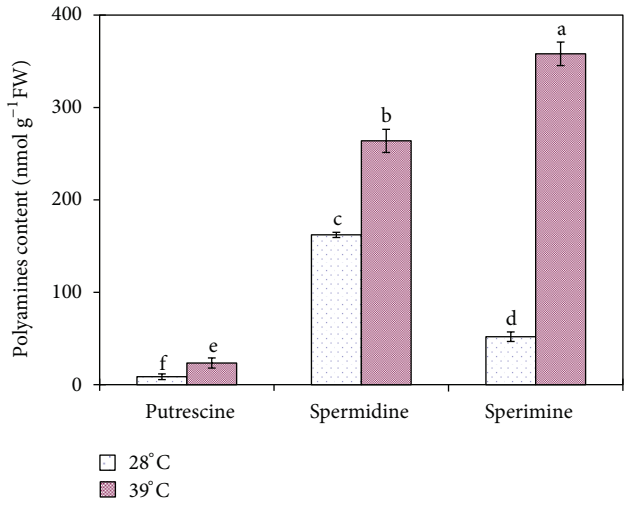

Figure 3: Variation of levels of free spermidine, spermine, and putrescine in rice seedlings under 39°C and 28°C treatments with HPLC analysis. Data from levels of free spermidine, spermine, and putrescine in rice seedlings are means of three replications ± SE. Dissimilar letters above bars differ significantly at $P \leq 0.05$.

Os06g0124900, *SRP14*, *Os02g0611200*, and *Os02g0788800*) expressions were increased under heat stress with one TDF (*Os07g0620200*) shown downregulated under the same condition.

4. Discussion

Previous genomic and proteomic studies on rice responses to heat stress have provided some valuable results. Genes [26] and proteins [27] of rice grain under heat stress conditions have been identified. Changes of proteomic profiles in rice seedling leaves at high temperature [4] have also been reported. In this study, responses of rice seedlings to heat stress environment (39°C day/30°C night) were investigated. Among TDFs, which were responsive to 39°C, 49 of them have been identified. The corresponding proteins or relative nucleotides of these identified TDFs were related to energy (photosynthesis and photorespiration) transportation, transcription, or translation, and other biological functions, respectively. Furthermore, unlike previous transcriptomic [26] and proteomic [4, 9, 27] studies, in our study 49 TDFs were identified as high-temperature-responsive genes. The identified genes provided valuable information by which the tolerance mechanisms of rice exposed to heat stress can be unveiled in detail.

4.1. Effect of Heat Stress on the Biological Process of the Rice Seedling. In this study, there are fourteen differential expression fragments, which were classified into carbohydrate metabolism group. The upregulated TDF (H51-4, Table 2) in response to heat stress is the chloroplast gene (*psbH*), which encodes a 9-10 kDa thylakoid membrane protein (PSII-H). PSII protein H is associated with photosysthem II and is subject to light-dependent phosphorylation at a threonine residue located on the stromal side of the membrane [28]. Thus, the up-regulation of *psbH* gene could protect the photosynthetic machinery in the high-temperature-stressed rice seedlings. Other two differential expression genes (N21-1, H53-1, Table 2), which are involved in carbohydrate metabolism, are also induced by the heat stress. TDF (N21-1, Table 2) corresponding to NAD dependent epimerase/dehydratase family protein is downregulated under 39°C treatment; TDF (H53-1, Table 2) corresponding to NADH dehydrogenase subunit 2 is upregulated by the stress treatment. NAD-dependent epimerase/dehydratase family protein takes part in the carbohydrate metabolic biological process, which includes the formation of carbohydrate derivatives by the addition of a carbohydrate residue to another molecule. Hence, downregulated TDF (N21-1, Table 2) suggested that heat stress may inhibit the carbohydrate derivatives formation in the seedling stage of seedling leaves. NADH dehydrogenase, which is responsible for the oxidative phosphorylation [29], is so-called entry-enzyme of the mitochondrial electron transport chain. The up-regulation of NADH dehydrogenase indicated that protective mechanism involved in electron transportation process may be stimulated in the heat-stressed rice seedling. In spite of down-regulation of NAD-dependent epimerase involved in carbohydrate derivatives formation, the more significant electronic transportation process regulated by NADH dehydrogenase showed upregulated trend indicating the protective capability of rice seedling confronted with heat stress.

In cellulose metabolism category, TDF (H46-3, Table 2 and Figure S1), which encodes cellulose synthase family protein, was also upregulated by the heat stress stimulation. The transcript expression of this enzyme in cDNA-AFLP was also correlated with the results in real-time RT-PCR (Figure 2). Earlier work concluded that cellulose synthase is the key enzyme involved in cellulose synthesis in cell wall [30]. The up-regulation of cellulose synthase during the heat stress indicated that high-temperature may improve the formation of cell wall and, hence, assumed the protective function against the damage generated by the temperature for the rice seedling.

Besides the most abundant groups mentioned above, there were still other TDFs, which were identified as functions involved in material transportation, cell structure and cycle, polyamine metabolism, and signal transduction, and so forth. In spite of fewer TDFs found in these groups, the accordance of the semiquantitative RT-PCR results (data not shown) and the real-time PCR results (Figure 2) with the cDNA-AFLP analysis (Figure S1) indicated that these fragments with corresponding functional groups were also affected severely by the increase of the temperature. Therefore, the identification of the TDFs will be a solid foundation for the future researches.

Five genes, were which, involved in material transportation were increasely expressed under heat stress, which implied a temperature-related activation of this process. In this study, TDF (H59-4, Table 2), identified as amino

acid permease, which could import serine and generate sphingoid bases during heat stress [31], was significantly upregulated at 39°C treatment. The activity change of TDF (H59-4), agreeing well with the change of mRNA expression of amino acid permease (Figure 2), confirmed that permease-regulated amino acid uptake could be increased by the stimulation of the heat stress. In addition, another TDF (H51-3, Table 2 and Figure S1) corresponding to triose phosphate translocator protein could also be included into material transporters group. Triose phosphate translocator protein is a membrane protein responsible for exchanging all carbohydrate products produced in photosynthesis in plants and, therefore, could mediate the export of fixed carbon in the form of triose phosphates and 3-phosphoglycerate from the chloroplasts into the cytosol [32]. So the up-regulation of TDFs (H51-3) indicated that the increase of the temperature could promote the carbohydrate products transportation produced in photosynthesis and, hence, provide the protective mechanism for the rice seedling leaves during the heat stress.

There are other two upregulated TDFs (H51-6, Figure S1 and Table 2; H57-5, Figure S1 and Table 2), which encode signal recognition particle subunit and S-adenosylmethionine decarboxylase, classified into two newly functional groups: signal transduction and polyamine metabolism. The up-regulation of TDF (H51-6, Figure S1 and Table 2), which encodes signal recognition particle subunit, suggested that high temperature could also improve the signal transduction process during the heat stress. S-Adenosylmethionine decarboxylase is an enzyme whose capability is to catalyze the conversion of S-adenosyl methionine to S-adenosylmethioninamine. S-adenosylmethionine decarboxylase plays an essential regulatory role in the polyamine biosynthetic pathway by producing the n-propylamine residue required for the synthesis of spermidine and spermine from putrescine [33, 34]. Spermidine and spermine synthase (SPDS; EC 2.5.1.16 and SPMS; EC 2.5.1.22) could synthesize higher spermidine and spermine by the successive addition of aminopropyl groups to putrescine [35]. Our result has also proved that the upregulated expression of S-adenosylmethionine decarboxylase gene agreed well with the contents of spermidine and spermine (Figures 2 and 3). In addition, one TDF (N60-4, Table 2, Figure S1) corresponding to heat shock protein (hsp) has been classified into stress response group. Its down-regulation function, which was agreeing well with the real-time RT-PCR result (Figure 2), indicated that the gene was affected obviously under heat stress condition.

In conclusion, this study reported 49 identified differential expression fragments in rice seedlings in respons to the heat stress. Meanwhile, ten functional groups classification containing 49 TDFs indicating different strategies were employed by rice seedlings exposed to different temperatures: the higher the temperature, the more effects could be observed. Our future research could be focused to reveal functions of the genes indentified under heat stress.

Authors' Contribution

Y. Cao and Q. Zhang contributed equally to this work.

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Review Article

Biotechnological Approaches to Study Plant Responses to Stress

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Multiple biotic and abiotic environmental stress factors affect negatively various aspects of plant growth, development, and crop productivity. Plants, as sessile organisms, have developed, in the course of their evolution, efficient strategies of response to avoid, tolerate, or adapt to different types of stress situations. The diverse stress factors that plants have to face often activate similar cell signaling pathways and cellular responses, such as the production of stress proteins, upregulation of the antioxidant machinery, and accumulation of compatible solutes. Over the last few decades advances in plant physiology, genetics, and molecular biology have greatly improved our understanding of plant responses to abiotic stress conditions. In this paper, recent progresses on systematic analyses of plant responses to stress including genomics, proteomics, metabolomics, and transgenic-based approaches are summarized.

1. Introduction

Plants and animals share some response mechanisms to unfavorable environmental conditions; however, plants, being sessile organisms, have developed, in the course of their evolution, highly sophisticated and efficient strategies of response to cope with and adapt to different types of abiotic and biotic stress imposed by the frequently adverse environment.

Stress can be understood as a stimulus or influence that is outside the normal range of homeostatic control in a given organism: if a stress tolerance is exceeded, mechanisms are activated at molecular, biochemical, physiological, and morphological levels; once stress is controlled, a new physiological state is established, and homeostasis is reestablished. When the stress is retired, the plant may return to the original state or to a new physiological situation [1].

In the last years, and because of the great interest for both basic and applied research, there has been an important progress in the understanding of the mechanisms and processes underlying abiotic stress adaptation and defense in different plant species [1, 2]. The sensing of biotic or abiotic stress conditions induces signaling cascades that activate ion channels, kinase cascades, production of reactive oxygen species (ROS), accumulation of hormones such as salicylic acid, ethylene, jasmonic acid, and abscisic acid. These signals ultimately induce expression of specific subsets of defense genes that lead to the assembly of the overall defense reaction [3].

The emergence of the novel "omics" technologies, such as genomics, proteomics, and metabolomics, is now allowing researchers to identify the genetic behind plant stress responses (Figure 1). These omics technologies enable a direct and unbiased monitoring of the factors affecting plant growth and development and provide the data that can be directly used to investigate the complex interplay between the plant, its metabolism, and also the stress caused by the environment or the biological threats (insects, fungi, or other pathogens). Plant responses to stress are mediated via profound changes in gene expression which result in changes in composition of plant transcriptome, proteome, and metabolome [4].

In this work, the main biotechnological approaches to study plant responses to stress are reviewed.

2. Genomics

A gene by gene approach has been typically used to understand its function. In Table 1, some of the genes involved in plant responses to stress are listed. Functional genomics

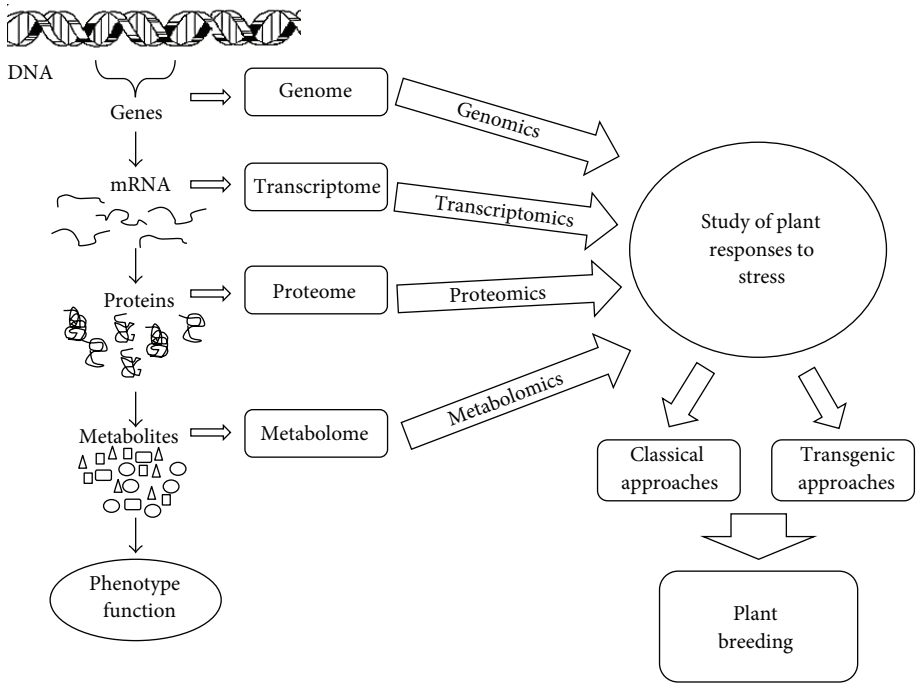

Figure 1: Plant response to abiotic stress factors. Genomics, transcriptomics, proteomics, and metabolomics have enabled active analyses of regulatory networks that control abiotic stress responses. Such analyses increase our knowledge on plant responses and adaptation to stress conditions and allow improving plant breeding.

Table 1: Genes involved in plant responses to stress.

	Stress	Reference
14.3.3 gene family (GF14b, GF14c)	Salinity, drought, fungal	[9]
MAPK	Abiotic and biotic stresses	[10]
MEKK1 and ANP1	Environmental stress	[11, 12]
MPK3, MPK4 and MPK6	Abiotic stress (pathogens) and oxidative stress	[10, 13]
CBF/DREB families (CBF1, CBF2, DREB2A)	Drought, cold, salinity	[47, 82]
HVA1	Salinity and drought	[83]
Glycerol-3-phosphate acyltransferase gene	Cold	[84]
ICS	Pathogens, UV light	[85]
LOX	Wounding, drought, and pathogens	[86, 87]
bZIPs family (e.g., ABF1, ABF2)	Drought, temperature, salinity	[24–33]
WRKY family (AtWRKY2, AtWRKY6, AtWRKY18)	Pathogens, wounding, salinity, temperature, drought, oxidative stress	[37]
ATAF	Wounding, drought, salinity, cold, pathogens	[88]

allows large-scale gene function analysis with high throughput technology and incorporates interaction of gene products at cellular and organism level. The information coming from sequencing programs is providing enormous input about genes to be analyzed. The availability of many plant genomes nowadays (reviewed in [5, 6]) facilitates studying the function of genes on a genomewide scale. The lack of information from other plant genomes will also be compensated in part by the availability of large collection of expressed sequence tags (ESTs) and cDNA sequences [7]. The basic interest behind these EST projects is to identify genes responsible for critical functions. ESTs, cDNA libraries, microarray, and serial analysis of gene expression (SAGE) are used to analyze global gene expression profiles in a functional genomics program. Large mutant collections are tools that complement large-scale expression studies. Gene iden-

tification through physical and chemical mutagens has become amenable for large-scale analysis with the availability of markers [8], but gene tagging is more promising for functional analysis on a wider scale. Moreover, the understanding of the complexity of stress signaling and plant adaptive processes would require the analysis of the function of numerous genes involved in stress response.

Numerous investigations show that plant defense response genes are transcriptionally activated by pathogens and also by different types of abiotic stress. It has been described that the induction of specific defense genes, in the response against certain pathogens, is dependent on specific environmental conditions, suggesting the existence of a complex signaling network that allows the plant to recognize and protect itself against pathogens and environmental stress [3]. Similar induction patterns of members of the 14.3.3 gene family (GF14b and GF14c) by abiotic and biotic stresses such as salinity, drought, ABA, and fungal inoculation have been documented in rice [9]. The rice GF14 genes contain cis-elements in their promoter regions that are responsive to abiotic stress and pathogen attack. The 14-3-3s family genes are also subject to the regulation by certain transcript factors [9].

On the other hand, kinase cascades of the mitogen-activated protein kinase (MAPK) class play a remarkably important role in plant signaling of a variety of abiotic and biotic stresses, and it is an essential step in the establishment of resistance to pathogens [10]. It has been described that in Arabidopsis MEKK1 and ANP1 act in the environmental stress response [11, 12], and MPK3, MPK4, and MPK6, are activated by a diversity of stimuli including abiotic stresses, pathogens, and oxidative stress [13].

Elucidating the molecular mechanism that mediates the complex stress responses in plants system is an important step to develop improved variety of stress tolerant crops. Many crop traits are quantitative, complex, and controlled by multiple interacting genes. Recent progress in molecular biology provides the tools to study the genetical make-up of plants, which allows us to unravel the inheritance of all traits whether they are controlled by single genes or many genes acting together, known as the quantitative trait loci (QTL). The molecular marker technologies available since the 1980s allows dissecting the variation in traits. With the progress of QTL mapping, new breeding approaches such as marker-assisted selection and breeding by design have emerged [14].

Advances in plant genomics research have opened up new perspectives and opportunities for improving crop plants and their productivity. The genomics technologies have been found useful in deciphering the multigenicity of biotic and abiotic plant stress responses through genome sequences, stress-specific cell and tissue transcript collections, protein and metabolite profiles and their dynamic changes, protein interactions, and mutant screens.

3. Proteomics

The adaptation of plants to biotic or abiotic stress conditions is mediated through deep changes in gene expression which result in changes in composition of plant transcriptome, proteome, and metabolome. Since proteins are directly involved in plant stress response, proteomics studies can significantly contribute to elucidate the possible relationships between protein abundance and plant stress acclimation. Several studies [15] have already proven that the changes in gene expression at transcript level do not often correspond with the changes at protein level. The investigation of changes in plant proteome is highly important since proteins, unlike transcripts, are direct effectors of plant stress response. Proteins not only include enzymes catalyzing changes in metabolite levels, but also include components of transcription and translation machinery (Table 2).

In the last years, there has been an important progress in the knowledge of several families of plant transcription factors linked to plant stress responses, such as responses to ultraviolet light, wounding, anaerobic stress, and pathogens [16]. The most important ones are as follows.

(i) The ethylene-responsive-element-binding factors (ERFs). This protein family has been linked to a wide range of stresses; the RNA levels of specific ERF genes are regulated by cold, drought, pathogen infection, wounding or treatment with ethylene, SA or JA [17]. ERF proteins are shown to function as either activators or repressors of transcription, which is of great relevance in all processes related to plant development and its responses to adverse growing conditions due to both biotic and abiotic factors [18]. It has been reported that ERF proteins from one plant species function in other plant species, enhancing their potential utility in increasing the stress tolerance of plants [19, 20]. However, constitutive overexpression of *ERF* genes generally causes deleterious effects. To overcome this problem, the use of stress-inducible promoters to control the expression of the *ERF* genes has been successfully used (reviewed in [21]).

(ii) NAC proteins are plant-specific transcription factors having a variety of important functions not only in plant development but also in abiotic stress tolerance [22]. NAC domain-containing proteins represent one of the largest TF families, firstly identified in model plants as Arabidopsis and rice but also recently characterized in woody fruit species [23].

(iii) Another important family of transcription factors is the called "basic-domain leucine-zipper (bZIP)" which are regulators of important plant processes such as organ and tissue differentiation [24], cell elongation [25], nitrogen/carbon balance control [26], pathogen defense [27], energy metabolism [28], unfolded protein response [29], hormone and sugar signaling [30], light response [31], osmotic control [32], and seed storage protein gene regulation [33]. One class of bZIP proteins that is linked to stress responses comprises the TGA/*octopine synthase* (*ocs*)-element-binding factor (OBF) proteins. These bind to the *activation sequence-1* (*as-1*)/*ocs* element, which regulates the expression of some stress-responsive genes [34]. A major advance was the discovery that

Table 2: Proteins and enzymes involved in plant responses to stress.

	Stress	Reference
ERF family	Cold, drought, pathogen infection, wounding, ET, SA, and JA	[19]
bZIPs family (e.g., ABF1, ABF2)	Drought, temperature, salt	[24–33]
WRKY	Pathogens, wounding, salinity, temperature, drought, oxidative stress	[36–39]
MYB family (AtMYB15, AtMYB30, AtMYB33 AtMYB60, AtMYB96, AtMYB101 AtMYB15, and AtMYB108)	Biotic and abiotic stress (pathogens, drought, cold)	[40–46]
ABF	Drought	[79]
NAC	Drought, salinity, cold	[22, 23]
MYC	Environmental stresses	[47]
LEA family (PMA 80, PMA 1959)	Salinity and drought	[71]
Heat shock proteins	Temperatures	[74, 75]
LOX family (e.g., LOX1)	Wounding, drought, and pathogens	[86, 87]
Glutathione peroxidase, superoxide dismutase, ascorbate peroxidases, and glutathione reductases	Oxidative stress	[68, 70]

TGA/OBF family members interact with nonexpressor of PR1 (NPR1), a key component in the SA defense signaling pathway [35].

(iv) WRKY proteins are a family of transcription factors that are unique to plants specific WRKY family members show enhanced expression and/or DNA-binding activity following induction by a range of pathogens, defense signals, and wounding (reviewed in [36]). Significant progress has been made in the past years in identifying target genes for WRKY factors. WRKY proteins bind to the W box, which is found in the promoters of many plant defense genes [37]. WRKY proteins also regulate the expression of regulatory genes such as receptor protein kinases [38]. Positive and negative regulation of *WRKY* promoters by specific WRKY proteins has been observed, and the promoters of many of the pathogen- and/or SA-regulated *AtWRK* genes are rich in W boxes [39].

(v) MYB proteins are key factors in regulatory networks controlling development [40], metabolism [41], and responses to biotic and abiotic stresses [42]. Since the Arabidopsis genome sequence was published, some years ago, an important amount of data has accumulated on the roles of MYB transcription factors in plants and some members of this family are involved in these responses. Therefore, *AtMYB30* encodes an activator of the hypersensitive cell death program in response to pathogen attack [43]; *AtMYB96* acts through the ABA signaling cascade to regulate water stress and disease resistance [44]. *AtMYB33* and *AtMYB101* are involved in ABA-mediated responses to environmental signals. *AtMYB15* is also involved in cold stress tolerance [45]. *AtMYB108* in both biotic and abiotic stress responses [46]. The elucidation of MYB protein function and regulation that is possible in Arabidopsis will allow predicting the contributions of MYB proteins to the responses to biotic and abiotic stress conditions in other plant species.

(vi) MYC proteins are involved in the response of plants to unfavorable environmental conditions. This transcription factor family plays a role in the induction of apoptosis, important in the hypersensitive cell death program in response to pathogen attack. Another putative MYC target is the ornithine decarboxylase gene, involved in polyamines synthesis. On the other hand, MYC proteins activate the major ABA-dependent stress response (reviewed in [47]).

Therefore, studies of plant reaction upon stress conditions at protein level can significantly contribute to our understanding of physiological mechanisms underlying plant stress tolerance. Proteomics studies could thus lead to identification of potential protein markers whose changes in abundance can be associated with quantitative changes in some physiological parameters related to stress tolerance (reviewed in [16]).

4. Metabolomics

The possibility of monitoring a complete set of metabolites could largely improve the understanding of many physiological plant processes. This systematic study, defined as "metabolomics," is intended to provide an integrated view of the functional status of an organism. Besides its use as a breeding or selection tool, metabolomics techniques have also been used to evaluate stress responses in barley [48], Citrus [49], *Medicago truncatula* [50], and *Arabidopsis thaliana* [51]. Some of the metabolites that have been involved in the plant responses to stress are listed in Table 3.

This technological tool, recently developed, includes different approaches, namely, targeted analysis, metabolic fingerprinting, and metabolite profiling.

Table 3: Metabolites and hormones involved in plant responses to stress.

	Stress	Reference
Abscisic acid, jasmonic acid, salicylic acid, polyamines, and others	Drought, salinity, cold	[3, 58, 65–67]
Proline, glycine-betaine, and other compatible osmolytes	Environmental stresses: drought, salinity, osmotic	[62–64]
Phytoalexins	Microbial pathogens	[89]
Terpenes	Toxins and pathogens	[89]
Phenolic compounds (coumarin, lignin, flavonoids, tannins, isoflavonoids)	Pathogens, oxidative stress, UV light	[89, 90]
Alkaloids	Pathogens (predators)	[87]
Unsaturated fatty acids	Environmental stresses	[73]
ROS, malondialdehyde	Biotic and abiotic stresses	[3, 58, 68]
Phytochelatins and metallothioneins	Heavy metal intoxication	[76, 77]

Targeted analysis is the most developed analytical approach in metabolomics [49]. It is used to measure the concentration of a limited number of known metabolites precisely, by using either gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS) or nuclear magnetic resonance spectroscopy (NMR).

Other approaches using high throughput metabolite analysis focus on a subset of useful information while avoiding the difficulties of comprehensive metabolite characterization; *metabolic fingerprinting* uses signals from hundreds to thousands of metabolites for rapid sample classification via statistical analysis [52]. In the last years, *metabolite profiling* attempts to identify and quantify a specific class or classes of chemically related metabolites that often share chemical properties that facilitate simultaneous analysis (reviewed in [53]).

The metabolome represents the downstream result of gene expression and is closer to phenotype than transcript expression or proteins. Extensive knowledge on metabolic flows could allow assessment of genotypic or phenotypic differences between plant species or among genotypes exhibiting different tolerance to some biotic or abiotic stresses. In addition, target metabolites have been analyzed as nutritional and/or agronomical biomarkers to classify different crop cultivars or to optimize growth conditions [54].

In contrast to high throughput methodology for the analysis of DNA, RNA, and proteins, current strategies for metabolite characterization still face significant obstacles. These challenges are largely caused by the high degree of chemical diversity among metabolite pools as well as the complexity of spatial and temporal distribution within living tissues. Plant metabolomics methodology and instrumentation are being developed at a rapid pace to address these analytical challenges [55].

Like other functional genomics research, metabolomics generates large amounts of data. Handling, processing, and analyzing this data is a clear challenge for researchers and requires specialized mathematical, statistical, and bioinformatic tools [56]. Further developments in this area require improvements in both analytical science and bioinformatics. Development of new analytical techniques is largely focused on increasing resolution and comprehensiveness, increasing speed and throughput of analytical assays and equipment miniaturization.

5. Transgenic Approaches: From the Study of Stress Tolerance Mechanisms in Plants to Crop Genetic Improvement

Use of modern molecular biology tools for elucidating the control mechanisms of stress tolerance and for engineering stress tolerant plants is based on the expression of specific stress-related genes. To date, successes in genetic improvement of environmental stress resistance have involved manipulation of a single or a few genes involved in signaling/regulatory pathways or that encode enzymes involved in these pathways [57].

The plant hormone abscisic acid (ABA) regulates the adaptive response of plants to environmental stresses such as drought, salinity, and chilling via diverse physiological and developmental processes [58]. The ABA biosynthetic pathway has been deeply studied, and many of the key enzymes involved in ABA synthesis have been used in transgenic plants to improve abiotic stress tolerance [59]. Transgenic plants overexpressing the genes involved in ABA synthesis showed increased tolerance to drought and salinity stress [59]. Similarly, many studies have illustrated the potential of manipulating *CBF/DREB* genes to confer improved drought tolerance [60].

Another mechanism involved in plant protection to osmotic stress associated to many abiotic stresses such as drought and salinity implies the accumulation of compatible solutes involved in avoiding oxidative damage and chaperoning through direct stabilization of membranes and/or proteins [61]. Many genes involved in the synthesis of these osmoprotectants have been explored for their potential in engineering plant abiotic stress tolerance [61]. The amino

acid proline is known to occur widely in higher plants and normally accumulates in large quantities in response to environmental stresses [62]. The osmoprotectant role of proline has been verified in some crops by overexpressing genes involved in proline synthesis [63]. The results of transgenic modifications of biosynthetic and metabolic pathways in most of the previously mentioned cases indicate that higher stress tolerance and the accumulation of compatible solutes may also protect plants against damage by scavenging of reactive oxygen species (ROS) and by their chaperone-like activities in maintaining protein structures and functions [64].

Polyamines, being polycationic compounds of low molecular weight, are involved in many cellular processes, such as replication, transcription, translation, membrane stabilization, enzyme activity modulation, plant growth, and development [65]. It has been reported that stress results in an accumulation of free or conjugated polyamines, indicating that polyamine biosynthesis might serve as an integral component of plant response to stress [66, 67].

Polyamines metabolic pathways are regulated by a limited number of key enzymes, among them ornithine decarboxylase (ODC) and arginine decarboxylase (ADC). Transgenic plants overexpressing *ADC* gene showed increase in biomass and better performance under salt stress conditions. It has also been described that genetic transformation with genes encoding ADC improved environmental stress tolerance in various plant species [66].

A common factor among most stresses is the active production of reactive oxygen species [2]. ROS are not only toxic to cells but also play an important role as signaling molecules. Under normal growth conditions, there is equilibrium between the production and the scavenging of ROS, but abiotic stress factors may disturb this equilibrium, leading to a sudden increase in intracellular levels of ROS.

In order to control the level of ROS and protect the cells from oxidative injury, plants have developed a complex antioxidant defense system to scavenge them [68]. These antioxidant systems include various enzymes and nonenzymatic metabolites that may also play a significant role in ROS signaling in plants. A number of transgenic improvements for abiotic stress tolerance have been achieved through detoxification strategy [69]. These include transgenic plants over expressing enzymes involved in oxidative protection, such as glutathione peroxidase, superoxide dismutase, ascorbate peroxidases, and glutathione reductases [70].

LEA proteins, including several groups of high molecular weight, accumulate in response to different environmental stresses. It has been reported that constitutive overexpression of the HVA1, a group 3 LEA protein from barley, conferred tolerance to soil water deficit and salt stress in transgenic rice plants [71]. It has also been reported that plants expressing a wheat LEA group 2 protein (PMA80) gene or the wheat LEA group 1protein (PMA1959) gene resulted in increased tolerance to dehydration and salt stresses [69].

An important strategy for achieving greater tolerance to abiotic stress is to help plants to reestablish homeostasis under stressful environments, restoring both ionic and osmotic homeostasis. This is a major approach to improve salt tolerance in plants through genetic engineering, where the target is to achieve Na^+ excretion out of the root, or their storage in the vacuole [72].

Transgenic approaches also aim to improve photosynthesis under abiotic stress conditions through changes in the lipid biochemistry of the membranes. Genetically engineered plants overexpressing chloroplast glycerol-3-phosphate acyltransferase gene (involved in phosphatidyl glycerol fatty acid desaturation) showed an increase in the number of unsaturated fatty acids and a corresponding decrease in the chilling sensitivity [73].

The heat shock response is a highly conserved biological response, occurring in all organisms in response to heat or other toxic agent exposures [74]. Genetic engineering for increased thermotolerance by enhancing heat shock protein synthesis in plants has been achieved in a number of plant species. Some authors have reported the positive correlation between the levels of heat shock proteins and stress tolerance (reviewed in [75]).

A special case of study is the heavy metal contamination. In spite of the natural occurrence of heavy metals as rare elements, diverse anthropogenic practices have contributed to spread them in the environment. Plants have developed mechanisms that can protect cells from heavy metal cytotoxicity, as the cytosolic detoxification by binding to the metal-binding molecules as phytochelatins, and metallothioneins which play an important role in heavy metal detoxification and homeostasis of intracellular metal ions in plant tissues. Overexpression of phytochelatin synthase in Arabidopsis leads to enhanced arsenic tolerance but surprisingly to cadmium hypersensitivity [76]. Therefore, new approaches could contribute to uncovering the complexity of plant tolerance to heavy metal stress [77].

The transcription factors activate cascades of genes that act together in enhancing tolerance towards multiple stresses as indicated before. On the other hand, some stress responsive genes may share the same transcription factors, as indicated by the significant overlap of the gene expression profiles that are induced in response to different stresses [37]. Transcriptional activation of stress-induced genes has been possible in transgenic plants over expressing one or more transcription factors that recognize promoter regulatory elements of these genes [75, 78]. Two families, bZIP and MYB, are involved in ABA signaling and its gene activation. Introduction of transcription factors in the ABA signaling pathway can also be a mechanism of genetic improvement of plant stress tolerance. Constitutive expression of *ABF3* or *ABF4* demonstrated enhanced drought tolerance in Arabidopsis, with altered expression of ABA/stress-responsive genes, for example, *rd29B*, *rab18*, *ABI1*, and *ABI2* [79].

It is important to point that genetic modification of higher plants by introducing DNA into their cells is a highly complex process. Practically any plant transformation experiment relies at some point on cell and tissue culture. Although the development transformation methods that avoid plant tissue culture have been described for Arabidopsis and have been extended to a few crops, the ability to regenerate plants from isolated cells or tissues *in vitro* is needed for most plant

transformation systems. Not all plant tissue is suited to every plant transformation method, and not all plant species can be regenerated by every method [80]. There is, therefore, a need to find both a suitable plant tissue culture/regeneration regime and a compatible plant transformation methodology [81].

6. Conclusions

To understand how plants respond to stress, it must be considered that they are subjected to a combination of adverse conditions. This preliminary consideration is essential to understand the performance of plants under stress and also to identify strategies to improve stress tolerance.

The integration of the omics approaches is likely to enable researchers to reconstruct the whole cascade of cellular events leading to rapid responses and adaptation to the various abiotic stimuli. A well-focused approach combining molecular, physiological, and metabolic aspects of plant stress tolerance is required to increase knowledge on the effects of gene expression and to understand whole plant phenotype under stress. A better understanding of the underlying physiological processes in response to different abiotic stresses can drive the selection of the appropriate promoter or transcription factor to be used for transformation.

In addition, the use of genetic and genomic analysis to identify DNA molecular markers associated to stress resistance can facilitate breeding strategies for crop improvement. This approach is particularly useful when target characters are controlled by several genes, as in the case of abiotic stress tolerance. These omics approaches could be combined with the potential to map different QTLs contributing to a given agronomical trait and to identify linked molecular markers. This will open the possibility to transfer simultaneously several QTLs and to pyramid QTLs for several agronomical traits in one improved cultivar.

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Research Article

Characterization of the Newly Developed Soybean Cultivar DT2008 in Relation to the Model Variety W82 Reveals a New Genetic Resource for Comparative and Functional Genomics for Improved Drought Tolerance

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Soybean (*Glycine max*) productivity is adversely affected by drought stress worldwide, including Vietnam. In the last few years, we have made a great effort in the development of drought-tolerant soybean cultivars by breeding and/or radiation-induced mutagenesis. One of the newly developed cultivars, the DT2008, showed enhanced drought tolerance and stable yield in the field conditions. The purpose of this study was to compare the drought-tolerant phenotype of DT2008 and Williams 82 (W82) by assessing their water loss and growth rate under dehydration and/or drought stress conditions as a means to provide genetic resources for further comparative and functional genomics. We found that DT2008 had reduced water loss under both dehydration and drought stresses in comparison with W82. The examination of root and shoot growths of DT2008 and W82 under both normal and drought conditions indicated that DT2008 maintains a better shoot and root growth rates than W82 under both two growth conditions. These results together suggest that DT2008 has better drought tolerance degree than W82. Our results open the way for further comparison of DT2008 and W82 at molecular levels by advanced omic approaches to identify mutation(s) involved in the enhancement of drought tolerance of DT2008, contributing to our understanding of drought tolerance mechanisms in soybean. Mutation(s) identified are potential candidates for genetic engineering of elite soybean varieties to improve drought tolerance and biomass.

1. Introduction

Soybean (*Glycine max*) is one the world's leading economic oilseed crops, providing the largest source of vegetable oil, proteins, macronutrients, and minerals for human consumption and animal feed. The consumption of soybean as food products is increasing worldwide because of its beneficial effects including lowering of cholesterol and prevention of cancer, diabetes, and obesity [1–4]. In addition, soybean is also an attractive crop for the production of biodiesel which produces more usable energy and less greenhouse gases than corn-based ethanol [5].

Vietnam with its 161.200 ha of soybean cultivated land produces approximately 235.450 metric tonnes (~1.41 metric tonnes/ha in average as recorded for the year of 2009) which is sufficient for only 8–10% of soybean consumption in the country [6]. The low productivity of soybean in Vietnam is mainly attributed to drought stress [6] which also

affects soybean production worldwide [1, 7]. Therefore, in the last decade the Vietnamese soybean research community has spent much effort on the development of drought-tolerant soybean cultivars. Several elite soybean cultivars were obtained by using conventional breeding coupled with radiation-induced mutagenesis. One of the varieties, the DT2008, exhibited enhanced tolerance to various biotic and abiotic stresses, including drought, enabling the farmers to grow the DT2008 cultivar in three crops/year with high and stable yield across the country (~2–4 metric tonnes/year depending on the regions) [6].

Plants, including soybean, activate various mechanisms to cope with drought stress [8–10]. In the last 20 years, many genes, including both regulatory and functional genes, have been identified in important crops, such as rice (*Oryza sativa*) and soybean, to be involved in defence mechanisms that function to increase drought tolerance [7, 11–17]. One of the preferred approaches widely used to identify stress-responsive genes was large-scale expression profiling using microarray analysis or high-throughput qRT-PCR [18–23]. The genomic sequence of the model soybean cultivar Williams 82 (W82) has been completed two years ago [24], enabling us to carry out large-scale expression profiling of soybean gene families [15, 20] or design gene chips for genome-wide identification of drought-responsive genes [21, 25]. With the advance in proteomic and metabolomic technologies, an increasing number of literature has described the applications of these techniques in study of mechanisms and signalings related to drought responses in soybean [26, 27].

In this work, we compared the drought-tolerant phenotypes of DT2008 and W82 by examining the dehydration-induced water loss and membrane stability of the shoot parts of the young seedlings. The results suggest that the enhanced drought tolerance of DT2008 is, at least in part, associated with shoot traits. Further comparisons of shoot and root growth under normal and drought stress conditions indicated that DT2008 grew better than W82 under both conditions. Data on root growth suggest that the better drought tolerance of DT2008 is coincided with better root growth and development in comparison with W82. The differential drought-responsive phenotypes of DT2008 and W82 will enable us to identify mutation(s) which caused drought-tolerance of DT2008 using systems biology-based approaches, such as transcriptomics, proteomics, or metabolomics. The mutations and/or mutated gene(s) identified can be used to improve drought tolerance of soybean cultivars, which have high productivity but are sensitive to drought stress, by genetic engineering.

2. Materials and Methods

2.1. Measurement of Relative Water Content under Dehydration. DT2008 and W82 seeds were separately germinated in 6-litre pots (10 seeds/pot) containing vermiculite and grown under well-water conditions in greenhouse (continuous 30°C temperature, photoperiod of 12 h/12 h, 80 μmol m^{-2} s^{-1} photon flux density, and 60% relative humidity). Ten-day-old DT2008 and W82 plants were carefully removed from soil, and roots were gently washed to remove soil. The plants were subsequently transferred onto a filter paper and allowed to dry for 5 h under the following conditions: 60% relative humidity, 25°C temperature and 10 μmol m^{-2} s^{-1} photon flux light intensity. Dehydrated shoot samples (without cotyledon leaves and root) were individually weighed to determine sample weight (W) after 5 h of dehydration (n = 20). Individual dehydrated samples were then placed into 50 mL tubes and hydrated overnight in 40 mL of deionized water to full turgidity under normal room light and temperature. The samples were then removed from water, residual leaf moisture was gently removed with filter paper, and samples were immediately weighed to obtain a fully turgid weight (TW). Subsequently, the plants were dried in an oven at 65°C for 48 h, and dry weight was measured (DW). RWC was calculated as RWC (%) = [(W − DW)/(TW − DW)] × 100 [28].

2.2. Measurement of Relative Water Content under Drought Stress. Two DT2008 and two W82 seeds were germinated in each 6-litre pot containing vermiculite and grown under well-water conditions in greenhouse (continuous 30°C temperature, photoperiod of 12 h/12 h, 80 μmol m^{-2} s^{-1} photon flux density and 60% relative humidity). For drought treatment, water was withheld from 5-day-old plants for 15 or 20 days. Volumetric soil moisture contents were monitored at 5-day intervals during drought stress treatment using a Hydrosense soil moisture probe (Campbell Scientific Inc.) (n = 5). For control, plants were grown in parallel under well-watered conditions. Detached aerial parts of stressed plants (n = 10) were individually weighed to determine sample weight (W) after 15 or 20 days of water withholding. After the initial determination of the sample fresh weight, all the samples were fully dipped in deionized water overnight under normal room light and temperature for rehydration to full turgidity. The samples were then removed from water, residual leaf moisture was gently removed with filter paper, and samples were immediately weighed to obtain a fully turgid weight (TW). Subsequently, the plants were dried in an oven at 65°C for 48 h, and dry weight was measured (DW). RWC was calculated as RWC (%) = [(W − DW)/(TW − DW)] × 100 [28].

2.3. Root and Shoot Growth Assays of 5-D-Old and 10-D-Old Young Seedlings under Well-Watered Conditions. DT2008 and W82 seeds were separately germinated in 6-litre pots (10 seeds/pot) containing vermiculite and grown for 5 or 10 days under well-watered conditions in greenhouse as described in Section 2.1. The 5- and 10-day-old seedlings were carefully removed from soil, and roots were gently washed to remove soil. The fresh weight (FW) of shoot part of each seedling was separately measured, and the length of the shoot and primary root of each plant was also determined. The number of the secondary roots of the 5-day-old plants was also counted. Subsequently, all the shoot and root samples were individually dried in an oven at 65°C for 48 h, and the dry weight (DW) of each root or shoot sample was measured (n = 20).

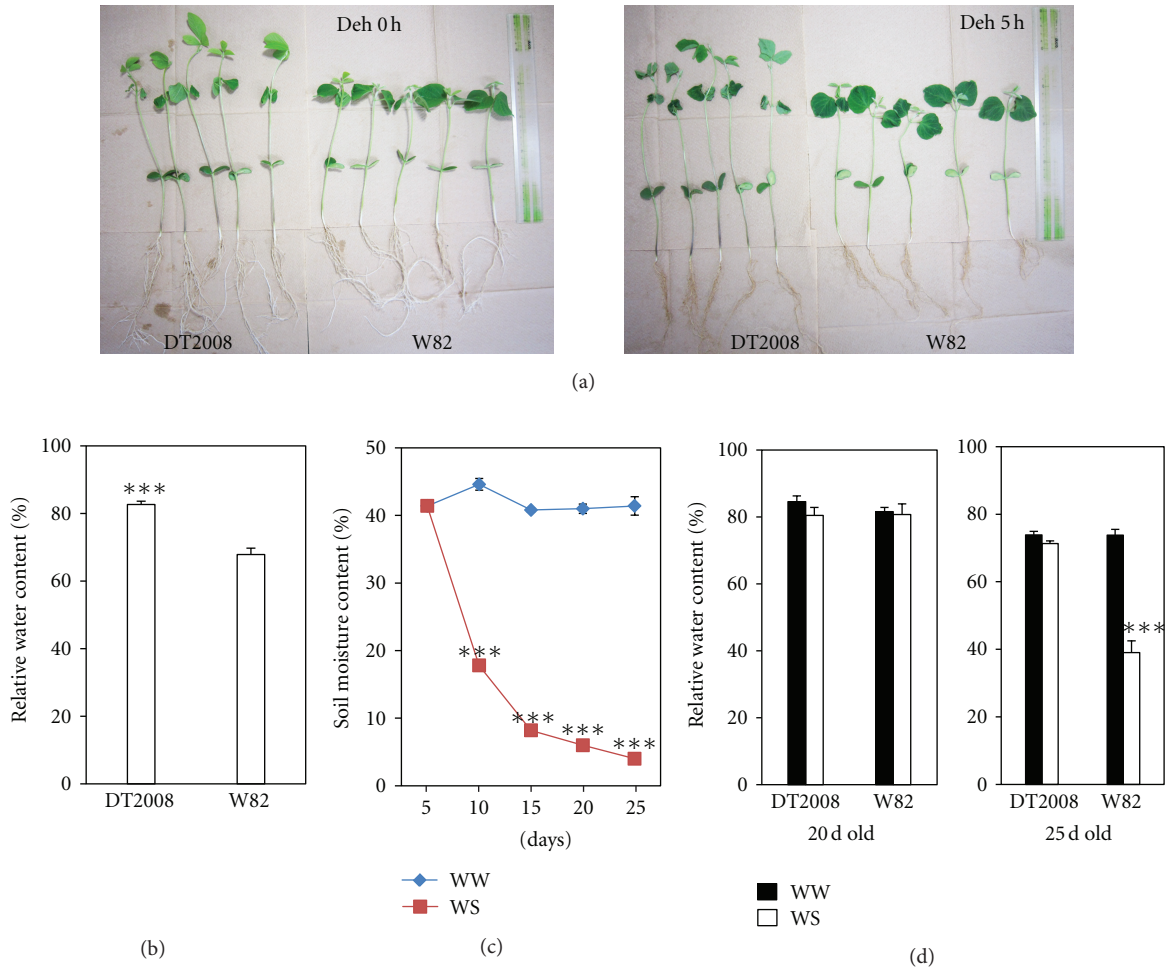

Figure 1: Relative water content (RWC) of DT2008 and W82 plants. (a) Ten-day-old plants were grown and exposed to dehydration stress as described in Section 2. (b) After 5 h of dehydration treatment, RWC was measured. Error bars represent standard errors ($n = 20$ plants/genotype). (c) Volumetric soil moisture contents were monitored during the drought stress treatment ($n = 5$). (d) Five-day-old plants were grown in pots and exposed to drought stress. After 15 or 20 days of water withholding, RWC was measured. Error bars represent standard errors ($n = 10$ plants/genotype). WW, well-watered control; WS, water stress. Asterisks indicate significant differences as determined by a Student's t-test ($^{***}P < 0.001$).

2.4. Root and Shoot Growth Assays of 20-D-Old and 25-D-Old Soybean Plants under Normal and Drought Conditions. DT2008 and W82 plants were grown, and drought stress treatment was performed as described in Section 2.2. The number of the trifoliate leaves of well-watered and stressed plants was counted at 5-day intervals during growth. For further comparison of root and shoot growths under drought stress, plants were carefully removed from soil, and roots were gently washed to remove soil after the drought treatment. The FW of shoot part of the well-watered and stressed plants was measured, and the length of the shoot of each plant was also determined ($n = 10$). Subsequently, the shoot and root samples were individually dried in an oven at 65°C for 48 h, and dry weight of each root or shoot sample was measured.

2.5. Statistical Analysis of the Data. Average values were used to plot figures, and error bars on each figure represent the standard errors. When appropriate, a Student's t-test (one-tailed, unpaired, and equal variance) was used to determine the statistical significance [20].

3. Results and Discussion

3.1. DT2008 Variety Has Higher RWC than the Model W82 Variety under Dehydration and Drought Stress Conditions. DT2008 soybean variety was produced in Vietnam by multiple hybridizations of local varieties and subsequent irradiation with Co^{60}. The significantly improved drought tolerance and productivity as well as yield stability of DT2008 have been examined in various regions in Vietnam in comparison with other local elite soybean cultivars which were used in production [6]. DT2008, therefore, is an important biological resource which can be used for the identification of mutations involved in the regulation of drought tolerance. Thus, in this study, we compared the drought-tolerant phenotypes

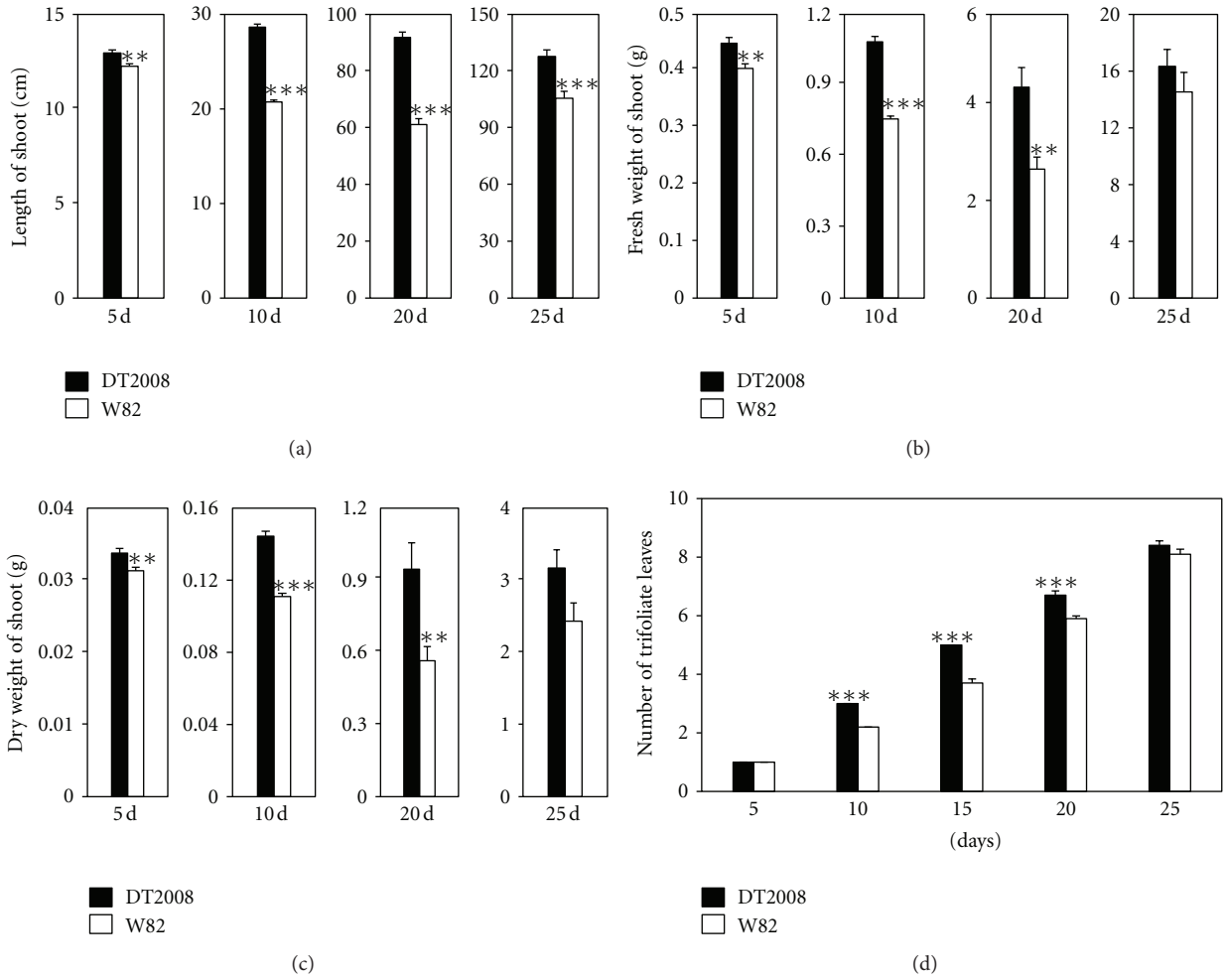

Figure 2: Comparison of shoot growth of DT2008 and W82 plants under well-watered conditions. (a) Comparison of shoot lengths of DT2008 and W82 plants after growth in pots for 5, 10, 20, and 25 days. (b) Comparison of fresh weights of 5-, 10-, 20-, and 25-day-old DT2008 and W82 plants. (c) Comparison of dry weights of 5-, 10-, 20- and 25-day-old DT2008 and W82 plants. For 5- and 10-day-old plants, $n = 20$ plants/genotype. For 20- and 25-day-old plants, $n = 10$ plants/genotype. (d) Comparison of numbers of trifoliate leaves of DT2008 and W82 plants at indicated time points ($n = 10$). Error bars represent standard errors. Asterisks indicate significant differences as determined by a Student's t-test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

of DT2008 and W82 to examine whether these two cultivars possess differential drought-tolerant phenotype, thereby providing research materials for identification of potential drought-related mutations in DT2008. These mutations may cause differential gene and protein expression or altered metabolomic pathways that can be identified using advanced omic approaches, such as transcriptomics, proteomics, or metabolomics. W82 was chosen because it is a soybean model plant whose genome sequence has been recently completed [24]. Thus, a large amount of genetic data has been available for this species, providing a basic foundation for comparative genomics of DT2008 and W82.

The drought-tolerant phenotypes of DT2008 and W82 were compared by means of comparing their water loss under dehydration or drought stress conditions. When 10-day-old plants were dehydrated as shown in Figure 1(a), the DT2008 seedlings could maintain higher RWC under dehydration in comparison with W82 plants (Figure 1(b)). When both DT2008 and W82 plants were subjected to a soil drying experiment (SMC was reduced to below 10%, Figure 1(c)), DT2008 plants had significantly lower water loss rate than W82 plants as well, specifically after 20 days of water withholding (Figure 1(d)). These results suggest that DT2008 has capacity to be tolerant better to drought stress than W82.

3.2. Differential Shoot Growth of DT2008 and W82 under Normal and Drought Conditions. The drought-tolerant phenotypes of DT2008 and W82 were also compared by evaluating their shoot growth under normal and drought stress conditions. For well-watered conditions, the length, FW and DW of the shoots of 5-, 10-, 20- and 25-day-old seedlings grown in soil pots were assessed under well-watered conditions. For drought stress treatment, seedlings were grown in pots for 5 day under well-watered conditions, subsequently subjected to 15 or 20 days of water-withholding, then the same

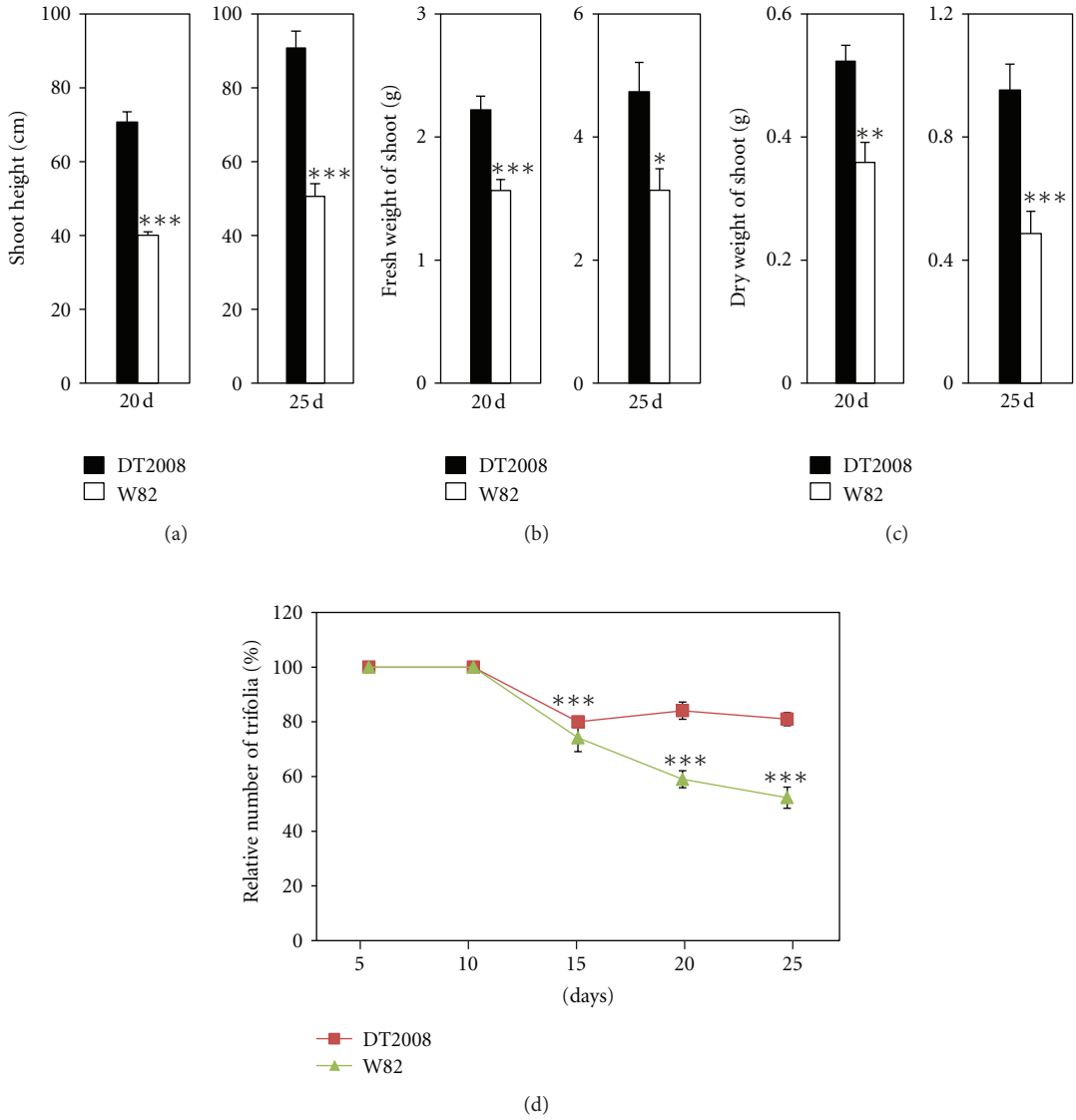

FIGURE 3: Comparison of shoot growth of DT2008 and W82 plants under drought stress. (a) Five-day-old DT2008 and W82 plants grown in pots were exposed to 15 or 20 days of water withholding, and their shoot lengths were compared. (b) Five-day-old DT2008 and W82 plants grown in pots were exposed to 15 or 20 days of water withholding, and their shoot fresh weights were compared. (c) Five-day-old DT2008 and W82 plants grown in pots were exposed to 15 or 20 days of water withholding, and their shoot dry weights were compared. (d) Numbers of the trifoliate leaves of well-watered and stressed DT2008 and W82 plants were counted at 5-day intervals during growth to determine relative number of trifolia at indicated time points for comparison. Error bars represent standard errors (n = 10 plants/genotype). Asterisks indicate significant differences as determined by a Student's t-test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

growth parameters were measured. For both well-watered and drought stress conditions, the numbers of the trifoliate leaves of DT2008 and W82 plants were also counted at 5-day intervals for comparison.

Under normal growth conditions we observed that the DT2008 plants exhibited higher shoot growth and more trifoliate leaves than W82 (Figure 2), suggesting that DT2008 possesses better shoot growth rate than W82. Thus, DT2008 is a potential variety for comparative genomics to identify genes or SNPs (single nucleotide polymorphisms) involved in regulation of shoot biomass. The increase in biomass production can be exploited as a mechanism to enhance plant productivity because increased yields have been shown to be associated with improved biomass production [29, 30].

As for comparison of shoot growth of DT2008 and W82 under drought stress conditions, we found that drought stress more drastically inhibited the growth of W82 than that of DT2008 as shown by higher decreases in the length, FW and DW of shoot as well as the numbers of trifoliate leaves observed for W82 when compared with DT2008 (Figure 3). These data further support that DT2008 is more strongly tolerant to drought stress than W82. Drought stress inhibits

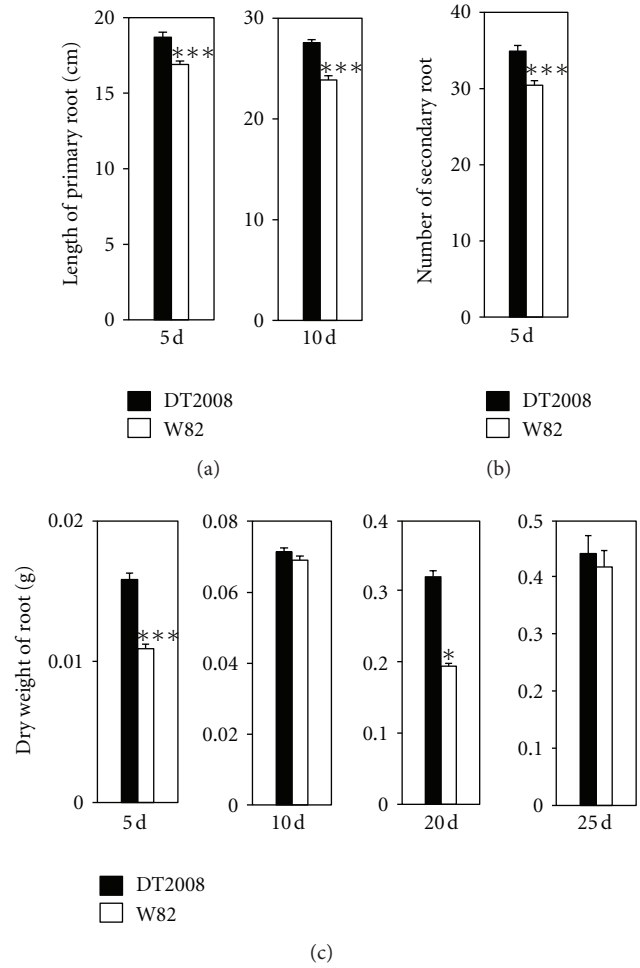

Figure 4: Comparison of root growth and development of DT2008 and W82 plants under well-watered conditions. (a) Comparison of primary root lengths of 5- and 10-day-old DT2008 and W82 plants. (b) Comparison of numbers of secondary root of 5-day-old DT2008 and W82 plants. (c) Comparison of dry weights of root biomasses of 5-, 10-, 20-, and 25-day-old DT2008 and W82 plants. Error bars represent standard errors. For 5- and 10-d-old plants, $n = 20$ plants/genotype. For 20- and 25-d-old plants, $n = 10$ plants/genotype. Asterisks indicate significant differences as determined by a Student's t-test ($^{*}P < 0.05$; $^{***}P < 0.001$).

shoot growth perhaps by limiting photosynthesis as reported previously [27]. Our result suggests that comparative and functional genomics of DT2008 and W82 will enable us to identify mutations responsible for improved biomass, thereby yield, under adverse conditions.

3.3. Differential Root Growth of DT2008 and W82 under Normal and Drought Conditions. Strong lines of evidence suggest that the degree of drought tolerance is positively correlated with root traits [1, 13]. Because the distribution of water within the rhizosphere is critical to maintaining function in different environmental conditions, an enhanced root development is an essential trait for drought tolerance. A long taproot will enable plants to reach lower soil layers where deep water is available, thereby helping plants adapt better to drought stress. An extensive secondary root system will allow plants to forage subsoil surface moisture [1]. Therefore, the root trait is a promising target for breeding or genetic engineering to develop improved drought-tolerant crops, including soybean [1, 28].

To examine the correlation between the root development and enhanced drought-tolerant phenotype of DT2008 in comparison with W82, we first compared the root growth rate of DT2008 and W82 under well-watered conditions. For comparison under well-watered conditions, three root growth-related parameters, namely, the length of primary root, the number of secondary roots, and the DW of the whole root systems were evaluated. DT2008 plants were found to display better root development under normal conditions when compared with W82. The 5- and 10-day-old DT2008 plants have longer primary root and higher number of secondary roots than W82 (Figures 4(a) and 4(b)). Additionally, all the 5-, 10-, 20-, and 25-d-old DT2008 soybean plants possessed larger root total biomass than W82 plants as evidenced by their DW that was measured (Figure 4(c)).

For the evaluation of root growth under drought stress conditions, the DWs of the total root biomasses of drought-

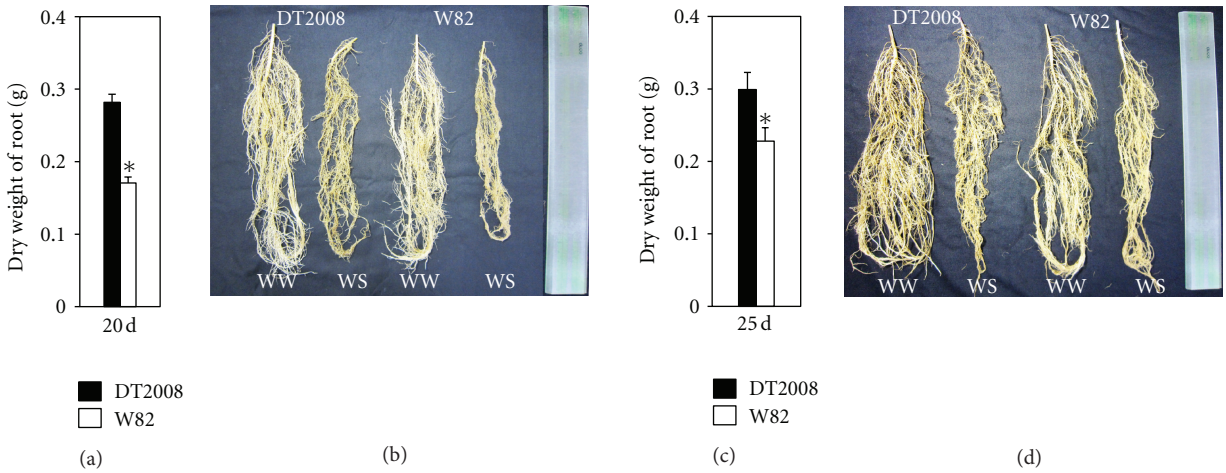

Figure 5: Comparison of root biomasses of DT2008 and W82 plants under drought stress. Five-day-old DT2008 and W82 plants grown in pots were exposed to 15 or 20 days of water withholding. (a) Comparison of dry weights of root biomasses of DT2008 and W82 plants after 15 days of drought stress. (b) Representative root samples of DT2008 and W82 plants grown under well-watered control or drought stress (15 days of stress) conditions. (c) Comparison of dry weights of root biomasses of DT2008 and W82 plants after 20 days of drought stress. (d) Representative root samples of DT2008 and W82 plants grown under well-watered control or drought stress (20 days of stress) conditions. Error bars represent standard errors ($n = 10$ plants/genotype). Asterisks indicate significant differences as determined by a Student's t test ($^*P < 0.05$).

stressed DT2008 and W82 plants were compared. DT2008 exhibited more sustainable root growth and development at reduced soil moisture conditions than W82 as much more notable reduction in the DW of roots was observed with W82 during drought stress treatment than with DT2008 (Figure 5). Collectively, our results suggest that the enhanced root systems of DT2008 may significantly contribute to its improved drought tolerance in relative to W82.

4. Conclusions

In this work, we have determined the differential drought tolerance phenotypes of DT2008 and W82 by comparing their capacity to maintain RWC as well as their shoot and root growths under normal, dehydration, and drought stress conditions. Our results indicated that DT2008 has stronger drought tolerance phenotype in comparison with W82. These two varieties can be used as genetic resources with contrasting drought-responsive phenotypes for the identification of mutations or mutated gene(s), which caused enhanced drought tolerance, using various omic approaches, such as transcriptomics, proteomics, and metabolomics, contributing to better understanding of drought response at molecular levels in soybean as well as providing candidate gene(s) for genetic engineering to improve drought tolerance of elite soybean cultivars.

In addition, it will also be interesting to examine whether enhanced development of the root system of DT2008 contributes to the improved drought tolerance of DT2008, when compared with other previously used elite cultivars [6], in the field conditions as a major trait. Extensive work is currently undergoing to elucidate this relationship.

Acknowledgment

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Research Article

Transcriptional Profiling of Canker-Resistant Transgenic Sweet Orange (*Citrus sinensis* Osbeck) Constitutively Overexpressing a Spermidine Synthase Gene

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Citrus canker disease caused by *Xanthomonas citri* subsp. *citri* (Xcc) is one of the most devastating diseases affecting the citrus industry worldwide. In our previous study, the canker-resistant transgenic sweet orange (*Citrus sinensis* Osbeck) plants were produced *via* constitutively overexpressing a spermidine synthase. To unravel the molecular mechanisms underlying Xcc resistance of the transgenic plants, in the present study global transcriptional profiling was compared between untransformed line (WT) and the transgenic line (TG9) by hybridizing with Affymetrix Citrus GeneChip. In total, 666 differentially expressed genes (DEGs) were identified, 448 upregulated, and 218 downregulated. The DEGs were classified into 33 categories after Gene ontology (GO) annotation, in which 68 genes are in response to stimulus and involved in immune system process, 12 genes are related to cell wall, and 13 genes belong to transcription factors. These genes and those related to starch and sucrose metabolism, glutathione metabolism, biosynthesis of phenylpropanoids, and plant hormones were hypothesized to play major roles in the canker resistance of TG9. Semiquantitative RT-PCR analysis showed that the transcript levels of several candidate genes in TG9 were significantly higher than in WT both before and after Xcc inoculation, indicating their potential association with canker disease.

1. Introduction

Citrus canker disease caused by a biotrophic bacterium *Xanthomonas citri* subsp. *citri* (Xcc) is one of the most devastating diseases in many citrus-producing regions. This disease results in defoliation, dieback, and premature fruit drop, leading to enormous loss of yield and fruit quality [1, 2]. Once the canker-free citrus producing areas are invaded by Xcc, all the suspected and infected trees should be uprooted and burned *via* an eradication programme, as has been done in Florida [3]. Because of the serious destruction and recalcitrance to management, citrus canker has been regarded as a quarantine disease in many countries. Every year, millions of dollars are spent on prevention, quarantines, eradication programs, and disease control in the world [3].

Current strategies for counteracting with canker disease are primarily directed to integrated approaches such as eradication programme and use of antibiotics or bactericides [2]. However, due to the disadvantages in labor investment, safety, consistency, and stabilization these strategies are not the ultimate solutions. Moreover, their applications are often compromised by inducing adverse environmental influence and change of pathogen strains. Therefore, the most effective and economical approach for controlling canker disease relies on the production of resistant cultivars. Genetic manipulation *via* transforming stress-related genes is a widely employed way to create disease-resistant germplasms that are otherwise impossible for classic breeding programme, especially in citrus. At present, antibacterial peptides, *R*-genes, pathogenic factors, and defense-related genes have

been applied to create canker-resistant germplasms in citrus [4, 5]. In our previous study, we produced transgenic sweet orange plants with less susceptibility to citrus canker *via* constitutively overexpressing a spermidine synthase (SPDS, EC 2.5.1.16) [6].

SPDS is a key enzyme involving in polyamine biosynthetic pathway, which converts putrescine (Put) to spermidine (Spd). Polyamines are low-molecular-weight aliphatic compounds that exist ubiquitously in all living organisms, mainly including diamine Put, triamine Spd, and tetraamine spermine (Spm). It has been well documented that polyamines are closely involved in a variety of physiological processes, including biotic stress responses. For example, the content of free and conjugated polyamines and the activities of polyamine biosynthetic and oxidative enzymes increased during the hypersensitive response (HR) of barley after the powdery mildew fungus attack [7], as well as during the formation of maize tumors induced by the the biotrophic pathogenic fungus *Ustilago maydis* [8]. The transcript levels of polyamine biosynthesis-related genes were also found to be accumulated in TMV-infected tobacco [9] and in *U. maydis*-infected maize [8]. In tobacco, polyamine oxidase (PAO) protein and the specific PAO enzymatic activities increased after infection with compatible plant-pathogenic bacterium *Pseudomonas syringae* pv *tabaci* [10]. The studies indicated that polyamines or PAO protein accumulation may be a common event for plant response to pathogens. Moreover, augmentation of endogenous polyamine level by exogenous application of polyamine enhanced host resistance to virus or to bacterial challenge [11–13]. The previous work provided numerous evidence showing that polyamines play important roles in plant pathogen responses.

The mechanisms underlying the role of polyamines in plant defense have been described in previous studies. In summary, two main mechanisms have been proposed. The first one relates to production of hydrogen peroxide (H_2O_2) due to PAO-mediated polyamine catabolism, triggering HR and induced tolerance to specific pathogens [6, 8–10, 12]. The second one points to the role of polyamines (especially for Spm) as signaling molecules to activate expression of pathogenesis-related proteins [14] and a subset of HR-specific genes [15, 16]. Mitsuya et al. [14] and Sagor et al. [17] found that a number of genes in *Arabidopsis* showed response to exogenous application of Spm and *Cucumber mosaic virus* (CMV) infection based on the super serial analysis of gene expression (SuperSAGE), implying that Spm-mediated signaling pathway might play a role in CMV response. Very recently, Gonzalez et al. [18] reported that a large number of differentially expressed genes were identified in Spm-overproducing transgenic *Arabidopsis* and Spm-decreased mutant by using microarray analysis. It should be pointed out that although many efforts have been invested, the underlying physiological and molecular mechanisms remain still elusive. The gene regulation network in polyamine involved plant pathogen response is largely unclear, particularly in perennial plants like citrus. In our previous study, ectopic expression of *SPDS* gene increases both Spd and Spm levels in the transgenic sweet orange and confers canker resistance. As Spm functions as a signaling molecule, it is hypothesized that the transgenic line might display an extensive transcriptional reprogramming. To address this issue and to gain new insights into the molecular mechanisms on the enhanced disease tolerance, genome-wide transcriptome analysis was conducted using the Affymetrix Citrus GeneChip microarray technology. The Affymetrix Citrus GeneChip contains 30,171 probe sets representing up to 33,879 citrus transcripts selected from citrus HarvEST EST and cDNA clustering database. The transcriptional profiling described here may contribute to explain molecular mechanism of polyamine in regulating plant pathogen response.

2. Methods and Materials

2.1. Microarray Hybridization and Data Analysis. The leaves were collected from untransformed line (WT) and the transgenic line (TG9) plant for hybridization with the Affymetrix Citrus GeneChip (Affymetrix, Santa Clara, CA, USA). In brief, 2 g leaves were sampled from uniform new flushes (about 20 days after sprout) of WT and TG9, and then immediately immersed in liquid nitrogen and stored at −80°C. All the other processes including the total RNA extraction (20 μg at least), cDNA and cRNA synthesis, cRNA fragmentation, hybridization, washing and staining, and scanning were performed by Gene Technology Company Limited of Shanghai in China. The detailed experimental procedures can be found in the GeneChip Expression Analysis Technical Manual (http://www.affymetrix.com/support/downloads/manuals/expression_analysis_technical_manual.pdf). To satisfy biological reproducibility requirements, the experiment was carried out using three independent biological replicates for both WT and TG9 (means both WT and TG9 were hybridized with microarray for three times).

The probe array was scanned with the Affymetrix GeneChip Scanner 3000, and the images were analyzed with the Affymetrix GeneChip Operating software (GCOS 1.4) to generate raw data, saved as CEL files. The CEL files were then imported into commercial Partek Genomic Suite 6.4 software (Partek Inc., St. Louis, MO) according to the way of RMA quantile normalization to obtain RMA data containing the expression values. Analysis of variance (ANOVA) was used to compare the statistical expression difference between TG9 and WT. Probe sets with a P value ≤ 0.05 and 2-fold change were considered as differentially expressed genes (DEGs) between the two groups at a statistically significant level.

2.2. Microarray Annotation and Functional Analysis. To assign putative functions of DEGs, Gene ontology (GO) term, Enzyme Commission (EC), and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation were performed using the Blast2GO [19] software. Blast2GO assigns GO annotation through three steps, blasting, mapping, and annotation. GO terms for each of the three main categories (biological process, molecular function, and cellular component) were obtained by using the combined graphs function of the software with default parameters. The KEGG analysis were performed by using the KEGG annotating function of

Blast2GO software, and the annotated KEGG pathways were further manually classified according to the published KEGG pathway lists (http://www.genome.jp/kegg/pathway.html).

2.3. Semiquantitative RT-PCR Analysis. Semiquantitative RT-PCR was employed to validate the microarray results using the same set of RNA samples for the hybridization experiments. Each RNA sample was pretreated with PCR amplification-grade RNase-free DNase I (Takara, Dalian, China) at 37°C to exclude DNA contamination. cDNA synthesis was done by the ReverTra Ace-α-kit (Toyobo, Japan) following the manufacturer's instructions. Specific primers of candidate genes were designed by Primer Premier 5.0 software (PRIMER Biosoft International, Palo Alto, CA) based on the citrus consensus sequences downloaded from Affymetrix website (Table 1). Each PCR reaction was composed of 200 ng cDNA, 2.0 μL 10× reaction buffer, 1.0 mM $MgCl_2$, 0.2 mM dNTP, 1.0 U of DNA polymerase (*Taq*, Fermentas) and 0.4 μM of each primer in a total volume of 20 μL. PCR amplifications were performed at 94°C for 5 min, followed by 28–32 cycles of 94°C for 40 s, 52°C for 40 s, 72°C for 40 s and 5 min extension at 72°C. An *Actin* gene (Table 1, [20]) was used as an internal positive control. Band intensity was quantified by Quantity One analysis software (Bio-Rad Laboratories), and the fold change was calculated by the signal intensity of TG9-specific product divided by the signal intensity of WT-specific product.

In another experiment, semiquantitative RT-PCR was performed to evaluate the expression patterns of several genes before or after *Xanthomonas axonopodis* pv. *citri* (Xcc) inoculation. For this purpose, the leaves sampled from uniform new flushes (about 20 days after sprout) of WT and TG9 were divided into two groups, respectively. One group of leaves without Xcc inoculation (uninoculated leaves) were immediately immersed in liquid nitrogen and stored at −80°C. And another group of leaves were subjected to a pinprick inoculation with Xcc bacterial suspension as described by Fu et al. [6]. Twenty-four hours after inoculation (hpi), the whole leaves of WT and TG9 were collected and stored at −80°C. The total RNA was isolated from uninoculated (0 hpi) and inoculated (24 hpi) leaves according to Liu et al. [21]. The other processes including RNA pretreatment, cDNA synthesis, PCR amplification, and quantification of band intensity were the same as mentioned above.

3. Results

3.1. Screening of the Differentially Expressed Genes and Verifying the Microarray Data. In our previous study, we produced a *SPDS*-overexpressed transgenic sweet orange line with higher levels of Spd and Spm and better resistance to canker disease [6]. To reveal the molecular mechanisms underlying canker resistance in TG9, the global transcriptional profiling of TG9 and WT were compared by citrus genome Genechip analysis. After statistical analysis, 666 genes with signal ratio fold change larger than 2 or smaller than 0.5 (P value ≤ 0.05) between the TG9 and WT were identified as differentially expressed genes (DEGs). Among these genes, 448 and 218 were upregulated and downregulated, respectively (see Supplemental Tables S1 and S2 in Supplementary Material available online at doi:http://dx.doi.org/10.1155/2013/918136).

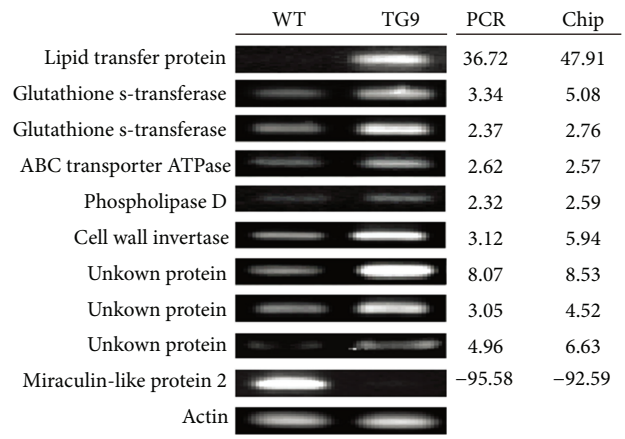

Figure 1: Validation of microarray results by semiquantitative RT-PCR. Ten probes putatively encoding lipid transfer protein (Cit.60.1.S1_at), glutathione s-transferase (Cit.6308.1.S1_at, Cit.9510.1.S1_s_at), ABC transporter ATPase (Cit.3246.1.S1_at), phospholipase d (Cit.6097.1.S1_s_at), cell wall invertase (Cit.5734.1.S1_at), miraculin-like protein 2 (Cit.57.1.S1_at) and unknown protein (Cit.19313.1.S1_at, Cit.5367.1.S1_at, Cit.1406.1.S1_s_at) were amplified with specific primers in WT and TG9 leaves, using *Actin* gene as an internal control for examining equal cDNA loading. The expression ratios between TG9 and WT were calculated by quantifying the band density using the Quantity One software.

In order to verify the reliability of the microarray data, 9 upregulated and 1 downregulated genes were randomly selected to analyze their expression levels in TG9 and WT *via* semiquantitative RT-PCR using gene-specific primers (Table 1). These genes putatively encode lipid transfer protein, glutathione s-transferase, ABC transporter ATPase, phospholipase d, cell wall invertase, miraculin-like protein 2, or unknown protein. As shown in Figure 1, transcript levels of the upregulated and the downregulated genes in TG9 were significantly higher or lower than in WT. The fold changes of these genes based on the calculation from semiquantitative RT-PCR results were largely consistent with the microarray data, suggesting that the microarray data are reliable.

3.2. Functional Annotation and Classification of the Differentially Expressed Genes. To further analyze the microarray data, the identified DEGs, including significantly upregulated and downregulated genes, were functionally annotated and classified using Blast2GO software. The annotated information of each gene such as sequence description, accession number of blasted gene, GO term annotation was listed in Supplemental Tables S1 and S2. The genes putatively encoding cysteine proteinase inhibitor (Cit.8163.1.S1_x_at, Cit.30421.1.S1_s_at, Cit.28011.1.S1_x_at) had the highest fold change (as high as 268.12), and the others such as lipid transfer protein (Cit.60.1.S1_at), lipid binding protein (Cit.19161.1.S1_at) and cytochrome p450 (Cit.26116.1.S1_at,

Table 1: Primers pairs used for Genechip verification and expression analysis of candidate genes.

Probe ID	Primers (5′–3′)	Fragment size
Cit.60.1.S1_at	F: TGGCGTATTGGGTGGGGCTG R: AGATACCCCCGCCCGTGCAA	258 bp
Cit.6308.1.S1_at	F: AGCCAGGGCTCGCTTTTGGG R: TCTTGCATCCAAGCTGACACCAGT	294 bp
Cit.19313.1.S1_at	F: GTGAGGTATTTCGGCGAGGGG R: GGCTTGCGATAACAGAGTGC	348 bp
Cit.5367.1.S1_at	F: GACAGCCACATTCCAAGCAG R: TGAGGCAAGTAGCGACAACG	430 bp
Cit.1406.1.S1_s_at	F: TGTTAGGTCTTTTGGTGTCTATTGTT R: CAGCCTCAGTTTGGGCATTG	369 bp
Cit.9510.1.S1_s_at	F: GCTTATGCTTCTCCCAAACGA R: ACCAGCCAAATACTTGCTCTTC	450 bp
Cit.3246.1.S1_at	F: GTGAAAGGAAACGCAACGAA R: TCCCAGGTCCAGTTACCAATG	328 bp
Cit.6097.1.S1_s_at	F: AGCCTATGTCAAAGCAATCCG R: GCTGCTGTCTACCCCGTCTAA	391 bp
Cit.5734.1.S1_at	F: GCTGCTCGCTTTGGCTTCA R: TTTCTTCATACTCCAGGCACTCA	367 bp
Cit.57.1.S1_at	F: GCTGGCGGCGGTGGAGTTAG R: CGAAAAGCGGCCAACGCTGC	408 bp
Cit.8163.1.S1_x_at	F: AGCGTGGAGAAGGCCTGGAC R: CCGCGAACTGCCCGATCTCC	217 bp
Cit.11548.1.S1_at	F: GCCTTACCTTCTCCTTCCTCAT R: AGTCGGTGGGCAAGTCTCA	590 bp
Cit.4425.1.S1_at	F: ACTCCAACACCTTTATTCCTTCAC R: CATCTCCGCTATTGCCCACT	337 bp
Cit.28117.1.S1_s_at	F: TAGACCGACTGACTGCACCAA R: TGCGAAATACAAAATGAACCC	239 bp
Cit.13055.1.S1_at	F: TGACTCCGCCGTTGTGAAGA R: CACCCGCCGACAACATACA	253 bp
Cit.20495.1.S1_at	F: TCACGGACAACGAAGACAAAG R: TCAACCAAAGCCGAGCAA	179 bp
Cit.5856.1.S1_at	F: GAAGCAACAGTTCCAGCAGC R: CACGAAGCCATCCAGTCAATA	264 bp
Cit.17124.1.S1_at	F: CAGAAGGCAGCCACGATGA R: GATGAGGATGACGAAGAAGAAGC	299 bp
Cit.2333.1.S1_at	F: GTGGAAGGGGTAACTGGGATT R: GCAGAAGTTATTGAAAATGGGTG	461 bp
Cit.6121.1.S1_at	F: AGATGAGTCACAAAGACCAGGAGG R: CACAGGCGTCAACCAATCAAG	205 bp
Cit.31932.1.S1_at	F: TGTAGTCGGTGGTGGCTGTAG R: TGAAAAGTGGGGTGGCATT	436 bp
Actin	F: CATCCCTCAGCACCTTCC R: CCAACCTTAGCACTTCTCC	190 bp

Cit.4425.1.S1_at) were also upregulated to a high level (Table S1). For the downregulated genes (Table S2), the gene with the maximum fold change was miraculin-like protein. The other downregulated genes with high fold change include DNA binding protein (Cit.8142.1.S1_at, Cit.30420.1.S1_x_at), early light-inducible protein (Cit.165.1.S1_s_at), and AP2/ERF domain-containing transcription factor (Cit.11068.1.S1_at, Cit.30607.1.S1_s_at).

The functional categorization was performed according to biological process, molecular function, and cellular component using Blast2GO software. As shown in Figure 2, the biological processes of these DEGs included mainly 15 categories such as cellular process, metabolic process, response to stimulus, localization and biological regulation, and among which the genes in response to stimulus and immune system process are of interest because they may participate in canker disease resistance directly. In addition, it is intriguing to find that most of these categories contained larger number of the upregulated genes than the downregulated genes, such as cellular process, metabolic process, response to stimulus, and so forth. In the immune system process, only upregulated genes were assembled to this group. Molecular functions were primarily related to binding activity, catalytic activity, transporter activity, electron carrier activity, transcription regulator activity, and others (Figure 2). Cellular component included cell, organelle, macromolecular complex, extracellular region, membrane-enclosed lumen, and envelope. Similar to the biological process category, in molecular function and cellular component the number of upregulated genes is larger than that of downregulated genes (Figure 2).

3.3. The Expression of Candidate Genes before and after Inoculation. According to the functional annotation and classification of the DEGs, and related studies in previous literatures, 11 genes putatively encoding cysteine proteinase inhibitor, thaumatin-like protein, cytochrome p450, aspartyl protease family protein, pyruvate kinase, pathogenesis-related protein, thioredoxin-like 5, AP2/ERF transcription factor, NADPH oxidase, TIR-NBS-LRR resistance protein, and Rubisco subunit binding protein were hypothesized to be involved in canker resistance of TG9. To answer this question and to identify canker responsive genes, the expression levels of these candidate genes were evaluated before (0 hpi) and 24 h after Xcc inoculation (24 hpi).

Before inoculation higher expression levels of the tested genes were detected in TG9 than in WT except those encode aspartyl protease family protein, NADPH oxidase, and TIS-NBS-LRR resistance protein which showed no difference between TG9 and WT (Figure 3). However, at 24 hpi the expression levels of all genes were higher in TG9 than in WT. Moreover, it is interesting to see that Xcc inoculation upregulated the genes in both TG9 and WT as compared with absence of Xcc inoculation, such as thaumatin-like protein, cytochrome p450, pathogenesis-related protein, thioredoxin-like 5, AP2/ERF transcription factor, NADPH oxidase, TIS-NBS-LRR resistance protein, and Rubisco subunit binding protein (Figure 3). Of note, the gene encoding cysteine proteinase inhibitor expressed at high levels in TG9 with or without Xcc inoculation, but it was not detected in the WT (Figure 3). Our data suggested that the expression of these candidate genes were constitutively upregulated in the transgenic line and can be further induced by the Xcc inoculation, which provides important information and evidence for its potential role in canker disease resistance.

4. Discussion

In our previous study, ectopic expression of a polyamine biosynthetic gene (*MdSPDS1*) in sweet orange confers citrus canker resistance, and transcript levels of several defense-related genes were induced in the transgenic line [6]. Therefore, we speculate that global transcriptional levels of transgenic plants are regulated due to the overexpression of *MdSPDS1*, which may explain at the transcriptional level the enhanced resistance in the transgenic plants. To confirm this hypothesis, global transcriptional profiling of WT and TG9 was compared through hybridizing with Affymetrix Citrus GeneChip in the present study. Genechip, a high-throughput and effective technology for studying global transcriptional profiling, has been widely used for deciphering molecular responses to abiotic and biotic stresses and comparing transcriptome under different treatments [22–24].

In the current study, 666 genes were identified as DEGs, accounting for 1.97% of all transcripts in the citrus genechip. Among these DEGs, the number of upregulated genes was about twice as that of the downregulated ones, in line with the microarray data overexpressing a spermidine synthase gene in *Arabidopsis* [25]. This result indicated that overexpression of a polyamine biosynthetic gene may lead to more prominent induction of the global transcript level. To further understand these DEGs, the functional annotation and classification were conducted using Blast2GO software. Out of the DEGs 60.66% were annotated, 39.34% upregulated, and 21.32% downregulated. Those genes without an annotation, including NoBLAST, NoMapping, and NoAnnotation, may be attributed to scarcity of enough amount of information of selected database or parameter setting. The phenomenon is not distinct as it has been also reported in previous studies [26, 27]. After GO annotation, all these DEGs were classified into 33 categories involved in biological process, molecular function, and cellular component. Moreover, the KEGG pathways of upregulated DEGs were annotated for further understanding participant metabolic and cellular processes. Three categories and annotated KEGG pathways were subjected to more detailed discussion as follow.

4.1. Genes Involved in Stimulus Response and Immune System Process. According to the GO annotation, 68 upregulated genes were shown to be involved in stimulus response and immune system process (Table 2). Based on this functional classification, we speculate that these genes have important relevance to canker disease. For instance, the gene (Cit.8878.1.S1_at) encoding major allergen pru had the highest fold change (8.63). This gene was also annotated as stress-related protein and pathogenesis-related (PR) protein 10, indicating that it may be associated with the pathogen defense. In this category, other genes include thaumatin-like protein, β-1,3-glucanase, AP2/ERF domain-containing transcription factor, ATP-binding cassette (ABC) transporter, copper/zinc superoxide dismutase, disease resistance protein, glutathione-*S*-transferase, and aspartyl protease family protein.

Thaumatin-like protein (TLPs), categorized under the PR5 family, can be induced by various stresses, such as

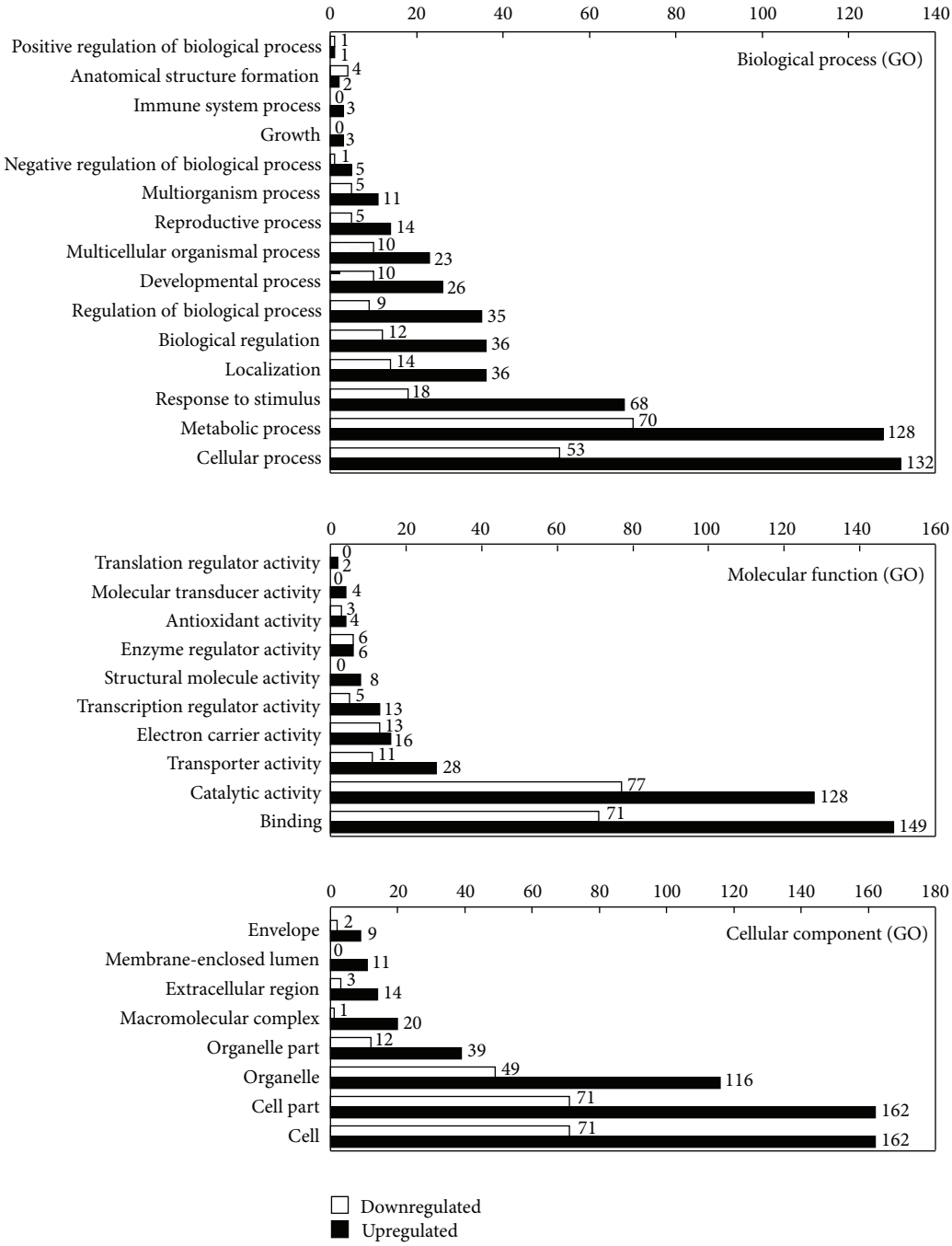

Figure 2: Functional categorization of upregulated and downregulated differentially expressed genes. 448 significantly upregulated and 218 significantly downregulated DEGs were categorized to biological process, molecular function, and cellular component based on GO annotation, and the represented number of each column was marked in the figure.

salinity, wound, and pathogen infection [28]. In addition, *in vitro* bioassays have shown that TLPs possess antifungal activity [29]. In the present study, expression analysis of TLPs before or after Xcc inoculation showed that TG9 had significant higher transcript levels than WT, in particularly after Xcc inoculation (Figure 3), indicating the function of TLPs on canker disease resistance. β-1,3-glucanase, hydrolyzing the 1,3-β-D-glucosidic linkages of β-1,3-glucan, belongs to PR2 family, has been shown to play a crucial role in plant pathogen defense [30–32]. AP2/ERF domain-containing transcription factor is an important plant-specific transcription factor, which has been suggested to play a critical role in stress response. Overexpression of AP2/ERF induced several PR genes expression and enhanced disease

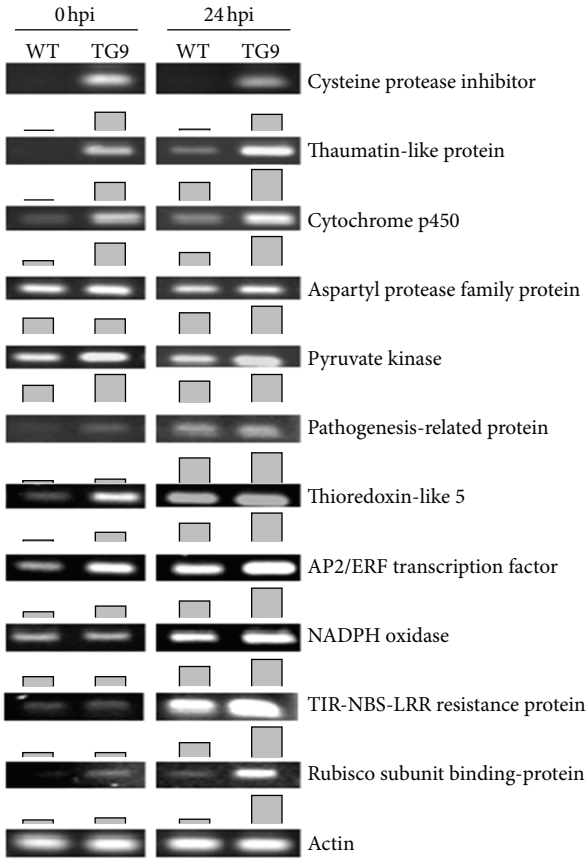

Figure 3: Expression of candidate genes in leaves of WT and TG9 infected or not with *Xanthomonas citri* subsp. *Citri* (Xcc). Eleven candidate genes putatively encoding cysteine proteinase inhibitor (Cit.8163.1.S1_x_at), thaumatin-like protein (Cit.11548.1.S1_at), cytochrome p450 (Cit.4425.1.S1_at), aspartyl protease family protein (Cit.28117.1.S1_s_at), pyruvate kinase (Cit.13055.1.S1_at), pathogenesis-related protein (Cit.20495.1.S1_at), thioredoxin-like 5 (Cit.5856.1.S1_at), AP2/ERF transcription factor (Cit.17124.1.S1_at), NADPH oxidase (Cit.2333.1.S1_at), TIS-NBS-LRR resistance protein (Cit.6121.1.S1_at), and Rubisco subunit binding protein (Cit.31932.1.S1_at) were assessed at 0 and 24 h post inoculation (hpi) in WT and TG9 leaves *via* semiquantitative RT-PCR. Column diagram was the quantification data of corresponding bands using Quantity One software.

resistance in tobacco [33, 34]. In this study, AP2/ERF was induced by Xcc inoculation, and TG9 had a higher transcript level than WT (Figure 3), suggesting that AP2/ERF domain-containing transcription factor may be presumably implicated in citrus canker disease. ABC transporter, a membrane protein, exists in bacteria, fungi, animals, and plants and acts on absorption and secretion of many substrates. Martinoia et al. [35] reported that ABC transporter possibly involved in secretion of antimicrobial compounds and detoxification of some toxic metabolites in plant defence.

4.2. Cell Wall-Related Genes. Plant cell wall is a battlefield of host-pathogen interaction because the invasion of pathogen must first break the physical barrier of plant cell wall, which constitutes the first line of pathogen defence [36–38]. In this study, 12 cell wall-related genes were significantly upregulated in TG9, including cysteine proteinase inhibitor, basic 7s globulin 2 precursor small subunit, glutathione-*S*-transferase, and some hypothetical cell wall proteins (Table 3). Cysteine proteinase inhibitor, a proteinase inhibitor, is categorized under the PR6 family, which plays important roles in plant defence [28, 39]. As shown in Figure 3, the gene encoding cysteine proteinase inhibitor (Cit.8163.1.S1_x) was upregulated in TG9 before and after Xcc inoculation, but it was not detected in WT. Solomon et al. [40] reported that cysteine proteinase inhibitor can regulate the process of programmed cell death (PCD), an important step in HR. Therefore, cysteine proteinase inhibitor can possibly enhance disease resistance *via* regulating HR in plants, in agreement with an apparent HR of TG9 leaves in our previous work [6]. Among the 12 cell wall-related genes, Cit.14918.1.S1_at was annotated as basic 7s globulin 2 precursor small subunit (the similarity was 100%) by blast2go or annotated as xyloglucan-specific endoglucanase inhibitor protein (XEGIP, the similarity was 99%) in NCBI database. XEGIP has been shown to be the newest class of plant-derived proteins that inhibit pathogen-secreted cell wall degrading enzymes [41, 42]. Cernadas et al. [22] also reported that canker disease inoculation induced upregulation of endoglucanase inhibitor protein in sweet orange. Therefore, we can speculate that the gene XEGIP is possibly involved in canker disease resistance *via* suppressing the plant cell wall degradation in the transgenic line. Glutathione-*S*-transferase (GST), a protein with multiple functions, is closely associated with detoxification of some hydrophobic and electrophilic compounds, transport of auxin and phenylpropanoids, and activation of phenylpropanoid metabolism as signalling molecules [43, 44]. GST has been proposed as a marker gene for pathogen reactions, and an increase in transcript level of GST gene has been shown to be relevant to pathogen challenge [28]. In this study, the transgenic line had higher expression levels of GST, suggesting that the former may exhibit a better detoxification and regulation capacity under biotic stress when compared with the wild type, leading to less serious damage.

4.3. Transcription Factors Pertinent to Pathogen Attack. It is well known that transcription factors (TFs) play a crucial role in abiotic and biotic stresses *via* regulating a series of downstream target genes. In this study, 13 TFs were identified in the upregulated genes, including AP2/ERF, MADS, BT4 (BTB and TAZ domain protein 4) protein, NAC, MYB, and several other unnamed TFs (Table 4). In an earlier work, Kasukabe et al. [25] reported that TFs like AP2/ERF, NAC, and MYB were upregulated in the transgenic *Arabidopsis thaliana* plants overexpressing *FSPDS* gene relative to the wild type. Moreover, 47 TFs, such as NAC, MYB, WRKY, and bZIP, were found to be upregulated in SPMS-overexpressing transgenic *Arabidopsis* [18]. Our data and earlier results demonstrate that modification of the polyamine synthesis may cause the transcription reprogramming in the transgenic plants, which may be ascribed to the regulatory role of

Table 2: Significantly upregulated genes in response to stimulus and involved in immune system process.

Gene ID	Seq. description	Hit ACC	Fold change
Cit.8878.1.S1_at	Major allergen Pru	ABK06393	8.63139
Cit.11548.1.S1_at	Thaumatin-like protein	Q9SMH2	5.84254
Cit.4426.1.S1_at	Homogentisate geranyl geranyl transferase	XP_002282953	5.63043
Cit.9703.1.S1_at	Beta-1,3-glucanase	CAA03908	5.45386
Cit.29880.1.S1_at	ATP binding	XP_002517441	5.3541
Cit.4504.1.S1_at	Thiamin biosynthesis protein	XP_002525602	4.83609
Cit.14918.1.S1_at	Basic 7s globulin 2 precursor small	XP_002517165	4.6577
Cit.21616.1.S1_at	abc transporter	CBI40242	4.6402
Cit.9706.1.S1_s_at	Beta-1,3-glucanase	ABQ45848	4.54013
Cit.38637.1.S1_at	Protein	XP_002517054	4.25433
Cit.28117.1.S1_s_at	Aspartyl protease family protein	ABK28718	3.93758
Cit.3949.1.S1_s_at	Copper zinc superoxide dismutase	ACC93637	3.85253
Cit.17438.1.S1_at	Protein	XP_002284819	3.69927
Cit.12005.1.S1_s_at	ATP-binding cassette	CBI40242	3.65824
Cit.6364.1.S1_s_at	Peptidase m	XP_002518664	3.64541
Cit.4504.1.S1_s_at	Thiamin biosynthesis protein	XP_002525602	3.60992
Cit.11209.1.S1_s_at	Nematode-resistance protein	XP_002268520	3.47979
Cit.17124.1.S1_at	AP2/ERF domain-containing transcription factor	NP_182011	3.3661
Cit.35636.1.S1_s_at	Hypothetical protein	XP_002262662	3.30867
Cit.9584.1.S1_x_at	Glutathione s-transferase	XP_002273830	3.00065
Cit.9587.1.S1_at	Glutathione s-transferase	XP_002273830	2.91727
Cit.916.1.S1_at	Protein	XP_002525204	2.89418
Cit.31147.1.S1_at	Disease resistance protein	CAN77656	2.82347
Cit.9704.1.S1_at	Beta-1,3-glucanase	ABQ45848	2.79617
Cit.20853.1.S1_at	Citrate synthase	ACU42176	2.79073
Cit.9510.1.S1_s_at	Glutathione s-transferase	XP_002530205	2.75554
Cit.17124.1.S1_s_at	AP2/ERF domain-containing transcription factor	NP_182011	2.72797
Cit.21717.1.S1_at	Wound-induced protein win2	XP_002319077	2.68663
Cit.28472.1.S1_at	Protein	XP_002320004	2.68478
Cit.17374.1.S1_at	Calcium binding protein	ABK06394	2.6752
Cit.23704.1.S1_at	Aspartyl protease family protein	ABK28718	2.64168
Cit.9587.1.S1_x_at	Glutathione s-transferase	XP_002273830	2.62727
Cit.2809.1.S1_s_at	AP2 domain-containing transcription factor	XP_002281709	2.62632
Cit.6280.1.S1_at	bt4 protein binding transcription regulator	XP_002304319	2.60135
Cit.12004.1.S1_at	ATP-binding cassette	CBI30263	2.57256
Cit.12589.1.S1_at	Syntaxin	XP_002326741	2.52495
Cit.32844.1.S1_s_at	Glutathione s-transferase	XP_002520166	2.52047
Cit.31254.1.S1_at	ATP-binding cassette	CAN77838	2.47584
Cit.12560.1.S1_s_at	erd15 protein	XP_002268033	2.45013
Cit.38633.1.S1_at	Transparent testa 12	XP_002314825	2.43996
Cit.24178.1.S1_at	sec12-like protein 1	CBI40184	2.43778
Cit.3550.1.S1_at	Ankyrin repeat-containing	XP_002526791	2.38623
Cit.28117.1.S1_at	Aspartyl protease family protein	ABK28718	2.32863
Cit.9584.1.S1_s_at	Glutathione s-transferase	XP_002273830	2.32824
Cit.13439.1.S1_at	DNA binding	XP_002512121	2.32431
Cit.31932.1.S1_at	Rubisco subunit binding-protein beta	XP_002514548	2.26026
Cit.4131.1.S1_at	Pyridoxal kinase	CBI33550	2.25534
Cit.7994.1.S1_at	Protein	XP_002511077	2.22751

Table 2: Continued.

Gene ID	Seq. description	Hit ACC	Fold change
Cit.5117.1.S1_at	Universal stress protein	XP_002515296	2.2129
Cit.4146.1.S1_at	Serine palmitoyltransferase	CBI15735	2.21272
Cit.10854.1.S1_s_at	Protein	XP_002514963	2.20543
Cit.21798.1.S1_at	Glucosyl transferase	ACS87992	2.17486
Cit.465.1.S1_s_at	Thiamin biosynthetic enzyme	XP_002305603	2.17393
Cit.1610.1.S1_at	Peptidyl-prolyl cis-trans isomerase-like protein	XP_002271056	2.17052
Cit.11040.1.S1_at	Hydroxyacylglutathione hydrolase	XP_002329233	2.1672
Cit.2333.1.S1_at	NADPH oxidase	XP_002511059	2.15579
Cit.13055.1.S1_at	Pyruvate kinase	NP_001065749	2.14823
Cit.26113.1.S1_at	Phototropic-responsive nph3 family protein	CAN63893	2.11134
Cit.30576.1.S1_at	Guanylyl cyclase	XP_002277052	2.11123
Cit.836.1.S1_s_at	Protein	XP_002520818	2.09287
Cit.14371.1.S1_at	Homogentisic acid geranylgeranyl transferase	BAH10642	2.08956
Cit.23824.1.S1_at	Protein	XP_002263043	2.08815
Cit.32832.1.S1_at	Protein	XP_002269885	2.07239
Cit.5555.1.S1_at	cop9 complex subunit	XP_002302493	2.05901
Cit.9144.1.S1_at	ATP-dependent clp	XP_002511102	2.05338
Cit.1554.1.S1_at	Membrane protein	CBI27668	2.03856
Cit.35569.1.S1_s_at	Syntaxin	XP_002326741	2.02263
Cit.2495.1.S1_at	RNA binding protein rp120	XP_002511064	2.01664

Table 3: Significantly upregulated cell wall-related genes.

Gene ID	Seq. description	Hit ACC	Fold change
Cit.8163.1.S1_x_at	Cysteine proteinase inhibitor	P37842	268.1240
Cit.30421.1.S1_s_at	Cysteine proteinase inhibitor	AAG38521	94.58230
Cit.28011.1.S1_x_at	Cysteine proteinase inhibitor	AAG38521	85.76650
Cit.14918.1.S1_at	Basic 7s globulin 2 precursor small subunit	XP_002517165	4.65770
Cit.35636.1.S1_s_at	Hypothetical protein	XP_002262662	3.30867
Cit.9510.1.S1_s_at	Glutathione s-transferase	XP_002530205	2.75554
Cit.14913.1.S1_at	Class III chitinase	XP_002276365	2.68317
Cit.30421.1.S1_x_at	Cysteine proteinase inhibitor	AAG38521	2.47166
Cit.9064.1.S1_x_at	40s ribosomal protein s9	XP_002511557	2.06885
Cit.9144.1.S1_at	ATP-dependent clp	XP_002511102	2.05338
Cit.2495.1.S1_at	RNA binding protein rp120	XP_002511064	2.01664
Cit.9064.1.S1_s_at	40s ribosomal protein s9	XP_002511557	2.00929

polyamines. TG9 contained higher level of spermine, which has been proposed as a signal molecule in previous studies [11, 14]. The upregulation of an array of the TFs suggests that the transgenic plants possess a robust system of transcriptional modulation towards the disease tolerance by regulating a large spectrum of relevant target genes of different TFs. Implication of the corresponding TFs in biotic stress has been experimentally corroborated in earlier studies [45–50]. For example, Nakashima et al. [48] reported transgenic rice transformed with an NAC gene displayed enhanced resistance to blight disease. In another work, Vailleau et al. [50] showed that overexpression of an *MYB* gene in *Arabidopsis thaliana* and tobacco conferred resistance to both bacteria and fungus.

Apart from the abovementioned TFs, it is interesting to find that the TFs involved in flowering regulation, such as MADS and FLC (flowering locus C) [51], were also upregulated in the TG9. Upregulation of MADS and FLC in TG9 suggests that overexpression of *MdSPDS1* has led to alteration of gene network associated with flowering in the transgenic plant. Although it will need time to compare the flowering dynamics between the transgenic line and WT, the polyamines have been shown to participate in the physiological process of flowering in plants [52, 53].

4.4. Potential KEGG Pathways Involved in Defence. To further understand the metabolic and cellular processes involved in defence, KEGG pathways of the upregulated DEGs were

Table 4: Significantly upregulated transcription factor related genes.

Gene ID	Seq. description	Hit ACC	Fold change
Cit.17124.1.S1_at	AP2/ERF domain-containing transcription factor	NP_182011	3.36610
Cit.15253.1.S1_s_at	MADS-domain transcription factor	XP_002273223	3.21612
Cit.17124.1.S1_s_at	AP2/ERF domain-containing transcription factor	NP_182011	2.72797
Cit.2809.1.S1_s_at	AP2 domain-containing transcription factor	XP_002281709	2.62632
Cit.6280.1.S1_at	BT4 (BTB and TAZ domain protein 4) protein binding transcription regulator	XP_002304319	2.60135
Cit.39092.1.S1_at	NAC domain protein; IPR003441	XP_002300866	2.30719
Cit.29898.1.S1_at	Unnamed protein product	CBI21863	2.20763
Cit.3005.1.S1_at	FLC-like 1 splice variant 4	ACB72865	2.14257
Cit.8950.1.S1_at	AP2/ERF domain-containing transcription factor	ABB89755	2.13449
Cit.30576.1.S1_at	Guanylyl cyclase	XP_002277052	2.11123
Cit.14044.1.S1_at	Transcription factor, putative	XP_002514876	2.06065
Cit.15941.1.S1_at	Chromatin remodeling complex subunit	ABA18099	2.05183
Cit.35206.1.S1_at	MYB family transcription factor	FR828559	2.00112

annotated by Blast2GO software. As shown in Supplemental Table S3, the annotated KEGG pathways included carbohydrate metabolism, energy metabolism, lipid metabolism, nucleotide metabolism, amino acid metabolism, cofactors and vitamins metabolism, biosynthesis of polyketides, terpenoids, alkaloids, hormones and other secondary metabolites, and phosphatidylinositol signaling transduction process.

Starch and sucrose not only serve as typical carbon and energy sources, but also play important roles in plant defense. Sucrose has been recognized as an endogenous signal to induce defense responses against pathogens [54]. Recently, Singh and Shah [55] reported that starch and sucrose contents significantly accumulated in the green peach aphid infested *Arabidopsis* and tomato leaves. Starch accumulation has been suggested to facilitate the host plant to generate a secondary sink that suppresses the insect to manipulate host metabolism [55]. Improved metabolism of starch and sucrose in TG9 possibly provides energy and signaling to the plant against Xcc attack. Over the past three decades, glutathione was gradually known to be involved in plant defense reactions and as a signaling molecule to induce various defense genes, and it has also been reported to crosstalk with a variety of hormone-related defense signaling, such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) [56]. Höller et al. [57] reported that enhanced glutathione metabolism was correlated with sulfur-induced resistance in *tobacco mosaic virus*-infected tobacco plants. Therefore, we could speculate that enhanced glutathione metabolism in TG9 is a potential mechanism for the enhanced Xcc resistance. On the other hand, biosynthesis of other compounds, such as cytochrome P450, phenylpropanoids, and plant hormones has been well documented to be involved in pathogen defense. For example, cytochrome P450 plays critical roles in the biosynthesis of defense-related compounds, hormones, and signaling molecules [58]. And it is interesting to find that a number of cytochrome P450-related genes were induced in Xcc-inoculated Meiwa kumquat (*Fortunella crassifolia*) in our previous study [59]. Phenylpropanoids, such as lignin and lignans, coumarins, and flavonoids, can function as preformed and inducible antimicrobial compounds or as signal molecules in plant-microbe interactions [60]. The phytohormones, including SA, JA, ET, and ABA, have been well known as important signaling molecular to induce plant defense reactions after pathogens attack [61]. Based on these illustrations, it is surmised that the pathways related to starch and sucrose metabolism, glutathione metabolism, metabolism of xenobiotics by cytochrome P450, biosynthesis of phenylpropanoids, and plant hormones may be significantly modulated in TG9, constituting an important defence against the pathogen attack.

5. Conclusion

Global transcriptional profiling was compared between WT and TG9 by hybridizing with Affymetrix Citrus GeneChip in this study. In total, 666 DEGs were identified, including 448 upregulated genes and 218 downregulated genes. After functional annotation and classification, the DEGs implicated in stimulus response and immune system process, cell wall and transcriptional regulation, and cellular and metabolism processes, such as starch and sucrose metabolism, glutathione metabolism, biosynthesis of phenylpropanoids, and plant hormones were hypothesized to play major roles in the canker resistance of TG9. Our data suggest that genetic engineering of a polyamine biosynthetic gene has a profound impact on the transcriptome of the transgenic plants. In the future, extra work is required to verify the function of the DEGs in the canker tolerance of the transgenic line. The present work lays groundwork for deciphering the molecular events of the transgenic line and for tapping desirable genes that hold great potential for genetic engineering aiming at improving biotic stress tolerance.

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Review Article

Roles of Organic Acid Anion Secretion in Aluminium Tolerance of Higher Plants

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Approximately 30% of the world's total land area and over 50% of the world's potential arable lands are acidic. Furthermore, the acidity of the soils is gradually increasing as a result of the environmental problems including some farming practices and acid rain. At mildly acidic or neutral soils, aluminium (Al) occurs primarily as insoluble deposits and is essentially biologically inactive. However, in many acidic soils throughout the tropics and subtropics, Al toxicity is a major factor limiting crop productivity. The Al-induced secretion of organic acid (OA) anions, mainly citrate, oxalate, and malate, from roots is the best documented mechanism of Al tolerance in higher plants. Increasing evidence shows that the Al-induced secretion of OA anions may be related to the following several factors, including (*a*) anion channels or transporters, (*b*) internal concentrations of OA anions in plant tissues, (*d*) temperature, (*e*) root plasma membrane (PM) H^+-ATPase, (*f*) magnesium (Mg), and (*e*) phosphorus (P). Genetically modified plants and cells with higher Al tolerance by overexpressing genes for the secretion and the biosynthesis of OA anions have been obtained. In addition, some aspects needed to be further studied are also discussed.

1. Introduction

Approximately 30% of the world's total land area and over 50% of the world's potential arable lands are acidic [1]. Furthermore, the acidity of the soils is gradually increasing as a result of the environmental problems including some farming practices and acid rain. Soil pH decreased significantly from the 1980s to the 2000s in the major Chinese crop-production areas [2]. Aluminium (Al) is the most abundant metal and the third most abundant element in the earth's crust after oxygen (O) and silicon (Si), comprising approximately 7% of its mass [3]. At mildly acidic or neutral soils, it occurs primarily as insoluble deposits and is essentially biologically inactive. In acidic solutions (pH < 5.0), Al becomes soluble and available to plants in the Al^{3+} and $Al(OH)^{2+}$ forms [4]. Micromolar concentration of Al^{3+} can rapidly inhibit root growth. The subsequent impairments on water and nutrient uptake lead to poor growth and productivity [5]. Therefore, in many acidic soils throughout the tropics and subtropics, Al toxicity is a major factor limiting crop productivity [6]. Many plants have evolved different mechanisms for detoxifying Al externally, including secretion of Al-chelating substances (e.g., organic acid (OA) anions, phosphate (Pi), and phenolic compounds) from the roots, increased pH in the rhizosphere, modified cell wall, redistribution of Al, and efflux of Al [6–9]. Increasing evidence shows that the Al-induced secretion of OA anions from roots is a major mechanism leading to Al tolerance in higher plants [6, 9–15]. In this paper, we review the roles of the Al-induced secretion of OA anions from roots in Al tolerance of higher plants.

2. Aluminium-Induced Secretion of Organic Acid Anions from Roots

The Al-induced secretion of OA anions, mainly citrate, oxalate, and malate, from roots is the best documented mechanism of Al tolerance in higher plants. Plants differ in the species of OA anions secreted, secretion patterns, temperature sensitivity, response to inhibitors, and dose response to Al (see [9, 13], Table 1). Since the first report on the Al-induced secretion of malate from wheat (*Triticum aestivum*) roots [16], increasing evidence shows that many Al-tolerant species or cultivars are able to secrete high levels of citrate, malate, and/or oxalate from roots when exposed to Al, including barley (*Hordeum vulgare*) [17], maize (*Zea mays*) [18], buckwheat (*Fagopyrum esculentum*) [19, 20], rye (*Secale cereale*) [21], soybean (*Glycine max*) [22, 23], *Citrus junos* [24], sorghum (*Sorghum bicolor*) [25], triticale(× *Triticosecale* Wittmark) [26], *Polygonum* spp. [27], *Paraserianthes falcataria* [28], *Lespedeza bicolor* [29], *Citrus grandis*, and *Citrus sinensis* [30, 31]. All these OA anions (citrate, oxalate, and malate) secreted from plant roots can form stable, nontoxic complexes with Al in the rhizosphere, thereby preventing the binding of Al to cellular components, resulting in detoxification of Al [9, 12]. Of the three OA anions, citrate has the highest chelating activity for Al followed by oxalate and malate [9]. The Al-induced secretion of OA anions is localized to the root apex, which is in agreement with the targeting site for Al toxicity [20, 32, 33] and their secretion is highly specific to Al, neither phosphorus (P) deficiency nor other polyvalent cations result in the secretion of OA anions [20, 30, 34–38]. Based on the timing of secretion, two patterns of Al-induced OA anion secretion have been proposed [10, 11]. In Pattern I plants, no discernible delay is observed between the addition of Al and the onset of OA anion secretion such as buckwheat [39], tobacco (*Nicotiana tabacum*) [40], and wheat [37]. In this case, Al may simply activate a transporter in the plasma membrane (PM) to initiate OA anion secretion, and the induction of genes is not required [9, 11]. In Pattern II plants, OA anion secretion is delayed for several hours after exposure to Al such as in rye [22], *Cassia tora* [39], *C. junos* [24], soybean [41], *L. bicolor* [29], and triticale [26]. In this case, Al may induce the expression of genes and the synthesis of proteins involved in OA metabolism or in the transport of OA anions [12]. Yang et al. investigated the effects of a protein-synthesis inhibitor (cycloheximide, CHM) on the Al-induced secretion of OA anions from the roots of buckwheat, a typical Pattern I plant, and *C. tora*, a typical Pattern II plants, suggesting that both *de novo* synthesis and activation of an anion channel are needed for the Al-activated secretion of citrate in *C. tora*, but in buckwheat the PM protein responsible for oxalate secretion preexisted [39]. Although the Al-induced secretion of OA anions has been well documented, there is a lack of correlation between OA anion secretion and Al tolerance in some plant species. For example, the Al-induced secretion of OA anions (citrate and oxalate) cannot account for the genotypic differences in Al tolerance in maize, soybean, and buckwheat cultivars [42–44]. Wenzl et al. observed that the secretion of OA anions from Al-treated signalgrass (*Brachiaria decumbens*) apices was three- to 30-times smaller than that from Al-treated apices of buckwheat, maize, and wheat (all much more sensitive to Al than signalgrass) [45]. Ishikawa et al. investigated the amount of malate and citrate in Al media of seven plant species (Al tolerance order: *Brachiaria brizantha*, rice (*Oryza sativa*), and tea (*Camellia sinensis*) > maize > pea (*Pisum sativum*) and *C. tora* > barley) and of two cultivars with differential Al tolerance each in five plant species (rice, maize, wheat, pea, and sorghum). They did not observe any correlation of Al tolerance among some plant species or between two cultivars in some plant species with the amount of citrate and malate in Al media [46]. Yang et al. showed that eight oxalate accumulator cultivars from four species including *Amaranthus* spp., buckwheat, spinach (*Spinacia oleracea*), and tomato (*Lycopersicon esculentum*) secreted oxalate rapidly under Al stress, but oxalate secretion was not related to their Al tolerance [47]. Therefore, it is reasonable to assume that some plant species may contain other (stronger) mechanisms, which mask the effect of OA anions and/or that the Al-induced secretion of OA anions is too low to be an effective mechanism [44, 48–50]. In this section, we will discuss several aspects that have been implicated in the regulation of the Al-induced OA anion secretion.

2.1. Anion Channels or Transporters. From the experiments with anion channel and carrier inhibitors, the Al-activated secretion of OA anions is mediated through anion channels and/or carriers [9, 20, 37, 69]. As early as 1995, Ryan et al. observed that inhibitors of anion channels inhibited the Al-activated secretion of malate from wheat roots, providing evidence that Al might activate malate secretion *via* a channel in the PM in the apical cells of Al-tolerant wheat cells [37]. Increasing evidence shows that the influence of anion channel inhibitors on the Al-activated secretion of OA anions depends on the species of OA anions secreted, plant species, inhibitor concentration, and species (see [13, 30, 37, 62], Table 1). Li et al. observed that two citrate carrier inhibitors (pyridoxal 5′-phosphate (PP) and phenylisothiocyanate (PITC)) effectively inhibited citrate secretion, meaning that the Al-activated citrate from rye roots is mediated by citrate carrier [21]. Yang et al. [36] and Li et al. [63] showed that the Al-activated secretion of citrate from rice bean (*Vigna umbellata*) and *Stylosanthes* spp. roots was inhibited by both anion channel and carrier inhibitors, indicating the possible involvement of both the citrate carrier and anion channel in the Al-activated citrate secretion. Although the use of inhibitors can be indicative of the type of transport protein involved in OA anion secretion, they do not provide definitive evidence because most inhibitors will eventually affect transport processes that can happen nonspecifically depending on the concentration and period of application. The use of patch clamp technique, which directly measures the transport activity, provides a much stronger evidence that anion channels are involved in the secretion of OA anions from roots under Al stress [69–72]. To date, two families of membrane transporters, the Al-activated malate transporter (ALMT) and the multidrug

Table 1: Characteristics of the aluminum- (Al-) induced secretion of organic acid (OA) anions from roots of different plant species.

Plant species	OA anions secreted	Secretion pattern	Dose response	Temperature sensitivity	Effective inhibitors	References
Acacia mangium	Citrate	NA	NA	NA	NA	[28]
Acacia auriculiformis	Oxalate, citrate	NA	P	NA	NA	[51]
Arabidopsis thaliana	Citrate, malate	NA	NA	NA	NA	[52, 53]
Barley (*Hordeum vulgare*)	Citrate	I	A	P	NIF, A9C	[17]
Buckwheat (*Fagopyrum esculentum*)	Oxalate	I	P	NA	PG	[19, 20, 39, 54]
Cassia tora	Citrate	II	P	NA	CHM	[34, 39, 46]
Citrus grandis and *Citrus sinensis*	Citrate, malate	I	P	P	CHM (malate), DIDS(1), A9C	[30, 31]
Citrus junos	Citrate	II	P	NA	NA	[24]
Deschampsia flexuosa	Malate	NA	NA	NA	NA	[55]
Eucalyptus camaldulensis	Oxalate, citrate	NA	P	NA	NA	[51]
Galium saxatile	Citrate	NA	P	NA	NA	[55]
Lespedeza bicolor	Citrate, malate	II	P (malate), A (citrate)	NA	A9C, CHM	[29]
Leucaena leucocephala	Citrate	NA	NA	NA	NA	[28]
Maize (*Zea mays*)	Citrate, malate	NA	P	NA	NIF, DIDS(2)	[18, 56]
	Oxalate	NA	A	NA	NA	[8]
Melaleuca cajuputi	Oxalate, citrate	NA	P	NA	NA	[51]
Melaleuca leucadendra	Oxalate, citrate	NA	P	NA	NA	[51]
Oat (*Avena sativa*)	Citrate, malate	NA	NA	NA	NA	[19]
Oryza glaberrima	Citrate	NA	NA	NA	NA	[46]
Paraserianthes falcataria	Citrate	NA	NA	NA	NA	[28]
Pea (*Pisum sativum*)	Citrate	NA	NA	NA	NA	[46]
Polygonum aviculare and *Polygonum lapathiifolium*	Oxalate	I	NA	NA	PG	[35]
Poplar *(Populus tremula*)	Oxalate, citrate	NA	P	NA	NA	[57]
Radish (*Raphanus sativus*)	Citrate, malate	NA	NA	NA	NA	[19]
Rape (*Brassica napus*)	Citrate, malate	NA	NA	NA	PG	[19, 58, 59]
Rice (*Oryza sativa*)	Citrate	NA	NA	NA	NA	[46]
Rice bean (*Viga umbellata*)	Citrate	II	NA	NA	A9C, NIF, MA, PITC, CHM	[36]
Rye (*Secale cereale*)	Citrate, malate	II	P	P	PP, PITC	[21]
Rye	Citrate, malate	I (malate), II (citrate)	P	P (citrate) A (malate)	NA	[60]
Rumex acetosella	Oxalate	NA	P	NA	NA	[55]
Snapbena (*Phaseolus vulgaris*)	Citrate	NA	NA	NA	NA	[61]
Soybean (*Glycine max*)	Citrate, malate	II	P	NA	NA	[22, 23, 41]
Soybean	Citrate	II	P	NA	A9C, CHM, MA	[62]
Sorghum (*Sorghum bicolor*)	Citrate	NA	NA	NA	NA	[53]

Table 1: Continued.

Plant species	OA anions secreted	Secretion pattern	Dose response	Temperature sensitivity	Effective inhibitors	References
Spinach (*Spinacia oleracea*)	Oxalate	I	P	NA	NA	[35]
Stylosanthes spp.	Citrate	II	P	NA	A9C, PITC, PG, NIF, DIDS(1), CHM	[63]
Sunflower (*Helianthus annuus*)	Citrate, malate	NA	NA	NA	NA	[64]
Taro (*Colocasia esculenta*)	Oxalate	NA	P	NA	NA	[65]
Tobacco	Citrate	I	P	NA	NA	[40]
Tomato (*Lycopersicon esculentum*)	Oxalate	I	A	NA	PG	[66]
Triticale (×*Triticosecale* Wittmack)	Citrate, malate	II	P	NA	NA	[26]
Veronica officinalis	Citrate	NA	P	NA	NA	[55]
Viscaria vulgaris	Oxalate	NA	P	NA	NA	[55]
Wheat (*Triticum aestivum*)	Malate	I	P	A	NIF, DPC, EA, A9C, NPPB, IAA-94	[21, 37, 67, 68]

A: absent; A9C: anthracene-9-carboxylic acid; CHM: cycloheximide; DIDS(1): 4,4′-diisothiocyanatostilbene -2,2′-disulfonic acid; DIDS(2): 4,4′-dinitrostilbene-2,2′-disulfonic acid; DPC: diphenylamine-2-carboxylic acid; EA: ethacrynic acid; IAA-94, (6,7-dichloro-2-cyclopentyl-2, 3-dihydro-2-methyl-oxo-1H-inden-5-yloxy) acetic acid; NA: not applicable; MA: mersalyl acid; NIF: niflumic acid; NPPB: 5-nitro-2-(3-phenylpropylamino)-benzoic acid; P: present; PG: phenylglyoxal; PITC: phenylisothiocyanate;. PP: pyridoxal 5′-phosphate; anion channel inhibitors: A9C, NIF, PG; Citrate carrier inhibitors: MA: PITC, PP; protein synthesis inhibitor: CHM. Two patterns of Al-induced OA anion secretion can be identified on the basis of the timing of secretion. In Pattern I plants, no discernible delay is observed between the addition of Al and the onset of OA anion secretion. In Pattern II plants, OA anion secretion is delayed for several hours after exposure to Al.

and toxin compounds extrusion (MATE) families, have been implicated in the secretion of OA anions from plant roots in response to Al. In 2004, Sasaki et al. first isolated the Al-activated OA anion secretion transporter from wheat (i.e., *Al-activated malate transporter 1, TaALMT1*) [73]. Electrophysiological studies show that TaALMT1 functions as a ligand-activated and voltage-dependent anion channel to facilitate malate secretion across the PM of root cells [67, 74, 75]. Following the cloning of the first Al-activated OA anion secretion transporter, *TaALMT1* homologs have been cloned from rape (*Brassica napus*; *BnALMT1* and *BnALMT2*) [76], *Arabidopsis thaliana* (*AtMALMT1*) [77], and rye (*ScALMT1*) [78]. Osawa and Matsumoto proposed that protein phosphorylation was associated with the Al-activated malate secretion from wheat root apex and that the OA anion-specific channel was possibly a terminal target that responded to Al signal mediated by phosphorylation [79]. Kobayashi et al. observed that the activation of AtALMT1 by Al was inhibited by staurosporine (kinase inhibitor) and calyculin A (phosphatase inhibitor), and that K252a (serine/threonine protein kinase inhibitor) inhibited the Al-dependent malate secretion without reducing gene expression [38]. Ligaba et al. provided evidence indicating that TaALMT1 activity was regulated by protein kinase C-mediated phosphorylation. They observed that TaALMT1 activity was disrupted when the serine residue at position 384 was replaced with an alanine,and concluded that the serine residue needed to be phosphorylated before TaALMT1 was activated by Al. These results suggest that the activation of ALMT1 by Al may involve reversible protein phosphorylation [80]. However, not all ALMT1-type transporters mediate Al-activated OA responses. For example, *ZmALMT1* isolated from maize was suggested to play a role in anion homeostasis and mineral nutrition, and the activity of this protein was independent of extracellular Al [81]. *ZmALMT2* isolated from maize is a root anion transporter that mediates constitutive root malate secretion and may play a role in mineral nutrient acquisition and transport but not Al tolerance [82]. Three ALMT-type transporters isolated from *A. thaliana* were expressed in leaf mesophyll (*AtALMT9*) or guard cells (*AtALMT6* and *AtALMT12*), implicating a primary role in malate homeostasis and guard cell function [83–85]. Recently, Gruber et al. demonstrated that HvALMT1 from barley likely contributed to the homeostasis of OA anions in the cytosol of guard cells and root cells by transporting them out of the cell or into cytosolic vesicles [86, 87].

In 2007, genes encoding citrate transporters that are members of a different transporter family, the MATE family, were isolated from barley (*HvAACT1,* also designated as *HvMATE1*) [88, 89] and sorghum (*SbMATE*) [53]. The MATE family of transporter proteins is a large and diverse group present widely in bacteria, fungi, plants, and mammals. Evidence shows that MATE proteins function as H^+ or Na^+ coupled antiporters for numerous substances such as flavonoid, anthocyanins, norfloxacin, ethidium bromide, berberine, acriflavine, nicotine, citrate, and Cd^{2+} [88, 90–92]. Plant MATEs can transport substrates other than citrate, which may also play a role in Al tolerance [88, 90–94]. Recently, MATE homologs involved in Al-activated citrate secretion were isolated from *A. thaliana* (*AtMATE*) [95], rye (*ScFRDL2*) [92], maize (*ZmMATE1*) [96], rice (*OsFRDL4*) [97], and rice bean (*VuMATE*) [98]. All these citrate transporters exhibit varying degrees of constitutive expression (i.e., in the absence of Al) except for *VuMATE*, and their expressions are upregulated by Al treatment except for *HvMATE*. Evidence shows that *OsFRDL4* isolated from rice and *AtMATE* isolated from *A. thaliana* are regulated by a C2H2-type zinc finger transcription factor ART1 and STOP1, respectively [95, 97].

In buckwheat, evidence shows that ABA is involved in the secretion of oxalate [99]. ABA activates the anion channel in stomatal guard cells and may play a similar role in the roots [9]. However, no oxalate transporter has been isolated from plants so far.

2.2. Internal Concentrations of Organic Acid Anions in Plant Tissues. The effects of Al on OA metabolism have been investigated in some plant species. In an Al tolerant maize single cross, exposure to increasing level of Al led to a strong (over 3-fold) increase in root tip citrate concentration and a significant activation of citrate secretion, which saturated at a rate close to 0.5 nmol citrate h^{-1} $root^{-1}$ occurring at 80 μM Al^{3+} activity, with the half-maximal rate of citrate secretion occurring at about 20 μM Al^{3+} activity [72]. Ligaba et al. demonstrated that Al-treated rape roots had increased *in vitro* activities of citrate synthase (CS, EC 4.1.3.7), malate dehydrogenase (MDH, EC 1.1.1.37) and phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31), and concentrations of citrate and malate, together with decreased respiration rate, concluding that the Al-induced accumulation and subsequent secretion of citrate and malate were associated with both increased biosynthesis and reduced catabolism [59]. In an Al tolerant tree species, *P. facataria*, the Al-induced increases in both secretion and accumulation of citrate were accompanied by increased mitochondrial CS (mCS) activity and enhanced *mCS* expression, indicating that the increased amount of citrate is produced in response to Al [28]. Aluminium treatments resulted in an increase in CS activity and a decrease in aconitase (ACO, EC 4.2.1.3) activity in the root tips of *C. tora*, accompanied by an increase in citrate concentration. However, the activities of NADP-isocitrate dehydrogenase (NADP-IDH, EC 1.1.1.42), MDH, and PEPC were unaffected by Al. It was suggested that Al-regulation of both CS and ACO activities might be responsible for the Al-induced increase in both secretion and accumulation of citrate [100]. Yang et al. reported that CS activity in soybean root apex was increased by 16% when exposed to Al, but the activities of PEPC and NADP-IDH and the concentration of citrate were unaffected. They suggested that the Al-induced increase in CS activity resulted in the increased secretion of citrate [41]. The unchanged concentration of citrate in the Al-treated roots likely reflected the balance between citrate synthesis and secretion in the root apex. In rye, the activity of CS in the root tip increased by 30% when exposed to Al, and the Al-induced increase in the synthesis of citrate appeared to be responsible for the enhanced

secretion of citrate from roots [21]. In an Al-tolerant soybean cultivar, mitochondrial MDH and CS activities increased and ACO activity decreased with the increasing of Al concentration and duration of Al treatment. The Al-induced citrate secretion was inhibited by the CS inhibitor suramin and enhanced by the ACO inhibitor fluorocitric acid. Transcript level of the mitochondrial *CS* increased in soybean roots in response to Al, whereas the expression of *ACO* showed no significant difference. These results indicate that Al triggers OA metabolic responses in mitochondria of soybean roots, which support the sustained secretion of citrate [101]. Based on the above results, it is reasonable to believe that altered OA metabolism is involved in the Al-induced secretion of OA anions. However, it is not immediately obvious that simply increasing internal OA will lead to increased secretion, because in any case some transport processes must somewhat be involved in the Al-induced secretion of OA anions. For example, Gaume et al. showed that the Al-tolerant maize cultivar had higher root concentrations and higher root secretion of citrate, malate, and succinate compared with the Al-sensitive one. Increased PEPC activity in root apices after Al exposure partially explained the differences of OA anion concentrations in the roots. However, the increased secretion was not proportional to the OA anion concentrations in the roots. The concentrations of citrate, malate, and succinate in the roots of both cultivars increased by a factor of 2 to 4, whereas the secretion of these anions increased by 2 to 20. They suggested that the secretion of OA anions might be mediated by transporters that are either activated or induced by Al [102]. In a study with two lines of triticale differing in the Al-induced secretion of malate and citrate and in Al tolerance, the concentrations of citrate (root apices and mature root segments) and malate (mature segments only) in roots increased in response to Al, but similar changes were observed in the two lines. The Al-induced changes in *in vitro* activities of CS, PEPC, NAD-MDH, and NADP-IDH were similar in the sensitive and resistant lines in both root apices and mature root segments. These results suggest that the Al-induced secretion of malate and citrate from triticale roots is not regulated by their internal levels in the roots or by the capacity of root tissues to synthesize them [103]. Yang et al. reported that the root concentration of oxalate was poorly related with its secretion among some oxalate accumulators such as *Amaranth* spp., buckwheat, spinach, and tomato [47]. Recently, we observed that Al decreased or did not affect the concentrations of malate and citrate in roots of two citrus species having different tolerance to Al, indicating that the Al-induced secretion of citrate and malate is poorly related to their internal levels in roots [30, 31, 104–106].

Transgenic plants and cells have provided additional evidence that OA metabolism can contribute to the Al-induced secretion of OA anions and Al tolerance [6, 107]. Modulation of OA metabolism enhanced Al tolerance and secretion of citrate and/or malate in transgenic tobacco and papaya (*Carica papaya*) plants overexpressing a *Pseudomonas aeruginosa CS* [108], rape plants [58], and carrot (*Daucus carota*) cells [109] overexpressing a mitochondrial *CS* (*mCS*) from *A. thaliana*, *Nicotiana benthamiana* plants overexpressing an *mCS* from *C. junos* [24], tobacco plants overexpressing a cytosolic *MDH* from *A. thaliana*, an *MDH* from *Escherichia coli* [110] and a *CS* from rice mitochondria [111], alfalfa (*Medicago sativa*) plants overexpressing a alfalfa nodule-enhanced form of *MDH* (*neMDH*) [112], and tobacco plants overexpressing *pyruvate phosphate dikinase* (*PPDK*, EC 2.7.9.1) gene from *Mesembryanthemum crystallinum* [113]. However, Delhaize et al. showed that the expression of a *P. aeruginosa CS* in tobacco did not result in enhanced citrate accumulation or secretion, despite generating transgenic tobacco lines that expressed the CS protein at up to a 100-fold greater level than the previously described CSb lines [40, 108]. They concluded that the activity of the *P. aeruginosa* CS in transgenic tobacco is either sensitive to environmental conditions or that the improvements in Al tolerance and P nutrition observed previously are due to some other variable [40].

In an Al-tolerant soybean cultivar, the Al-induced root secretion of citrate increased steadily when exposed to continuous light, and only low citrate secretion was observed under 24 h in the continuous dark. The rate of Al-induced citrate secretion decreased at 6 h after the shoots were excised. The rate of citrate secretion by shoot-excised roots was 3-times lower than that of their respective controls (Al treatment in plants with shoots) during the 6–9 h after 50 μM treatment and 6-times lower during the 9–12 h. These results indicate that the shoots play a role in the Al-induced citrate secretion through providing the carbon source and/or energy for citrate synthesis in the roots [41]. Neumann and Römheld reported that P deficiency strongly increased the concentrations of carboxylic acids in chickpea (*Cicer arietinum*) and white lupin (*Lupinus albus*) roots, but only had small effects on the accumulation of carboxylates in shoots, and suggested that the ability to accumulate carboxylic acids in roots depended on the partitioning of carboxylic acids or related precursors between roots and shoots [114]. Quantification of soybean root enzymes involved in OA metabolism displayed only a 16% increase in CS activity 6 h after Al treatment with no differences in other enzymes; hence citrate may be transported from the shoots to the roots [41].

2.3. Temperature. Yang et al. observed that the Al-induced secretion of citrate and malate by the roots of *C. grandis* and *C. sinensis* seedlings was inhibited by low temperature, indicating that an energy dependent process may be involved in the Al-induced secretion of OA anions [30]. A similar result has been obtained in Al-tolerant barley [17]. However, the Al-induced secretion of citrate in rye (Pattern II) was decreased by low temperature, but the Al-induced secretion of malate in wheat (Pattern I) was unaffected by low temperature [21]. Recently, Li et al. reported that, in rye, the Al-induced secretion of malate belonged to Pattern I and was not inhibited, while the Al-induced secretion of citrate belonged to Pattern II and was affected by low temperature [60]. Further research is needed to elucidate the mechanism.

2.4. Root Plasma Membrane H^+-ATPase. Since PM H^+-ATPase plays a critical role in energizing and regulating an array of secondary transporters [115, 116], the modulation

of PM H^+-ATPase activity may be involved in the Al-induced secretion of OA anions. In two soybean cultivars, the Al-induced activity of root PM H^+-ATPase paralleled the secretion of citrate. The Al-induced increase in PM H^+-ATPase activity was caused by a transcriptional and translational regulation. Both activity and expression of root PM H^+-ATPase were higher in the Al-tolerant than in the Al-sensitive cultivar. Aluminum activated the threonine-oriented phosphorylation of PM H^+-ATPase in a dose- and time-dependent manner. The relationship between the Al-induced secretion of citrate and the activity of PM H^+-ATPase was further demonstrated by an analysis of PM H^+-ATPase transgenic *A. thaliana*. When grown on Murashige and Skoog medium containing 30 μM Al, transgenic plants of *A. thaliana* overpressing PM H^+-ATPase secreted more citrate compared with wild-type *A. thaliana* [117]. Ahn et al. showed that after 4 h *in vivo* treatment with 2.6 μM Al, PM H^+-ATPase activity and H^+-transport rate were decreased and ζ potential was depolarized in PM vesicles from root tips of Al-sensitive wheat cultivar (ES8) but not of Al-tolerant ET8. They concluded that the Al-induced secretion of malate from wheat roots was accompanied by changes in PM surface potential and activation of H^+-ATPase [118]. However, the Al-induced changes of root PM H^+-ATPase activity were not associated with oxalate secretion in two tomato cultivars differing in the ability to secrete oxalate under Al stress [66]. Other studies showed Al inhibited root PM H^+-ATPase activity in barley [119], squash (*Cucurbita pepo*) [120], and rice bean [121].

2.5. Magnesium. Magnesium (Mg) can ameliorate Al toxicity, but the mechanism by which Al alleviates it remains obscure [122, 123]. Long-term secretion of OA anions requires continuous biosynthesis of OAs inside the root cells. In this regard, cytoplasmic Mg^{2+} is pivotal for the activation of many enzymes (e.g., CS, PEPC, IDH, malic enzyme (ME, EC 1.1.1.40), and MDH) involved in OA biosynthesis and degradation [122]. In soybean, micromolar concentration of Mg in the treatment solution alleviated Al toxicity by enhancing citrate biosynthesis and secretion by roots. Increased production and secretion by soybean roots in response to Mg might promote both external and internal detoxification by formation of Al-citrate complexes [123]. In rice bean, Mg could stimulate the Al-induced secretion of citrate from roots thus alleviating the inhibition of root growth by Al. The stimulation of citrate secretion by Mg might result from the restoration of root PM H^+-ATPase activity by Mg [121].

2.6. Phosphorus. Phosphorus deficiency is another major factor limiting plant growth in acidic soils [6]. Evidence has shown that Al toxicity can be alleviated by P supply in some plants, including *C. grandis* [30, 31], sorghum [124], maize [102], and *L. bicolor* [125]. There are several authors investigating the effects of P on the Al-induced secretion of OA anions from roots, but the results are somewhat different. Yang et al. showed that the Al-induced secretion of citrate and malate by excised roots from Al-treated *C. grandis* and *C. sinensis* seedlings decreased with increasing P supply, whereas P supply increased or had no effect on the concentrations of both citrate and malate in Al-treated roots [30, 31]. The decreased secretion of OA anions due to P application can be due to the amelioration of Al toxicity by P rather than due to decreased root accumulation of OA anions. In two maize cultivars, the Al-induced increases in root activity of PEPC, root concentrations, and secretion of OA anions were decreased in plants pretreated with higher P concentrations during the 21 days prior to Al treatment [102]. In two cowpea genotypes of contrasting Al tolerance, Al enhanced malate secretion from root apices of both genotypes. Phosphorus deficiency increased the Al-induced secretion of malate by roots only in the Al-tolerant genotype IT89KD-391 [126]. In an Al-tolerant leguminous shrub, *L. bicolor*, the Al-induced secretion of citrate and malate under P sufficiency was less than that under P deficiency [125]. The above results indicate that the enhancement of Al tolerance by P is not associated with an increased secretion of OA anions from roots. However, P-sufficient rape plants displayed more pronounced Al-induced accumulation and secretion of citrate and malate in roots than P-deficient plants. Interestingly, the degree of inhibition of Al-induced root elongation was more or less the same in both P-sufficient and P-deficient plants. It was suggested that the severity of Al toxicity in P-deficient plants was masked by the stimulating effect of P deficiency on root elongation [59]. Using four soybean genotypes differing in P efficiency, Liao et al. investigated the effects of Al and P interactions on OA anion secretion by roots grown in homogeneous and heterogeneous nutrient solutions. In the homogenous solution experiments, P enhanced Al tolerance in four soybean genotypes, but greatly decreased the Al-induced citrate and malate secretion by roots. The two P-efficient genotypes displayed more Al tolerance than the two P-inefficient genotypes under high-P condition, but no significant genotypic difference was found in the secretion of OA anions under both low- and high-P conditions. The secretion of OA anions in a homogenous solution may not reflect the ability of soybean plants to detoxify exogenous Al. At the early stages of the heterogeneous nutrient solution experiment, P greatly increased the rates of the Al-activated citrate and malate secretion from the taproot tips of the four genotypes and the Al tolerance for the two P-efficient genotypes, and the two P-efficient genotypes secreted more malate from the taproot apices under high-P condition. They concluded that, at the early stage of heterogeneous nutrient solution experiment, P might increase the Al-activated secretion of OA anions, thus enhancing Al tolerance [23]. In two soybean cultivars and one rye cultivar, P deficiency did not increase the Al-induced secretion of citrate by roots [60, 127]. In soybean, short-term P deficiency (4 days) followed by Al treatment led to 50% increase in the Al-induced citrate secretion, while longer-term (10 days) P deficiency followed by Al treatment reduced the Al-induced citrate secretion to trace amounts [128]. However, in another study with soybean, Yang et al. showed that application of Al induced a greater citrate secretion rate in the Al-tolerant cultivar than in the Al-sensitive cultivar independently of the P status of the plants [22]. Dong et al. showed that long-term (14 days)

P deficiency followed by Al stress (7 h) had no effect on the Al-induced secretion of citrate from soybean roots [127]. This disagreement was attributed to the differences in the plant materials and experimental methods used [128]. Thus, it appears that the influence of P on the Al-induced secretion of OA anions depends on the time of exposure to Al, growth conditions, and plant species or cultivar.

2.7. Other Factors. Yang et al. showed that sodium nitroprusside (SNP, a nitric oxide (NO) donor) increased the Al-induced secretion of malate and citrate by excised roots from Al-treated *C. grandis* seedlings and that the stimulatory effects of SNP on the Al-induced secretion of malate and citrate might be involved in the SNP-induced amelioration of Al toxicity [105]. There are several papers reporting that NO regulates K^+ and Ca^{2+} channels in plants [129, 130]. The stimulation of OA anion secretion by SNP might result from its direct effect on anion channels, because SNP did not enhance the accumulation of malate and citrate in the roots [105]. Chen et al. demonstrated that H_2S played an ameliorative role in protecting soybean plants against Al toxicity by increasing citrate secretion and citrate transporter gene expression and enhancing the expression of PM H^+-ATPase [131].

3. Genetic Engineering Technology for the Secretion and Biosynthesis of Organic Acid Anions

A common agricultural practice for acidic soils is to apply lime to raise soil pH. However, the option is not economically feasible for poor farmers, nor is it an effective strategy for alleviating subsoil acidity [132]. A complementary approach to liming practice is to tailor plants to suit acidic soils by identifying and/or developing plants with improved tolerance to Al in acidic soils. The best documented mechanism for plant Al tolerance is the Al-induced secretion of OA anions from roots [6, 9], although it is not the only tolerance mechanism [43, 77]. The production of transgenic plants with an enhanced ability to secrete OA anions appears to be an appealing strategy to produce Al tolerant plants. The two main approaches for increasing OA anion secretion are to increase OA synthesis and to increase OA anion transport across the PM [107]. Table 2 summarizes the attempts to obtain transgenic plants or cells with higher Al tolerance by overexpressing genes involved in the biosynthesis and the secretion of OA anions.

The early attempts to enhance Al tolerance focused on OA synthesis because the genes encoding transporters for OA anions were not cloned at the time. The first influential report of such an approach was from De La Fuente et al. [108] who overexpressed a *P. aeruginosa CS* in tobacco and papaya. Overproduction of citrate was shown to result in Al tolerance in transgenic tobacco and papaya through increasing citrate secretion. Delhaize et al. [40] could not repeat the original study of De La Fuente et al. [108] in the same and other tobacco lines expressing the *CS* from *P. aeruginosa* at higher levels. The authors also observed that *CS*-expressing alfalfa did not show an improved Al tolerance. They concluded that the expression of the *CS* in plants is unlikely to be a robust and easily reproducible strategy for enhancing the Al tolerance of crop and pasture species. Recently, Barone et al. assessed the Al tolerance of 15 transgenic alfalfa overexpressing the *CS* from *P. aeruginosa* by *in vitro* root growth, hydroponics, or soil assay. They deemed that *CS* overexpression could be a useful tool to enhance Al tolerance, but the type of assay used was critical to properly evaluate the transgenic phenotype [133]. Overexpression of mitochondrial *CS* also resulted in increased citrate secretion and enhanced Al tolerance in *A. thaliana* [134], rape [58], *N. benthamiana* [24], and tobacco [111]. In tobacco, overexpression of cytosolic *MDH* genes from *A. thaliana* and from *E. coli* led to increased malate secretion and enhanced Al tolerance [110]. Zhang et al. reported that overexpression of a *C. junos MDH* in tobacco conferred Al tolerance [135]. In alfalfa, overexpression of an alfalfa nodule-enhanced form of *MDH* (*neMDH*) resulted in increased synthesis and secretion of OA anions and enhanced Al tolerance. However, the transgenic alfalfa overexpressing an alfalfa *PEPC* did not show increased accumulation and secretion of OA anions [112]. Trejo-Téllez et al. showed that overexpression of an *M. crystallinum PPDK* in tobacco roots increased the exudation of OA anions, with a concomitant decrease in plant Al accumulation [113]. It should be noted that OA metabolism may not be a limiting factor for Al-induced secretion of OA anions in some plant species because only a small portion of internal OAs is secreted in response to Al [9]. In addition, the Al-induced secretion of OA anions must somewhat be associated with some transport processes [9, 107]. Therefore, the effect that modifying internal OA concentrations has on the level of plant Al tolerance may be small or not observed in some plant species.

Transgenic plants overexpressing genes encoding transporters for OA anions have been widely studied since the first major gene (*TaALMT1*) was cloned from wheat [73]. *TaALMT1* expression in rice, cultured tobacco cells, barley, and *A. thaliana* led to increased Al-activated malate secretion and enhanced Al tolerance for all except for rice [73, 136, 137]. Overexpression of *TaALMT1* in wheat conferred greater Al-activated malate secretion from the roots and improved Al tolerance, which was kept in the T1 and T2 generations [138]. This is the first report of a major food crop being stably transformed for greater Al tolerance. Homologs of *TaALMT1* cloned from *A. thaliana*, rape, barley, and rye [76–78, 87] could also be utilized to increase plant Al tolerance. For example, expression of *BnALMT1* and *BnALMT2* in tobacco cultured cells [76], *HvALMT1* in barley [87], and *AtALMT1* in *A. thaliana* [107] resulted in increased malate secretion and enhanced Al tolerance. Increases in Al tolerance have been achieved by overexpressing citrate transporters. Expression of *SbMATE1* [53], *FRD3* [94], and *ZmMATE1* [96] in *A. thaliana*, *HvAACT1* in tobacco [88], and *VuMATE* in tomato [98] enhanced the secretion of citrate and Al tolerance.

Arabidopsis thaliana has one gene encoding for a type I H^+- pyrophosphatase (AVP1, *Arabidopsis* Vacuolar Pyrophosphatase 1) and another gene encoding for a type II H^+- pyrophosphatase (AVP2) [139, 142]. Yang et al. reported

Table 2: Transgenic plants or cells with higher aluminum- (Al-) tolerance overexpressing genes for the biosynthesis and the secretion of organic acid (OA) anions.

Genes	Origins	Transgenic plants or cells	Increased secretions of OA anions	Al tolerance	References
Citrate synthase (*CS*)	*Pseudomonas aeruginosa*	Papaya (*Carica papaya*)	NA	+	[108]
		Tobacco (*Nicotiana tabacum*)	Citrate	+	[108]
		Tobacco	No	No	[40]
		Alfalfa (*Medicago sativa*)	No	No	[40]
	Arabidopsis thaliana	Alfalfa	NA	+	[133]
		Rape (*Brassica napus)*	Citrate	+	[58]
		Carrot (*Daucus carota*) cell	Citrate	+	[109]
	Citrus junos	*Nicotiana benthamiana*	Citrate	+	[24]
	Carrot	*A. thaliana*	Citrate	+	[134]
	Rice (*Oryza sativa*)	Tobacco	Citrate	+	[111]
Malate dehydrogenase (*MDH*)	*C. junos*	Tobacco	NA	+	[135]
	A. thaliana, *Escherichia coli*	Tobacco	Malate	+	[110]
Nodule-enhanced form *of MDH (neMDH)*	Alfalfa	Alfalfa	Citrate, oxalate, malate, succinate, acetate	+	[112]
*Phospho*enol*pyruvate carboxylase (PEPC)*	Alfalfa	Alfalfa	No	No	[112]
Pyruvate phosphate dikinase (PPDK)	*Mesembryanthemum crystallinum*	Tobacco	Citrate, malate	+	[113]
TaALMT1	Wheat (*Triticum aestivum*)	Tobacco cells	Malate	+	[73]
		Rice	Malate	No	[73]
		Barley	Malate	+	[136, 137]
		Wheat	Malate	+	[138]
		A. thaliana	Malate	+	[107]
AtALMT1	*A. thaliana*	*A. thaliana*	Malate		[107]
BnALMT1 and *BnALMT1*	Rape	*Tobacco cells*	Malate	+	[76]
HvALMT1	Barley	Barley	Malate	+	[87]
HvAACT1	Barley	Tobacco	Citrate	+	[88]
SbMATE	Sorghum (*Sorghum bicolor*)	*A. thaliana*	Citrate	+	[53]
FRD3	*A. thaliana*	*A. thaliana*	Citrate	+	[94]
ZmMATE1	Maize	*A. thaliana*	Citrate	+	[96]
VuMATE	*Vigna umbellata*	Tomato	Citrate	+	[98]
Plasma membrane H^+-*ATPase*	*A. thaliana*	*A. thaliana*	Citrate	NA	[117]
Type 1 H^+-*pyrophosphatase (AVP1)*	*A. thaliana*	*A. thaliana*, tomato, and rice	Malate	+	[139]

NA: not applicable; No: no change in secretion of OA anions or Al tolerance.

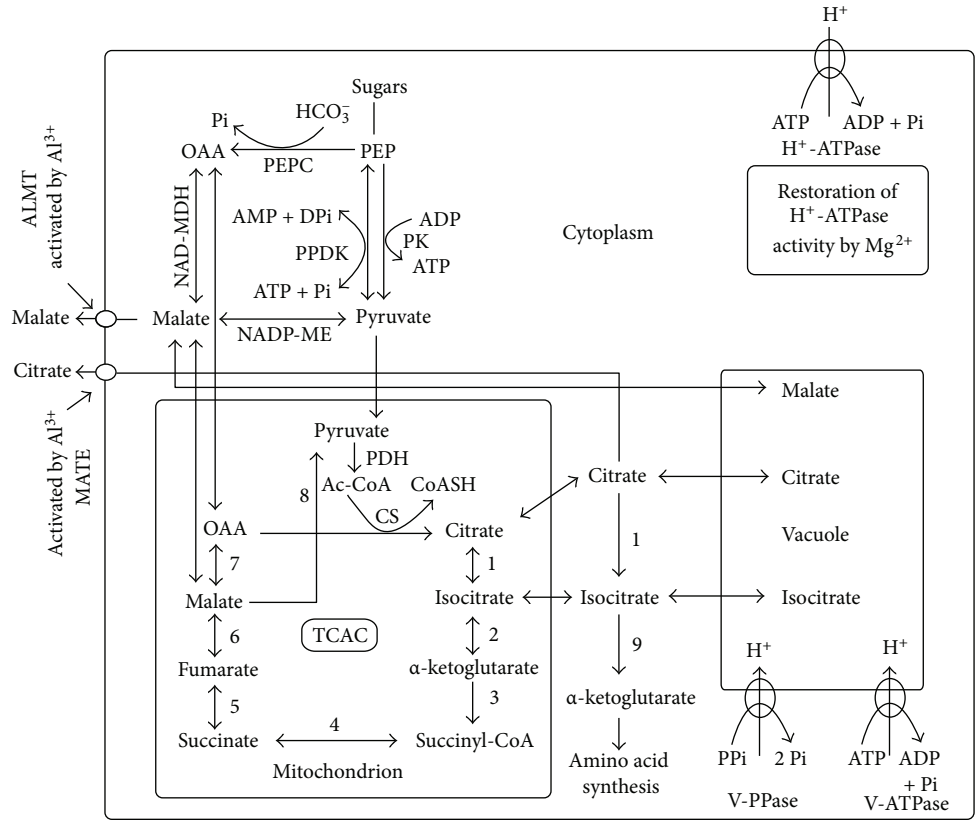

Figure 1: A diagram showing the reactions and processes involved in the accumulation and secretion of organic acid (OA) anions in aluminium- (Al-) treated plants. Ac-CoA: acetyl-CoA: ALMT, Al-activated malate transporter; CS: citrate synthase; DPi: diphosphate; MATE, multidrug and toxin compounds extrusion; NAD-MDH: NAD-malate dehydrogenase; NADP-ME: NADP-malic enzyme; OAA: oxaloacetate; PDH: pyruvate dehydrogenase; PEP: phospho*enol*pyruvate; PEPC: PEP carboxylase; Pi: phosphate; PK: pyruvate kinase; PPDK: pyruvate Pi dikinase; PPi: pyrophosphate; TCAC: tricarboxylic acid cycle; V-ATPase: tonoplast adenosine triphosphatase; V-PPiase, tonoplast pyrophosphatase; 1, aconitase (ACO); 2, NAD-isocitrate dehydrogenase (NAD-IDH); 3, α-ketoglutarate dehydrogenase; 4, succinate thiokinase; 5, succinate dehydrogenase; 6, fumarase; 7, NAD-MDH; 8, NAD-malic enzyme (NAD-ME); 9, NADP-IDH (redrawn from Delhaize et al. [14], Anoop et al. [58], Bose et al. [122], Lin et al. [140], and Mariano et al. [141]).

that overexpression of *AVP1* in *A. thaliana*, tomato, and rice exhibited greater tolerance to Al and higher level of citrate and malate secretion compared with controls and that increased AVP1-dependent H^+ extrusion appeared to be charge balanced by the enhancement of K uptake and the release of OA from roots [139].

4. Concluding Remark

Aluminium toxicity is one of the most deleterious factors for plant growth in acidic soils, which comprise approximately 30% of the world's total land area and over 50% of the world's potential arable lands [1]. In recent years, there has been significant progress in our understanding of the physiological and molecular mechanisms of Al tolerance in higher plants. In particular, the Al-induced secretion of OA anions from roots has been widely studied by many researchers in different plants because it is a major mechanism leading to Al tolerance in higher plants, but the mechanisms which lead to the accumulation and secretion of OA anions are not fully understood (Figure 1). Modulation of OA metabolism and activation of anion channels have been suggested to be involved in the Al-activated secretion of OA anions and transgenic plants or cells overexpressing genes for biosynthesis and secretion of OA anions have displayed increased secretion of OA anions and enhanced Al tolerance (Table 2). It is clear that both ALMT1 proteins from wheat, rye, *A. thaliana*,and rape and MATE proteins from barley and sorghum require Al to activate their function [107]. However, the mechanisms of this activation remain unclear, although evidence shows that the induction of *ALMT1* expression by Al may involve reversible phosphorylation [79, 80, 107] and that *OsFRDL4* and *AtMATE* are regulated by a C2H2-type zinc finger transcription factor ART1 and STOP1, respectively [95, 97]. Although anion channels may play an important role in the Al-induced secretion of oxalate in some plants [47], no oxalate transporter has been isolated from plants so far. Organic acid anions secreted from the roots of P-deficient plants have been shown to mainly result from increased PEPC activity in the shoots [143], but relatively few studies have investigated the roles of shoots in the Al-induced secretion of OA anions. Although some transgenic plants

overexpressing *PEPC* or *CS* did not show enhanced accumulation and secretion of OA anions [40, 112], genetically modified plants with higher Al tolerance by overexpressing genes for the secretion and the biosynthesis of OA anions may still be a potentially rewarding area of research in the future. Recent work showed that the Al-activated malate and citrate transporters from the MATE and ALMT families functioned independently to confer Al tolerance of *A. thaliana* [95] and that overexpression of *TaALMT1* in barley, which is very sensitive to Al and does not possess an Al-activated secretion of malate, had an Al-activated secretion of malate with properties similar to those of Al-tolerant wheat and enhanced Al tolerance [136, 137]. Therefore, an additive or a synergistic effect on Al tolerance may be achieved by overexpressing two or more anion transporters regulating the secretion of different OA anions at the same time. If the secretion of OA anions is limited by their supply, genes for OA anion synthesis can be cotransformed with genes for OA anion transport, which may produce transgenic plants with higher levels of Al tolerance. Finally, many transgenic plants with significantly increased Al tolerance will be produced through the collaboration between plant breeders and plant physiologists.

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Review Article

The Role of Canonical and Noncanonical Pre-mRNA Splicing in Plant Stress Responses

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Plants are sessile organisms capable of adapting to various environmental constraints, such as high or low temperatures, drought, soil salinity, or pathogen attack. To survive the unfavorable conditions, plants actively employ pre-mRNA splicing as a mechanism to regulate expression of stress-responsive genes and reprogram intracellular regulatory networks. There is a growing evidence that various stresses strongly affect the frequency and diversity of alternative splicing events in the stress-responsive genes and lead to an increased accumulation of mRNAs containing premature stop codons, which in turn have an impact on plant stress response. A number of studies revealed that some mRNAs involved in plant stress response are spliced counter to the traditional conception of alternative splicing. Such noncanonical mRNA splicing events include *trans*-splicing, intraexonic deletions, or variations affecting multiple exons and often require short direct repeats to occur. The noncanonical alternative splicing, along with common splicing events, targets the spliced transcripts to degradation through nonsense-mediated mRNA decay or leads to translation of truncated proteins. Investigation of the diversity, biological consequences, and mechanisms of the canonical and noncanonical alternative splicing events will help one to identify those transcripts which are promising for using in genetic engineering and selection of stress-tolerant plants.

1. Introduction

Being sessile, plants have to adapt to unfavorable environmental conditions via activation of the molecular machinery which increases the chance of plant survival under these conditions. Most adaptations that lead to acquisition of stress tolerance require changes in gene expression, which is regulated transcriptionally and posttranscriptionally [1, 2]. Pre-mRNA splicing, along with mRNA stability, mRNA export from the nucleus, and mRNA translation, are the steps of the important mechanism regulating gene expression at the posttranscriptional level. The multitude and diversity of pre-mRNA splicing events quantitatively and qualitatively affect the mRNA population of a variety of genes. Plant pre-mRNAs, as pre-mRNAs of other eukaryotes, typically include noncoding fragments, introns, which are removed, and coding fragments, exons, which have to be spliced together to form the mature mRNAs. Pre-mRNA splicing is an important step of pre-mRNA processing, which also includes 5′ capping and 3′ polyadenylation. Alternative splicing (AS) is a process when the coding and noncoding fragments of a gene are rearranged in various ways by the spliceosomes at different splice sites, thus generating several mRNA transcripts from the same pre-mRNA molecule (for a recent review of AS mechanisms see [3, 4]). In turn, the mRNA transcripts govern the synthesis of several structurally and functionally distinct protein isoforms, thereby increasing the composition of a plant proteome using a limited number of genes.

Recent genome-wide studies have revealed that AS is highly pervasive in plants. Genome-wide mapping of the *Arabidopsis* transcriptome using the Illumina RNA sequencing (RNA-seq) and high-throughput sequencing of a normalized cDNA library indicated that at least ~42% [5] or ~61% [6] of intron-containing genes in *Arabidopsis* are alternatively spliced, which is considerably higher than previous data (~12–22%) obtained from the cDNA/EST sequencing [7, 8]. Recent studies suggest that environmental stresses can induce AS or alter its efficiency and fidelity in a number of genes

playing a role in plant stress response and tolerance [5, 7, 9, 10]. There is also increasing evidence that environmental stresses induce extensive AS, affecting several exons of a mRNA and often lead to the generation of "nonproductive" splice variants of plant stress-inducible gene transcripts [5, 11–13]. These extensive AS events may lead to the production of "nonproductive" transcripts and, thus, decrease gene expression levels or prevent increases in the levels of a full-length protein despite transcriptional stimulation. In addition, there were some studies where noncanonical splicing-like events have been reported for plant genes involved in plant stress signaling pathways. Altogether, the latest data suggest that AS, including noncanonical splicing, might be important in plant stress adaptation. However, despite the phenomenon of AS is known for more than two decades, the list of biological roles for splice variants generated from a single gene in plant stress is not completed and poorly understood. The purpose of this paper is to put together the recent data on AS of plant genes involved in stress signaling and focus on the occurrence, properties, and functional consequences of unconventional splicing and splicing-like events in plants.

2. Types and Mechanisms of Pre-mRNA Splicing in Plants

2.1. Types and Mechanisms of Canonical AS in Plants. During pre-mRNA splicing, the spliceosome, a large flexible RNA-protein complex, splices out the noncoding sequences and stitches the coding sequences together [14]. Spliceosome consists of several small nuclear ribonucleoprotein particles (snRNPs) and numerous protein factors which recognize specific sequences at the 5′ and 3′ splice sites important in manufacturing the mature RNA. In plants, as in other eukaryotes, the spliceosome recognizes conserved dinucleotides at the ends of introns: the majority of introns start with a GT at the 5′ end, donor site, and end with an AG at the 3′ end, acceptor site [4, 15]. Such labeled introns are removed by the major class U2-type spliceosome. A minority of introns (<1% in *Arabidopsis* and humans), which are removed by the minor U12-type spliceosome, often start with AT and terminate with AC dinucleotides at the 5′ and 3′ ends, respectively. Even more rarely, pre-mRNA splicing occurs at GC–AG or GT–GG donor–acceptor pairs of sites. Altogether, these four pairs of the donor and acceptor sites can be found in the majority of all splicing events and serve as splicing-specific signs. Splice sites in the *Arabidopsis* DNA and DNA of other eukaryotes can be detected using the GeneSplicer Web Interface [16] found at http://www.cbcb.umd.edu/software/GeneSplicer/ or the NetPlantGene Server [17] found at http://www.cbs.dtu.dk/services/NetPGene/. To compile and visualize the evidence for alternative splicing in plants, a comprehensive web-interfaced database ASIP [8] found at http://www.plantgdb.org/ASIP/ was created.

The common types of AS that are generally recognized and are widespread in plants are shown in Figures 1(b), 1(c), 1(d), and 1(e). These AS forms comprise intron retention, exon skipping, alternative 5′ or 3′ site selection, and mutually exclusive exons [4, 18, 19]. It has been generally believed that intron retention (Figure 1(b)) is the most frequent type of AS in plants, representing about 60% of the AS events [8]. However, the latest data reveal that the frequency of intron retention events in the *Arabidopsis* transcriptome is ~40%, while the frequency of AS events that do not include intron retention is ~51% [6]. The authors suggest that the significance of intron retention in generating transcript diversity in plants has been generally overestimated in previous studies.

Such types of AS as exon skipping and mutually exclusive exons (Figures 1(c), and 1(e)) lead to the production of proteins with different rearrangements of their domains or with a deletion of a domain. These rearrangements often result in changed binding properties, activity and stability, and subcellular localization of a protein [4, 19]. Intron retention events and alternative 5′ and 3′ splice sites (Figures 1(b), and 1(d)) more easily lead to the production of mRNAs with premature stop codons (PTCs), which is referred to as "unproductive splicing". The occurrence of the PTCs may lead either to mRNA degradation via the nonsense-mediated mRNA decay (NMD) mechanism or to the translation of truncated proteins [10, 13]. Besides elimination of the PTC containing aberrant mRNAs, AS coupled to NMD represents a mechanism to control the amount of functional transcripts in the cell through the targeted degradation of specific alternatively spliced isoforms [13, 20], which could have specific biological roles in a living organism. The truncated proteins might be detrimental or energy costly for the cell, and therefore most of PTC carrying mRNAs are eliminated by the NMD machinery before they reached ribosome [10, 21]. However, recent data show that the truncated proteins derived from the PTC containing mRNAs are not necessarily functionless compared to the full-length protein. A number of studies demonstrate that the truncated proteins perform important functions in plant stress adaptation [22, 23].

2.2. Types and Possible Mechanisms of Noncanonical AS and AS-Like Events in Plants. At present, there are several studies, which will be enumerated and discussed in the Section 3.2, where unusual posttranscriptional processing of plant mRNAs has been reported. These noncanonical AS and AS-like events comprised "variations affecting multiple exons", which refer to deletions of large coding sequences spanning several exons (Figure 2(a)); intraexonic deletions (Figure 2(b)); generation of chimeric mRNAs, which join together portions of two separate mRNA molecules (Figure 2(c)); frameshifting, which was caused by an intron excision (Figure 2(d)). This unusual pre-mRNA processing was often coupled to extensive canonical AS events, including intron retention, exon skipping, or alternative splice site selection and often occurred at noncanonical splice sites.

The noncanonical splice sites were usually represented by short 4–8 nt long direct repeated (SDR) sequences and were different from those splice signals classically recognized by the U2- or U12-type spliceosomes. Recently, Niu et al. [11] identified and analyzed potential SDR-containing sequences in various plant species and described features

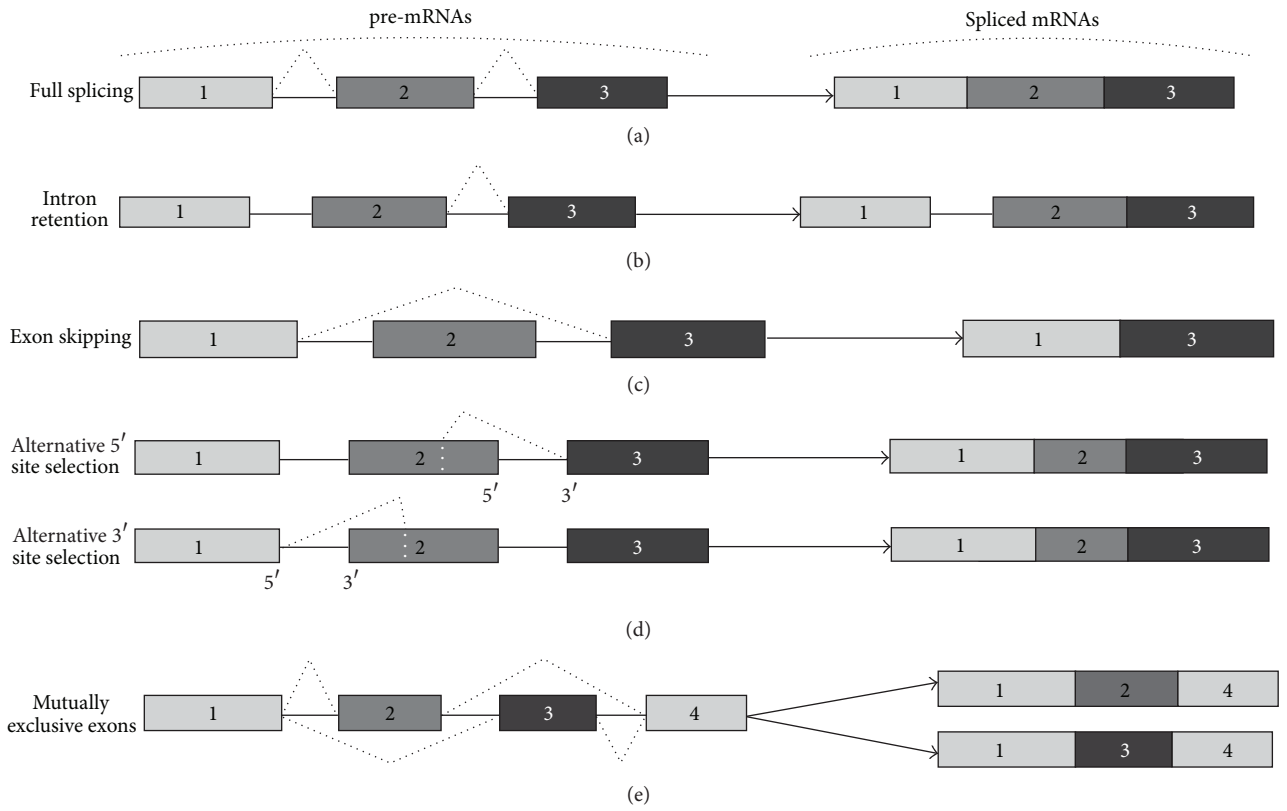

Figure 1: Common types of pre-mRNA splicing in plants. Exons are represented by grey, dark grey and black boxes, and introns-by horizontal solid lines between the boxes. Dashed lines above and below the exons and introns depict AS events. (a) full splicing of a pre-mRNA; (b) intron retention; (c) exon skipping; (d) alternative 5′ or 3′ splice site selection; (e) mutually exclusive exons.

of the SDRs. They found that the plant SDRs were heavily rich in G and C nucleotides. According to the analysis, GC, CG, CC, and GG dinucleotides were prevalent in the plant SDRs. However, according to the authors, a universal consensus sequence for all the SDRs could not be identified. It has been shown that SDR sequences are indispensable elements for the noncanonical splicing events, essential both for chimeric mRNA transcripts and transcripts with a variety of noncanonical deletions [11, 24, 25].

The molecular mechanism for generation of the noncanonically processed mRNAs is not known. In case the process involves participation of the spliceosome, such mRNAs could be considered as a product of a noncanonical AS process (Figure 3(a)). However, it is also possible that the noncanonical "alternatively spliced" transcript variants with intraexonic deletions and deletions affecting multiple exons are generated via transcriptional slippage mechanism that require SDR sequences for reannealing of dissociated pre-mRNA molecule from its DNA template strand (Figure 3(b)). In the case of transcriptional slippage, the transcriptional complex (RNA polymerase II and transcription factors), along with the newly synthesized pre-mRNA, could dissociate from its DNA template during transcription and subsequently reassociate at an identical SDR sequence. Ritz et al. [26] suggested that this mechanism was used for generation of intraexonic deletions and deletions affecting multiple exons, which have been discovered in their study in the human SGCE gene, associated with the neurological movement disorder myoclonus dystonia. Ritz et al. [26] supported their data by analyzing SGCE gene expression in zebrafish, rats, and mice. The noncanonical splicing-like events, which were discovered in the plant stress-inducible genes and are described in the Section 3.2, exhibit similar features to the noncanonical transcript variants described by Ritz et al. [26] and, therefore, could be also generated by the intramolecular slippage mechanism.

A number of mechanisms have been suggested to produce chimeric mRNA transcripts: (1) *trans*-splicing (Figure 3(a); [27]), when two different mRNAs are spliced together by the spliceosome and, presumably, some other *trans*-acting protein factors participate in this process; (2) the read-through mechanism (Figure 3(c); [28]), when the RNA polymerase transcribes two adjacent genes by reading through the intergenic DNA sequence; (3) the transcriptional slippage mechanism (Figure 3(b); [25]), when the RNA polymerase dissociates from SDR sequences on the DNA template with subsequent reassociation at a homologous SDR. It has been suggested that mRNA fusion transcripts with canonical splice signals are spliced via the *trans*-splicing mechanism, while fusion transcripts lacking canonical splice sites and harboring SDRs could be spliced via the transcriptional slippage [25, 26].

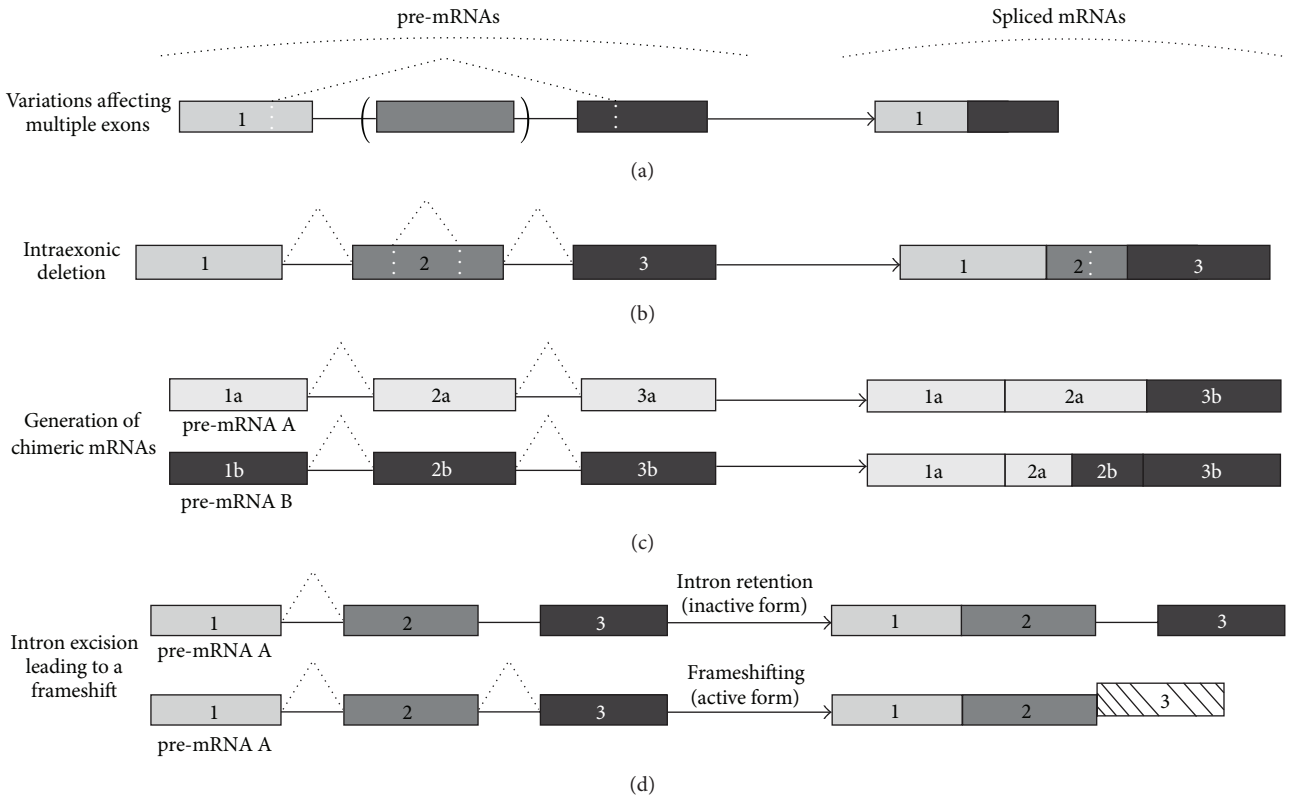

Figure 2: The types of noncanonical pre-mRNA splicing and splicing-like events in plants. (a) variations affecting multiple exons; (b) intraexonic deletion; (c) generation of chimeric mRNAs; (d) frameshifting as a result of intron excision. Exons are represented by grey, dark grey and black boxes, and introns-by horizontal solid lines between the boxes. Dashed lines above and below the exons and introns depict excision events. The dashed box in part (d) outlines a frameshift caused by an intron excision (details can be found in the Section 3.2). In this case, the spliced mRNA produces a protein with a different amino acid sequence at the C-terminus.

It is reasonable to hypothesize that the dissociation and subsequent association of the newly transcribed mRNAs with the DNA template, that is, transcriptional slippage, are not spontaneous and depend on environmental conditions. Ritz et al. [26] suggested that, in stress situations, transcriptional regulation becomes less strict, and this results in enhanced transcriptional slippage and accumulation of the low frequency noncanonical splicing-like transcript variants.

3. The Role of AS in Plant Stress Response

3.1. Canonical AS and Plant Stress Response. A number of AS events have been reported to occur in plants in response to biotic and abiotic stresses. Genome-wide studies demonstrate that various environmental stresses change AS profiles and increase the number of alternatively spliced transcripts in *Arabidopsis* [5, 7]. Some of the alternatively spliced transcripts were induced in response to biotic stresses and were shown to encode regulatory proteins with important functions in plant pathogen response pathways. These regulatory genes, whose mRNAs are alternatively spliced in response to pathogen attack, include pathogenesis-related (PR) genes, such as chitinase [29]; defense-related genes, such as isochorismate synthase [30]; genes encoding cyclotides-defence peptides with insecticidal and antimicrobial activities [31]; the plant resistance (R) genes encoding nucleotide-binding site-leucine-rich repeat (NBS-LRR) proteins, which are responsible for the intracellular detection of pathogen-related elicitors [22, 32, 33].

In response to abiotic stresses, AS also affects a range of regulatory genes that are presumed to function in abiotic stress adaptation of plants. These genes include mitogen-activated protein kinases (MAPKs) [34–36]; several groups of stress-related transcription factors, for example, MYB and CBF/DREB transcription factors [23, 37–39]; ubiquitin ligases and transcription factors, which are considered to be involved in protein degradation in response to environmental cues [40, 41]; the serine/arginine (SR) proteins [42, 43], which are important splicing regulators in the plant development and stress responses [44]. The examples of AS events concerning regulatory genes involved in biotic and abiotic stress response and mentioned above were enumerated and discussed in detail in a recent review by Mastrangelo et al. [10].

Some other gene families playing important roles in plant adaptation to environmental stresses are reported to be alternatively spliced in plant cells, for example, dehydrin genes in *Vitis riparia* and *V. vinifera* [45], whose protein products accumulate in response to various dehydrating

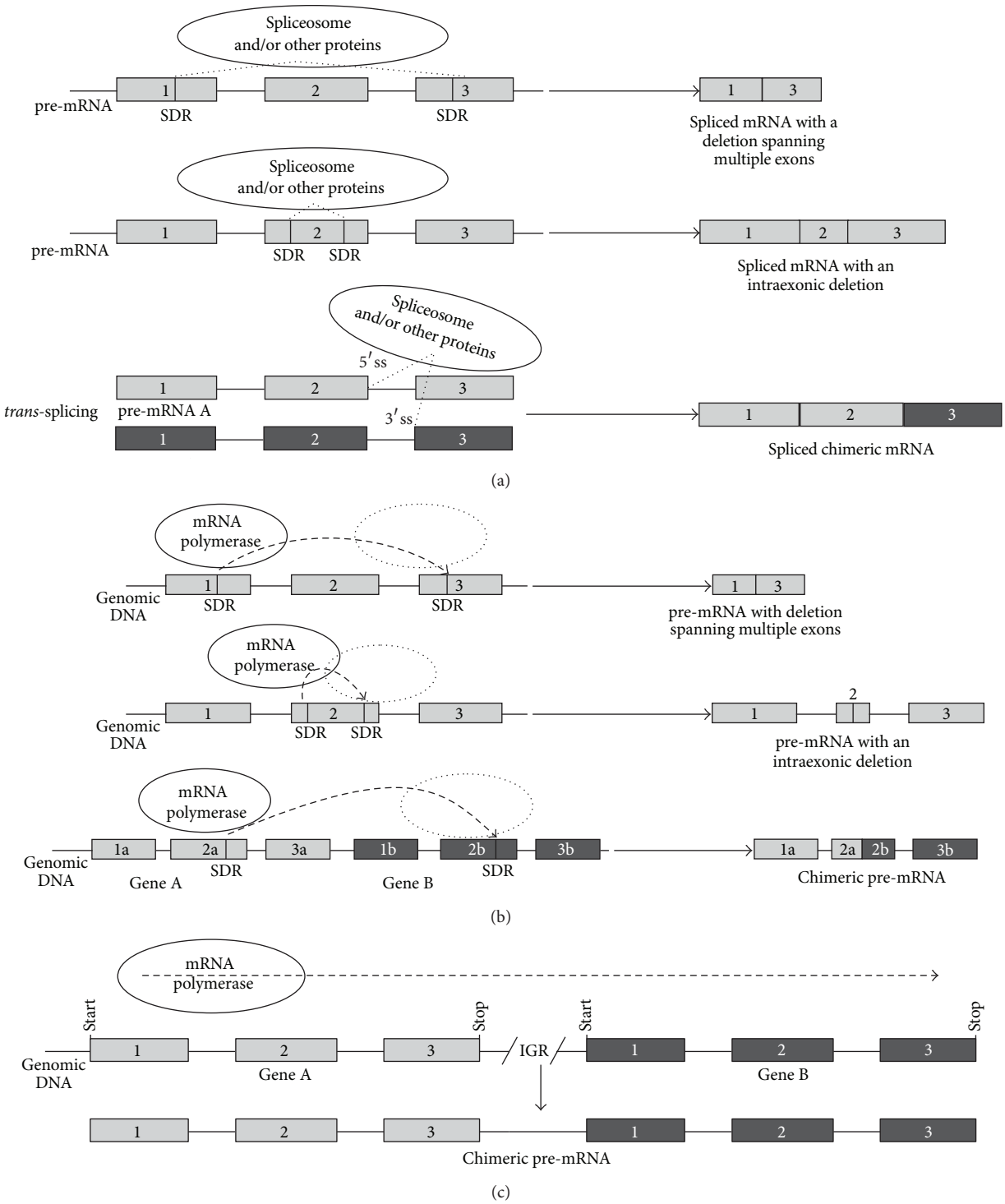

Figure 3: Hypothetical mechanisms of noncanonical pre-mRNA splicing-like events. (a) noncanonical alternative splicing; (b) transcriptional slippage; (c) polymerase read through. Exons are represented by grey or dark grey boxes, and introns—by horizontal solid lines between the boxes. Dashed lines above and below the exons and introns depict AS events. Dashed arrows above the exons and introns depict the direction of RNA polymerase movement. IGR—intergenic region.

stress conditions; potato invertase genes, which are responsible for cold-induced potato sweetening [46]; Δ1-pyrroline-5-carboxylate synthetase1 (*P5CS1*) gene, whose AS contributes to the natural variation in proline accumulation between different *Arabidopsis* accessories [47]; the genes of JASMONATE ZIM-domain (JAZ) proteins, which regulate jasmonic acid signaling in response to biotic stress [48]; the genes of wound-responsive RNA-binding UBA2 proteins [49]; the L-myo-inositol-phosphate synthase (*MIPS*) genes, induced in response to a variety of abiotic stresses [50]; the rice *Sultr* genes encoding sulphate transporters, which regulate sulphur status during stress conditions [51].

A considerable part of the AS events resulted in the occurrence of PTC carrying mRNAs which cannot not be translated into full-length proteins. As it is currently believed, such mRNAs can be either degraded by the NMD machinery or translated into truncated proteins lacking some active domains [10]. There is growing evidence that AS coupled to NMD not only serves to eliminate aberrant mRNAs but also plays a certain role in plant stress response (see Section 4 of the present review). There are several studies demonstrating important biological role of truncated proteins in plant stress adaptation. For example, the transcripts of plant R genes are alternatively spliced to produce truncated protein forms with different combinations of functional domains whose existence has been confirmed by Western blotting and other methods [22, 32], and it has been shown that the truncated proteins induce hypersensitive response-like cell death and, thus, help plants combat bacterial pathogens [22]. Special attention attracts the report by Matsukura et al. [23] that truncated nonfunctional transcription factors OsDREB2 were generated in the absence of stress in rice; however, when stress conditions were applied, the functional full-length *OsDREB2* transcript was generated via stress-inducible alternative pre-mRNA splicing and then performed its function to activate expression of stress-responsive genes. Thus, it is possible that generation of a nonfunctional protein in the absence of stress allows plants to avoid the detrimental effects and high metabolic cost for producing the proteins conferring resistance to biotic and abiotic stresses. Mastrangelo et al. [10] also suggest that generation of a full-length protein in response to stress by changing splicing pattern of the corresponding gene could prevent spending time on transcriptional activation and accumulation of the necessary mRNAs.

A number of studies describe extensive AS in plant genes when splicing events affected multiple exons in response to environmental stress. The phenomenon of extensive missplicing in plant genes has been first described for the In1 and Vp1 transcription factors in maize and wheat, respectively [52, 53]. R1/b1 transcription factors, including In1 transcription factor, control anthocyanin biosynthesis, which have been shown to protect plant tissues from photoinhibition, or high-light stress [54]. The In1 transcription factor encodes a repressor of anthocyanin biosynthesis in maize seeds [52]. The missplicing events resulted in the production of a nonfunctional In1 protein. This might be a mechanism ensuring that normal anthocyanin production was not suppressed. McKibbin et al. [53] demonstrated that extensive missplicing of wheat Vp1 transcription factor transcripts contributes to the susceptibility of wheat to an environmentally triggered disorder, preharvest sprouting. The disorder occurs when grains mature under cool and damp conditions. There was a variety of deletions of different length in several exons of the gene which resulted in the loss of a functional domain, introduction of PTCs, and shifted reading frame.

Filichkin and Mockler [12] demonstrated that extensive unproductive AS is a widespread phenomenon, which frequently generates mRNA isoforms harboring in-frame PTCs, among plant circadian clock genes. Plant circadian clock and abiotic stress are regarded as firmly interconnected processes [55]. Filichkin and Mockler [12] reported that the relative ratios of the PTC carrying mRNAs encoding for several key circadian clock regulators can be considerably shifted under abiotic stress conditions. In particular, their results indicate a potential role of extensive AS and nonsense transcripts of the CCA1/LHY-like subfamily of MYB transcription factors in the regulation of circadian rhythms. According to Yuan et al. [30], isochorismate synthase of *Populus trichocarpa* encoded by a single gene undergoes extensive AS, and this results in the generation of at least 37 splice variants where intron retention and alternative acceptor splice sites predominated as AS types. A significant portion of the transcripts was generated by several types of AS events and resulted in the loss of multiple exons. Similarly to the phenomenon described by Filichkin and Mockler [12], the majority of the transcripts contained PTCs and might be targeted for degradation via NMD.

Taken together, these data indicate that AS does not represent "noise" of the cellular stress. It is rather a mechanism playing an important role in plant adaptation to unfavorable environmental conditions through degradation of specific mRNA transcripts or generation of new truncated protein forms. This class of events can be classified as expansion of molecular basis for search of new ways of adaptations. The effect of AS on plant stress response is probably still underestimated, and a number of not yet known AS-based mechanisms are likely to play a role in the plant adaptation to adverse environmental conditions.

3.2. Noncanonical AS and AS-Like Events in Plant Stress Response. There are several studies where extensive missplicing of plant stress-responsive gene products has been reported [11, 24, 56–61]. The missplicing events often included intron retention; exon skipping, alternative $5'$ and $3'$ splice sites selection (Figures 1(b), 1(c), and 1(d)) and were coupled to the occurrence of multiple noncanonical pre-mRNA processing events, which resembled alternative splicing (Figures 2(a), 2(b), and 2(c)).

Several cases of such unusual posttranscriptional pre-mRNA processing have been recently reported for choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH) genes in rice [24, 56]. The SDR-mediated unusual processing of *BADH* transcripts was also detected in other cereal crops, including maize (*Zea mays*), wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*) [56]. CMO and BADH are responsible for glycine betaine (GB)

synthesis in plants, which are capable of synthesizing this compound, so-called GB accumulators [62]. GB, a quaternary ammonium solute, is a metabolite playing a crucial role in developing osmotic tolerance [63]. Rice is defined as non-GB accumulator, since there is no evidence of detectable GB accumulation in rice plants [64]. Luo et al. and Niu et al. [24, 56] have shown that most rice *CMO* and *BADH* transcripts are processed incorrectly, generating transcripts with retained introns, with a variety of deletions of coding sequences, or with insertions of exogenous gene sequences. The unusual deletion events occurred at the SDR sequences which were present at $5'$ and $3'$ splicing junctions that were distinct from conventional (U2/U12-type) splicing boundaries.

Luo et al. and Niu et al. [24, 56] proposed that the SDRs are required for the recognition of the deletion sites in response to stress conditions. The unusual SDR-mediated posttranscriptional processing events included partial exon deletions, exon fragment repetitions, deletions affecting multiple exons (Figure 2(a)), intraexonic deletions (Figure 2(b)), and generation of chimeric mRNAs (Figure 2(c)), which resulted in either partial loss of an exon or unusual exonic sequence rearrangements within or between RNA molecules. These noncanonical mRNA modifications were coupled to extensive AS events of common types, such as intron retention or selective exon inclusion (Figures 1(b) and 1(e)). According to Niu et al. [56], the site selection for the deletions/insertions was altered in response to the stress conditions. At the protein level, canonical and noncanonical splicing events resulted in the removal of the translation initiation codon, deletion of a functional domain, and frameshifts with premature termination of translation by introducing PTCs. Taken together, these findings indicate that a lack of precise *CMO* and *BADH* mRNA transcripts contributes to the variation of GB-synthesizing capacities among various plant species and the absence of GB in rice [24, 56]. In support of the data, Fan et al. [57] described similar extensive missplicing of the rice VP1 transcription factors which contribute to the development of stress-mediated preharvest sprouting disease in rice. The SDR sequences were again present at the junctions of the unusual excision sites in the *VP1* splice variants.

Using the GenBank database, Niu et al. [11] predicted that 24 plant candidate genes involved in diverse functional pathways and belonging to both monocots (wheat, barley, and maize) and dicots (*Arabidopsis* and tobacco) potentially possess SDR-mediated posttranscriptional processing. These potential SDR-mediated processing events were experimentally detected by the authors using the known SDR-containing sequences as probes for RT-PCR and subsequent DNA sequencing. The authors also demonstrated that the paired presence of SDRs is necessary but not sufficient in SDR-mediated splicing in transient and stable transformation systems.

The sunflower *sf21C* gene is a member of a small plant gene family related to the human N-myc downstream-regulated gene family (NDRG), which is involved in stress and hormone responses [65, 66]. Lazarescu et al. [58] characterized 20 splice isoforms of the *sf21C* gene and reported that five identified variants were generated by splicing at novel splice sites, different from those classically recognized by U2- and U12-type spliceosomes. These non-canonical splice sites were again represented by the SDR sequences. In addition, twelve transcript variants contained PTCs, which resulted in frameshifts and possible translation of truncated proteins of different length and structure. Although the *sf21C* transcripts with PTCs are potential candidates for NMD, they were abundant and occurred in distinct combinations in some sunflower organs and, therefore, the transcripts could be translated into truncated proteins and exert a certain biological function. The authors suggest that the structural modifications of the proteins may affect their compartmentalization (nucleus or other cell organelles) or binding properties.

Zou et al. [59] described a similar case of extensive AS with noncanonical splice variants when studying the structure of the prolyl 4-hydroxylase (*P4H*s) genes and their expression patterns in maize seedlings under waterlogging. In plants, P4Hs are suggested to function as oxygen sensors under hypoxia stress [67, 68]. Zou et al. [59] found that *ZmP4H* genes displayed different expression patterns under waterlogging, and many *P4H* transcripts were spliced using nonconventional splicing sites at the exon/intron junctions containing SDRs. Many of the *P4H* splice variants contained PTCs. The authors propose that the splicing events might be important in the regulation of *ZmP4H* genes under waterlogging stress in plants.

A new type of a noncanonical intron, again with SDR sequences at $5'$ and $3'$ splice sites, was found in some of the alternatively spliced transcript variants of the R2R3-MYB transcription factor in *Arabidopsis* and rice [60]. Thirty-eight *Arabidopsis* and rice genes were found to have this type of noncanonical intron, and most of the genes were suggested to undergo AS. In addition, a noncanonical intron flanked by SDRs has been reported for *FCA* [61], an *Arabidopsis* gene controlling flowering time, which is a stress-dependent event. The authors detected four transcript variants of the *FCA* gene. Transcript δ, representing approximately 10% of the *FCA* transcripts, was alternatively spliced at one of its introns which was flanked by two 6 bp repeated sequences. The authors also noted that neither of the repeats incorporates canonical splice sites.

It is well established that protein folding in the endoplasmic reticulum (ER) is disturbed in plants upon environmental stress [69]. The Unfolded Protein Response (UPR) is a cellular stress response mechanism which is activated in response to the accumulation of unfolded or misfolded proteins in the lumen of ER and is aimed to alleviate the ER stress by arresting protein translation and activating signaling pathways to increase the production of molecular chaperones [70, 71]. It has recently been established that in response to the ER stress the IRE1 kinase catalyzes unconventional splicing of the bZIP60 transcription factor to produce active form of the protein in *Arabidopsis thaliana* [72]. The active bZIP60 transcription factor moves to the nucleus and activates transcription of the genes responsible

for the UPR. During the ER stress response, a 23 nt intron is spliced out from the *bZIP60* mRNA (Figure 2(d)). The intron excision leads to a frameshift and a PTC (Figure 2(d)), and this, in turn, generates a different amino acid sequence in the C-terminal region of the protein. Due to the splicing event, the bZIP60 protein loses the domain that anchors it on the ER membrane. The orthologue of the bZIB60 transcription factor in *Oryza sativa*, OsbZIP74 (also known as OsbZIP50), has been shown to be similarly activated by the unconventional splicing leading to a frameshift [73, 74].

Currently, there is accumulating evidence that *trans*-splicing is a mechanism of modification of gene expression in mammals, flies, and nematodes [27, 75], which is to a certain part analogous to horizontal gene transfer, considering that it occurs at the molecular level and in a single cell. In a recent study, Zhang et al. [76] demonstrated the high complexity of the rice transcriptome using deep RNA sequencing at single base-pair resolution. Unexpectedly, the authors identified 234 putative chimeric transcripts (Figure 2(c)), containing parts of two different genes, which seem to be produced by *trans*-splicing (Figure 3(a)). However, there is also a likelihood that chimeric mRNAs might be produced by a mechanism that does not involve participation of a spliceosome, for example, via transcriptional slippage (Figure 3(b)) or read-through transcription (Figure 3(c)).

Although there is compelling evidence for a relatively high number of *trans*-splicing events in mammals, flies, and nematodes [27, 75], there is scarce information on the occurrence of fused transcripts of such origin in plants. To our knowledge, there is clear evidence for only two *trans*-splicing events observed during transcription of plant genes. Kawasaki et al. [77] demonstrated that two RNAs were independently transcribed from the *SPK-A* and *SPK-B* genes of *O. sativa* and joined in a chimeric mRNA, possibly by *trans*-splicing. The rice *SPK* genes encode calcium-dependent protein kinases (CDPKs), which are known to play an important role in mediating plant biotic and abiotic stress responses [78]. He et al. [79] have found and provided evidence that the nodule specific *MsHSF1c* transcript of a heat-shock transcription factor may be generated by *trans*-splicing mechanism. In addition, in our research, we identified extensive noncanonical splicing-like events, including intraexonic deletions, an insertion of extraneous 35 bp sequence of unknown origin in the kinase domain of grape *CDPKs*, or generation of chimeric *CDPK* transcripts, when analyzing expression of the *CDPK* genes in cell cultures of *V. amurensis* and Panax *ginseng* with different levels of tolerance to salt stress [80, 81]. Similar posttranscriptional modifications were also observed for *CDPK* genes in somatic embryos of *P. ginseng* [82, 83]. Brummell et al. [84] provided evidence for the generation of chimeric transcripts encoding potato invertase inhibitors and their active accumulation in response to cold stress. The potato invertase inhibitors are known to prevent invertase-induced hexose accumulation in potato tubers (potato sweetening) in response to low temperatures. However, *trans*-splicing is probably not the case for the chimeric mRNAs of the invertase inhibitor, since these mRNAs are generated using noncanonical splice sites represented by SDRs.

In summary, these experimental data suggest the existence of molecular mechanisms that do not follow conventional notion of splice site selection in plant AS and indicate the involvement of the noncanonical splicing and splicing-like events in plant stress responses. Further research is needed to uncover mechanistic consequences of the presence of SDRs in a variety of plant stress-related genes and physiological roles of the SDR-mediated noncanonical splicing-like events.

4. The Role of AS Coupled to NMD in Plant Stress Response

NMD surveillance machinery is a mechanism that identifies cellular mRNAs carrying PTCs and targets these mRNAs for degradation, thereby preventing accumulation of truncated proteins, which probably could have deleterious effects on the cell metabolism (for recent reviews on NMD mechanisms, see [85–87]. Briefly, NMD is triggered by exon junction complexes (EJCs; components of the assembled ribonucleoprotein particles) that act during the mRNA splicing process and recruit the key NMD *trans*-acting protein factors, UPF proteins, thereby leading to formation of functional NMD complexes and rapid degradation of the PTC-containing mRNA. In plants, both introns and long $3'$-UTRs operate as *cis*-acting elements, which are recognized by the *trans*-acting NMD factors. NMD is a widespread phenomenon and is conserved among different eukaryotes, including plants [21]. Genome-wide studies revealed that up to 20–30% of the *Human*, *Drosophila*, and *Caenorhabditis* mRNA transcripts can be targeted to degradation via NMD [88–90]. Surprisingly, according to a recent genome-wide mapping of AS in *A. thaliana* [5, 13], alternatively spliced transcript isoforms with PTCs comprised the majority (~45–78%) of alternatively spliced transcripts in the analyzed *A. thaliana* transcriptome. About 13–18% of intron-containing genes are potentially regulated by AS coupled to NMD [13]. Although little is known about the functional roles of NMD in plants, there is increasing evidence that AS coupled to the NMD-mediated mRNA degradation not only removes aberrant mRNAs but also acts as a mechanism to regulate gene expression, including expression of genes playing essential roles in plant stress adaptation.

Filichkin et al. [5] demonstrated that the relative ratios of the PTC containing mRNAs can be significantly shifted for several key regulatory genes under abiotic stress treatments in *A. thaliana*. At present, a number of genes known to be involved in stress response pathways in plants have been shown to undergo AS coupled to NMD. Palusa and Reddy [91] provided evidence that a significant part of PTC containing splice variants of the SR proteins, which are known to regulate plant AS in response to environmental stress, are subject to degradation by the NMD mechanism. Sugio et al. [92] showed that the specific heat shock factors (HSFs), notably HSF2A, are also regulated by AS coupled to NMD. NMD-deficient *Arabidopsis* mutants were shown to possess constitutive pathogenesis-related (PR) gene expression, salicylic acid (SA) accumulation, and increased resistance to

pathogens [93]. Furthermore, a recent study by Rayson et al. [94] has found that the majority of NMD-targeted transcripts in *A. thaliana* mutants deficient in NMD are associated with response to pathogens. Those NMD mutants, where the NMD-targeted transcripts were elevated, exhibited partial resistance to *Pseudomonas syringae*. This finding indicates that gene expression regulation via AS coupled to NMD possesses specific biological roles. Rayson et al. [94] proposed that plants may employ NMD-controlled gene expression as a means to coordinate pathogen responses. The findings by Rayson et al. [94] suggest that understanding the biological consequences of AS coupled to NMD is prospective and necessary for developing new approaches in crop protection.

In a recent genome-wide study, Kalyna et al. [13] showed that AS coupled to NMD affects the transcript abundance and thus regulates expression of many stress-responsive genes in *A. thaliana*, including transcription factors (e.g., At-Di19-5 and Zn finger B-box type protein), protein kinases (e.g., CRK18, CPK28, the SNF1-like protein kinase, and AtKIN11YY), calcium sensors (e.g., SOS2 and SOS3/CBL4), various temperature, drought and salt response factors (e.g., SRF2 and HSF2A), and some other genes involved in stress adaptation. Unexpectedly, Kalyna et al. [13] have also found that a considerable part of the alternatively spliced transcripts retaining introns, which are known to be the most common AS type in plants [5, 6], do not trigger NMD even though they possess PTCs and other classical NMD-inducing features. Thus, the question of the destiny and biological roles of the PTC containing transcripts in plant stress response and other cellular processes remains open. It is highly likely that the intron-retaining transcripts, which are not targeted to NMD, are translated into truncated proteins playing essential roles in plant development, metabolism, and stress adaptation.

There is compelling evidence that AS coupled to NMD regulates expression of plant genes involved in plant stress response. Although the precise roles for the phenomenon are not known, it appears that unproductive, in terms of protein translation, AS is exploited by plants to adapt to changing environmental conditions. Further research is needed to clarify the involvement of AS coupled to NMD in plant stress responses.

5. Rare Noncanonical Transcriptional Events or Artifactual RT-PCR "Splicing"?

It has long been known that during reverse transcription of the retroviral genome, reverse transcriptase makes a number of template switches in a homology-dependent manner, which is necessary for the retroviral viability and variability [95, 96]. During reverse transcriptase template switching, the growing cDNA strand can dissociate from the RNA template and reassociate with a homologous region on the same RNA template (intramolecular switching) or with a homologous region on a different RNA template (intermolecular switching) thus implementing the *cis*- and *trans*-events, respectively. The homologous regions are usually represented by short 8 nt long direct repeats. Not surprisingly, during cDNA synthesis *in vitro* reverse transcriptase can also switch from one template to another and, thereby, generate artificially deleted or chimeric cDNA transcripts [96–99]. Thus, during intramolecular reverse transcriptase template switching, cDNAs bearing artificial deletions are generated [97]. These cDNAs can be misinterpreted as alternatively spliced mRNA transcripts. Intermolecular template switching often results in the synthesis of artificial chimeric cDNAs, which can be misinterpreted as *trans*-spliced mRNAs [99].

A number of studies provided clear evidence that some deleted or chimeric cDNAs, previously identified as alternatively spliced or *trans*-spliced, are artifacts of reverse transcription and are often present in cDNA databases [97, 99–103]. For example, Cocquet et al. [97] searched for the template-switching artifacts in cDNA databases by scanning a collection of human splice sites (Information for the Coordinates of Exons, ICE database). The authors discovered that artifacts represent a significant portion of the noncanonical "spliced" transcripts deposited in the database. These artifactual transcripts did not possess canonical splice signals and contained SDR sequences instead, which indicates that these cDNAs could be examples of the reverse transcriptase switching. According to Cocquet et al. [97], the template-switching events were also present, but rare, among transcripts with canonical splice sites. The authors experimentally confirmed several cases of the putative template switching events. The observations by Delviks and Pathak [104], Mader et al. [101], and Cocquet et al. [97] demonstrate that long sequences between the SDRs and the propensity of the sequences to form stem-loop secondary structures both increase the probability of onset of template-switching events.

Houseley and Tollervey [99] recommend applying methods which do not include reverse transcription in order to determine whether a truncated or chimeric transcript is a true splicing event or an artifact. *In vitro* transcription, northern blotting or RNase protection assay can be used for this purpose [97, 99]. Also, it is possible to use a reverse transcriptase enzyme with increased thermal stability, since the reverse transcriptase template switching is usually not detected anymore in this case [97, 99]. However, according to Houseley and Tollervey [99], using a heat-stable reverse transcriptase does not completely eliminate the probability of reverse transcriptase template switching.

Although there is compelling evidence for the capability of reverse transcriptase to generate artifactual splicing-like products *in vitro*, it appears reasonable to admit that there is also likelihood that the noncanonical splicing-like events in the plant stress-responsive genes are generated via the transcriptional slippage mechanism or a noncanonical AS process *in vivo*. In case of transcriptional slippage, RNA polymerase might dissociate from the DNA template *in vivo* similarly to reverse transcriptase. Support for this assumption may be found in a recent genome-wide study by Ritz et al. [26] demonstrating that the transcriptional regulation is much more complex than previously believed and that the rare unusual splicing events, including deletions affecting parts of adjacent exons, intraexonic deletions, and

chimeric transcripts, were not artifacts of PCR, sequencing, or reverse transcription. The authors provided evidence for the existence of such noncanonical transcripts not only in the human tissue but also in mouse, rat, and zebrafish. They proposed that the transcript variants were generated as a result of intramolecular slippage. Recently, Zhang et al. [76] conducted a comprehensive study of the rice transcriptome using high-throughput paired-end RNA-seq and found a large number of chimeric transcripts which appeared to arise as a result of *trans*-splicing. The chimeric RNAs showed partial alignment to two genes, which often are present on different chromosomes. Unfortunately, the RNA-seq approach again employs reverse transcription to prepare cDNA library and sequence a transcriptome, and therefore care should be taken when arriving at a conclusion on the RNA-seq results. Nevertheless, several cases of the unconventional splicing-like events using noncanonical splice signals in the plant stress-related genes were verified using such alternatives to RT-PCR as RNase protection assay or *in vitro* transcription (e.g., [61, 68, 77, 79]).

Since the noncanonical AS events in the plant stress-responsive genes in the majority of experiments have been identified using RT-PCR and subsequent sequencing of the amplified RT-PCR products (e.g., [11, 24, 56, 58, 59]), there is likelihood that at least some of the reported alternatively spliced transcripts were artifacts of reverse transcription. It is particularly likely for the noncanonical splicing events, where SDRs are present instead of the canonical splice signals. Thus, one should admit that the phenomenon of reverse transcriptase template switching may hamper the identification of truly alternatively spliced mRNAs, and it raises the question of what portion of the identified noncanonical splicing and splicing-like events in plant stress-related genes is genuine.

Numerous stipulations of authors of primary investigations, which we conserved deliberately in our review, seem to cast doubt on the very existence of such complex events in plants and other higher organisms. However, the validity of search in this direction is based on the following reasons.

(i) The first and most general reason arises from the notion about the hierarchical self-similarity of biological world. As far as we know, the mixing and rearrangement of genetic material are key events in living systems development (see, e.g., [105–107]), so their occurrence can be expected at the all levels of presentation of biological world. Indeed, symbiogenesis and horizontal gene transfer events correspond to taxon level of presentation and sex reproduction, chromosome pairing, and crossover-to the organismal, chromosomal, and gene levels, respectively. The life is very sophisticated in its invention of new devices and representations: a recently discovered human gut microbiome with its collective gene set is a good example. So the mixing of more small fragments of genetic material (including mixing and rearrangement via *cis*- and *trans*-splicing) seems reasonable to correspond to subgene level.

(ii) The NMD mechanism occurrence in higher organisms is well proved, and this fact supposes the availability of substrate for its operation. Noncanonical splicing can supply the NMD mechanism with this substrate.

(iii) The results of *in vitro* experiments (where chimeric cDNAs synthesized by switching of reverse transcriptase were similar to splicing products) compel one to search the analogy in native cell. The full analogy seems to be impossible since there is no clear evidence of significant activity of reverse transcriptase in the healthy plant cells. However, some quantities of extragenomic DNA can appear supposedly in a plant cell in some way, for instance, in a way similar to that in which the fetus DNA has appeared in maternal plasma [108]. This extra DNA being modified in aggressive surroundings could serve as matrices for synthesis of unusual transcripts. However, the plasma system analog in plants, apoplast, represents an effective barrier for long-distance DNA transport [109]. The mobile small RNAs can move from cell to cell inducing gene silencing and some other effects [110], but these molecules are too small to imitate the products of noncanonical splicing. So, the splicing hypothesis is the simplest for *in vivo* context.

(iv) Finally, the unusual transcripts were detected in plant cell, and their contents were shown to depend on the physiological state of cells. This dependence is in agreement with logics about the behavior of plant in the stress conditions, whereas no stress correlations can be expected in the case of method errors.

6. Conclusion

On reviewing the literature it may be stated that environmental stresses have an impact on AS, which, in turn, affects plant stress responses and might promote plant stress tolerance to adverse environmental conditions. AS enriches the response capacity of cells by enabling them to synthesize structurally and functionally different proteins from a gene and contributes to the complexity of both transcriptome and proteome to increase the survival potential of a phenotype in various physiological conditions. Much remains to be discovered about the molecular mechanisms of the various AS and AS-like events that increase the plasticity of plant transcriptome and proteome in such a tremendous rate. In addition, it is intriguing why plants spend so much energy to produce numerous "unproductive" splice variants containing PTCs and then just "kill" them in the recently discovered NMD degradation pathway. This might represent a still obscure but important evolutionary strategy of living to substitute in parts the natural selection of phenotypes by molecular selection of genetic elements. Studies of the transcript diversity generated in response to environmental stresses as well as uncovering-specific biological consequences of some principal splicing events are perspective for plant biotechnology in terms of developing new strategies for crop breeding and protection.

Abbreviations

AS:	Alternative splicing
BADH:	Betaine aldehyde dehydrogenase
CMO:	Choline monooxygenase
GB:	Glycine betaine
ORF:	Open reading frame
NMD:	Nonsense-mediated mRNA decay
pre-mRNAs:	Intron-containing precursor-mRNAs
SDRs:	Short direct repeated sequences
PTCs:	Premature stop codons
RNA-seq:	Illumina RNA sequencing.

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