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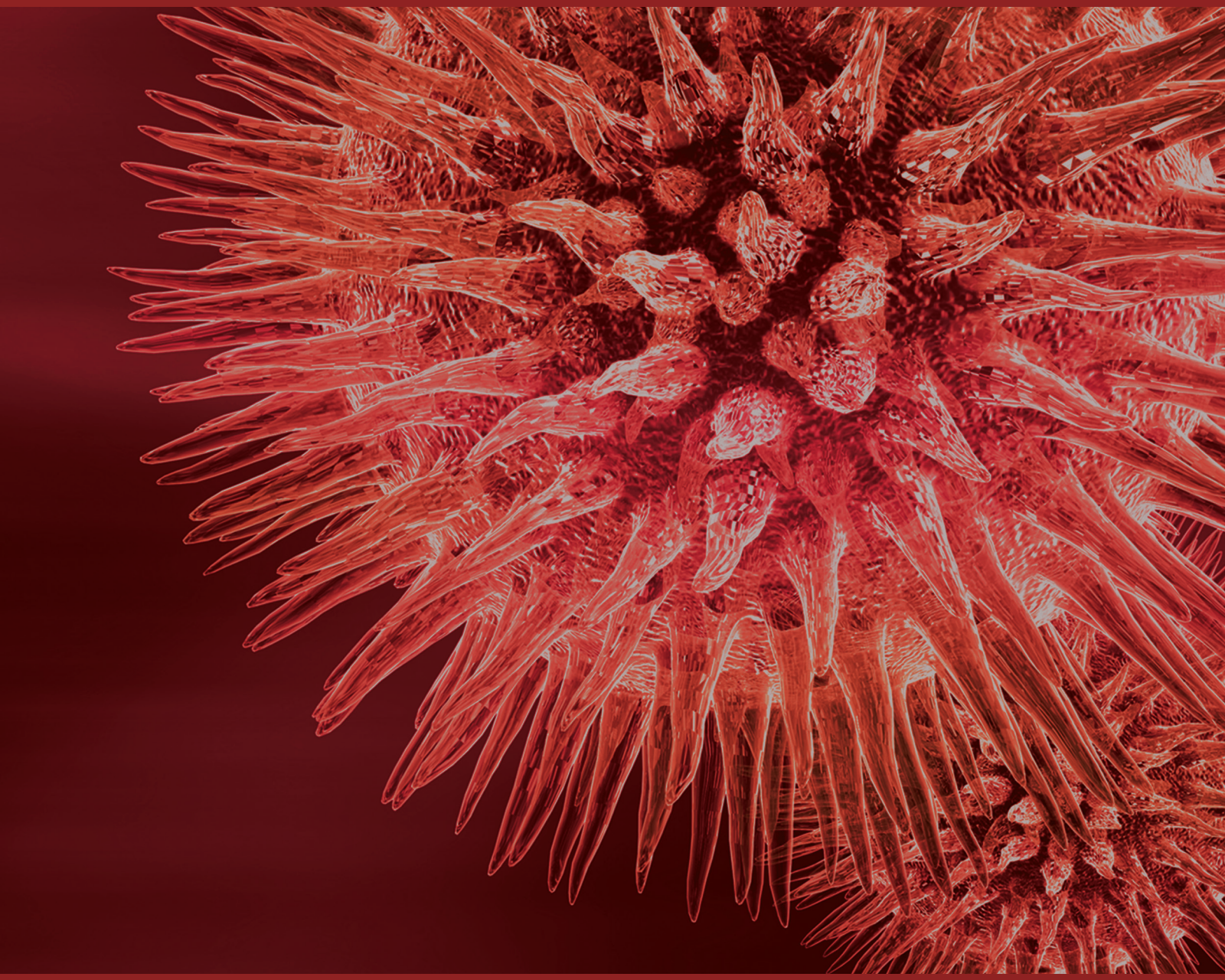
Guest Editors: Hatem Rouached, Sikander Pal, Shimon Rachmilevitch, Marc Libault, and Lam-Son Phan Tran



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Editorial

Plants Coping Abiotic and Biotic Stresses: A Tale of Diligent Management

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Plants unlike other living forms are sessile thereby facing severe biotic and abiotic stresses. Plants have evolved different efficient defence responses which thrive upon a number of intrinsic factors, such as genotypic and phenotypic constitutions and developmental circumstances, and extrinsic factors like severity and duration of the stresses. Stress management uses molecular and biochemical level controls, the competence, and speed, at which a stress signal is perceived and transmitted to generate stress signal molecules and activate stress-protective mechanisms. A well-concerted action of the plants' competence at morphological, physiological, biochemical, and molecular strata regulates numerous adaptive responses to biotic and abiotic stresses. Genetic manipulations of signalling networks have been widely used to improve plant productivity under stressful conditions. Advanced biotechnological application will enable maintaining agriculture in a sustainable manner. In this special issue, we present two reviews and four research papers which address genomic, molecular, and physiological regulations as well as signalling networks dealing with plant responses to abiotic and biotic factors.

Climate change, desertification, and the rise in human population have put a severe load on agriculture and are deteriorating crop productivity. In recent years, numerous molecular and metabolic pathways involved in plant responses and adaptation to various types of environmental stresses have been identified and reported. Among hundreds of metabolic pathways identified, the role of polyamines in stress management to enhance plant acclimation and adaptation is emerging rapidly. In this special issue, a timely review by P. Rangan et al. (2014) summarizes our knowledge on biosynthesis and catabolism of polyamines and highlights recent progress in elucidating the functions of polyamines in regulation of plant responses to abiotic stresses. Given a huge genetic variation among plant species, the authors also discuss a systematic approach based on polyamine-mediated enhancement of stress tolerance which might be used as a potential strategy for screening and identification of natural variants within existing crop species. The identified genotypes that possess compatible allelic variants could be then used for the improvement of stress tolerance.

Plant-microbe interactions are at the core of symbiotic, parasitic, or mutualistic plant-microbe relationships. These interactions have displayed a unique way of mutualistic communications for a resource sharing. To shed light on the topic, a detailed review of M. Libault (2014) explains

the unique mechanisms in using legumes to interact with bacteria. Leguminous plants have developed a mutualistic symbiotic relationship with rhizobium (a type of soil bacteria). Upon bacterial infection, a new root organ called nodule is developed that enables the leguminous plants to access a steady source of nitrogen through the fixation and assimilation of the atmospheric N_2 by the symbiotic bacteria. In return, the bacteria also get benefit from the symbiotic plants that provide photosynthesis product to bacteria as source of carbon. Environmental stress or climate change, which influences the concentration of the atmospheric carbon dioxide (CO_2), will have a significant impact on plant photosynthesis. As a consequence, this will affect the nitrogen and carbon metabolism, leading to altered nitrogen fixation efficiency. The key regulatory mechanisms controlling carbon/nitrogen balances with particular attention to legume nodulation are reviewed by coeditor M. Libault in his review article. In addition, readers can also get an overview about the effect of the change in CO_2 level on nitrogen fixation efficiency through this review, giving rise to idea as to how we could mitigate the impact of the change in atmospheric CO_2 concentration.

Besides the change in atmospheric CO_2 level, water deficit is one of the major constraints for nodulation, and, as a consequence for plant productivity. This topical issue is presented here by a research article of S. Sulieman et al. (2014). In their study, the authors assessed the growth and nodulation attributes of two soybean varieties DT2008 and Williams 82 (W82), which have contrasting drought-tolerant capacity, in a symbiotic association with *Bradyrhizobium japonicum* under drought and subsequent rehydration. The authors aimed to understand the correlation between N_2 fixation efficiency and differential drought-responsive phenotypes of DT2008 and W82. Their results also provide genetic resources and basis foundation for further genomic research that would lead to better understanding of mechanisms involved in regulation of N_2 fixation in soybean under drought at molecular level.

Heavy metal pollution has been a matter of grave concern. Until recently, efforts have been mainly restricted to phytoremediation of soils using plant species with high metal uptake capacity such as *Brassica* species. Chromium (Cr) is a highly phytotoxic heavy metal affecting crop productivity and human health via entering the food chain. Phytoremediation of Cr-polluted soils has been mostly demonstrated in using herbaceous plants, whereas use of cotton cultivars in Cr phytoremediation is least explored. In the present issue, M. K. Daud et al. (2014) have shown the potentials of two transgenic cotton cultivars (J208 and Z905) and their hybrid line (ZD14) in Cr phytoremediation using a multiple biomarker approach. Their work showed that these cotton cultivars and hybrid line could effectively uptake and sequestrate Cr in dead parts of the plants, such as vacuole and cell wall, besides having a more highly accelerated antioxidant system. This study thus proposes a new role of cotton cultivars in phytoremediation of Cr-polluted soils.

The interactions between macro- and micronutrient homeostases have been reported in many physiological and nutritional situations. N. Bouain et al. (2014) studied the interaction between phosphate (Pi) and zinc (Zn) homeostasis in two lettuce varieties. The results revealed that the variation in Pi and Zn supplies affects the biomass and photosynthesis as well as the dynamics of Pi transport in a contrasting manner between the two varieties, indicating a genetic basis for such physiological responses. On the basis of their results, the authors proposed a working model of how Pi and Zn signalling pathways are integrated into functional networks to control plant growth.

Salinity is a major abiotic stress worldwide claiming agriculture lands and affecting productivity. Research paper by A. Matsui et al. (2014) investigated salt stress in *Arabidopsis*. The authors reported that the tasiRNA-ARF pathway is involved in controlling floral architecture in plants under drought and high salinity. The ta-siRNA biosynthesis mutants and mutated *ARF3*-overexpressing plants showed short stamen under drought and salt stress, which hampered self-pollination. This study reports for the first time that a type of ta-siRNAs (tasiRNA-ARF) plays an important role in maintaining normal stamen growth and fertilization under drought and high salinity.

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Research Article

DT2008: A Promising New Genetic Resource for Improved Drought Tolerance in Soybean When Solely Dependent on Symbiotic N_2 Fixation

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Water deficit is one of the major constraints for soybean production in Vietnam. The soybean breeding research efforts conducted at the Agriculture Genetics Institute (AGI) of Vietnam resulted in the development of promising soybean genotypes, suitable for the drought-stressed areas in Vietnam and other countries. Such a variety, namely, DT2008, was recommended by AGI and widely used throughout the country. The aim of this work was to assess the growth of shoots, roots, and nodules of DT2008 versus Williams 82 (W82) in response to drought and subsequent rehydration in symbiotic association as a means to provide genetic resources for genomic research. Better shoot, root, and nodule growth and development were observed in the cultivar DT2008 under sufficient, water deficit, and recovery conditions. Our results represent a good foundation for further comparison of DT2008 and W82 at molecular levels using high throughput omic technologies, which will provide huge amounts of data, enabling us to understand the genetic network involved in regulation of soybean responses to water deficit and increasing the chances of developing drought-tolerant cultivars.

1. Introduction

Soybean (*Glycine max* (L.) Merr.) has been classified among the most important commercial oilseed crops worldwide [1]. It can substantially provide oils, micronutrients, minerals, and vegetable proteins suitable for livestock feed and human consumption. In addition, soybean has supplied materials for industrial uses, such as biodiesel, plastics, lubricants, and hydraulic fluids. Currently, world production of soybean is greater than any other oilseed crop. Globally, it accounts for approximately 68% of global crop legume production and 57% of world oilseed production [2]. Collectively, soybean production occupies around 6% of the world's available land [3].

As a leguminous plant, soybean has a superior potential capability to fix atmospheric N_2 in association with highly specialized soil bacteria. Under most conditions, soybean meets 58–68% of its nitrogen (N) demand through symbiotic association, but it can fulfill up to 100% with the aid of this vital process [4–6]. Moreover, a large portion of the fixed N can be readily accessible for the subsequent crops in the rotation systems or the natural ecosystems. Therefore, the soybean-rhizobia relationship represents a vital option to sustain agricultural development due to its superior N_2

fixation, enabling us to reduce the dependence on N fertilizers and thus avoiding the overexploitation of natural resources. Optimizing this association can upgrade soybean production and enhance soil fertility, whilst reducing the production costs and environmental impacts associated with N-chemical fertilizers [5]. Nevertheless, nodulating soybean plants growth and production are highly sensitive to adverse environmental conditions, particularly water scarcity in soils [7, 8].

In Vietnam, soybean occupies an important front position in the structure of agricultural crops throughout the country [9]. Recently, Vietnam's soybean production continues to fall well below the demand for food, feed, and vegetable oil industry. According to the 2012's statistical data, Vietnam imported 1.29 million metric tonnes of soybeans which represents a 26% increase over the previous year [10]. Due to high prices in the global market, soybean importation value had reached $776 million in 2012 (41% increase over the past year). Currently, the Vietnamese Government's Master Plan for Oilseeds has further development priorities for the sector with an objective of 350000 ha of soybean-cultivated land and a production of 700000 metric tonnes by 2020 (http://www.thecropsite.com/reports/?id=3701&country=VN). However, drought has a tremendous effect on soybean growth and development, thus negatively affecting the projected expansion of crop production [11]. In recent years, drought has occurred more and more commonly as a result of global warming and climate change [12]. Therefore, selective breeding for high drought-tolerant soybean cultivars and investigating the mechanisms to improve the drought tolerance of soybean have become top priority for many scientific researchers. On this basis, the soybean breeders at the Agriculture Genetics Institute (AGI) of Vietnam have initiated a long-term soybean breeding program to construct and release various drought-tolerant soybean cultivars through conventional breeding and radiation-induced mutagenesis. One of the newly developed prospective cultivars, the DT2008, revealed enhanced drought tolerance capability and yield stability (~2–4 metric tonnes per ha) under various field growing conditions [13]. Thus, we have started a joint project to fully characterize this cultivar under drought and various N regimes. Under nonnodulation conditions, we have recently documented that DT2008 has higher drought tolerance ability against the soybean reference cultivar Williams 82 (W82) [14].

In this report, we have extended our previous approach by comparing the drought-tolerant cultivar DT2008 and W82 based on their potential symbiotic association under drought and rehydration treatments. Results of this study demonstrated that DT2008 has a better drought tolerance and higher recovery level than W82.

2. Materials and Methods

2.1. Biological Materials. The soybean variety DT2008 was basically created by multiple hybridizations of local cultivars and subsequent irradiation with gamma rays Co^{60} – 18 Gy + F4 (DT2001/IS10) [15]. It has a wide adaptability to various harsh conditions and is suitably cultivated in 3 crops per year with a growth duration ranging from 110 to 120 days and relatively higher yield potentiality (2.5–4.0 tonnes/ha). In addition to its superior drought and thermotolerance, DT2008 has comparatively higher level of resistance against three kinds of diseases, namely, rust, downy mildew, and bacterial pustule [15]. Thus, in this study, we have intended to examine this promising drought-tolerant variety versus the widely used soybean reference cultivar W82 that was used to produce the reference genome sequence of soybean [16]. Accordingly, these materials would provide an efficient platform for omic analyses to identify new single nucleotide polymorphisms (SNPs) and promising candidate genes for genetic engineering. For testing the potential symbiotic capability under drought and rehydration conditions, both cultivars were inoculated with the microsymbiont *Bradyrhizobium japonicum* strain USDA110. Owing to its superior symbiotic N_2 fixation activity and full determination of its genome sequence, *B. japonicum* USDA110 has been widely used for the purpose of physiology, molecular genetics, and ecological studies [17].

2.2. General Plant and Bacterial Growth Conditions. Seeds of DT2008 and W82 were separately germinated in 6-litre pots containing autoclaved vermiculite as rooting substrate in a controlled greenhouse conditions (continuous 30°C temperature, 60% relative humidity, 12/12 h photoperiod, and 150 μmol m^{-2} s^{-1} photon flux density). Seeds were inoculated with *B. japonicum* USDA110 grown in yeast mannitol broth (YMB) (mannitol 2 g L^{-1}; yeast extract 0.4 g L^{-1}; K_2HPO_4 0.5 g L^{-1}; $MgSO_4 \cdot 7H_2O$ 0.2 g L^{-1}; NaCl 0.1 g L^{-1}; pH 6.8) for 48 h at 28°C. Cultures were diluted with water and added at a rate of ~10^8 cells mL^{-1} after the seeds were sown in the vermiculite. Plants were watered to field capacity three times a week with full-strength Herridge's nutrient solution [18] until the stress treatments were imposed. The basal nutrient solution contained 0.25 mM $CaCl_2$; 0.25 mM KCl; 0.5 mM $MgSO_4 \cdot 7H_2O$; 0.13 mM KH_2PO_4; 0.13 mM K_2HPO_4; 23.5 μM Fe (III)-EDTA; 71.5 × 10^{-2} mg L^{-1} H_3BO_3; 45.3 × 10^{-2} mg L^{-1} $MnCl_2 \cdot 4H_2O$; 2.8 × 10^{-2} mg L^{-1} $ZnCl_2$; 1.3 × 10^{-2} mg L^{-1} $CuCl_2 \cdot 2H_2O$; and 0.6 × 10^{-2} mg L^{-1} $NaMoO_4 \cdot 2H_2O$.

2.3. Drought and Recovery Treatments. Drought was imposed on 21-day-old plants by withholding water. The plants were randomly separated into two main sets (control and drought) containing four biological replicates each. Control (well-watered) plants were watered every day, whereas drought was imposed by withholding water for either 4 or 7 days (4 D or 7 D). Recuperation was carried out by rewatering the stressed plants for 3 days (7 D + 3 W). Both water-stressed (WS) and well-watered (WW) plants were harvested at set time points: 4, 7, and 10 D after the onset of drought-rehydration treatments. At each time point, soil volumetric moisture contents (VMC) were monitored using a HydroSense soil moisture probe (Campbell Scientific, Inc.). Measurements of various growth and nodulation parameters were performed at

the end of the stress and rehydration periods. At each harvest, plants were fractioned into shoots, roots, and nodules. Shoot and root tissues were dried at 65°C for a minimum of 48 h and weighed for dry matter (DM) determination.

2.4. Statistical Analysis. Means and standard errors (SEs) were used to plot figures and evaluate treatment responses. All statistical analyses were performed using statistical tools imbedded in Microsoft Excel 2010. The significance of difference between means was determined by Student's t-test where the values of $P < 0.05$ were considered significant and those of $P < 0.01$ and $P < 0.001$ were highly significant.

3. Results and Discussion

Drought is a recurring problem limiting nodulation and N_2 fixation in crop production particularly in tropical and semiarid tropical areas [19, 20]. Under the present scenarios of climate change, drought is more likely to occur, leading to ultimate growth and productivity reductions for most important economic crops, including soybean. Although access to irrigation can be used partially to alleviate drought impact, the usage of soybean drought-tolerant cultivars remains the most practically promising strategy to adopt. To cope with water deficit, drought-tolerant cultivars have developed a number of strategies that are genetically encoded [1, 9]. Thus, it is important to elucidate these striking adaptive mechanisms developed in such tolerant cultivars in order to improve the agronomic performance of soybean and other plant species by genetic engineering [21, 22]. Indeed, many physiological and biochemical responses to drought are shared amongst various tested plant species [23].

Our adopted strategy in the improvement of drought-tolerant cultivars is based on establishing an integrated approach involving conventional breeding and radiation-induced mutagenesis program and subsequently analyzing the internal adaptive mechanisms, which underlie plant responses to drought, through molecular biology techniques. A long-term, multidisciplinary research program was started to produce high-yielding adaptive cultivars for limited water conditions which exploited the drought-tolerant traits of DT2008. This biological resource was basically produced by multiple hybridizations of local cultivars and irradiation exposure [14]. A question was then raised whether DT2008 is able to maintain N_2 fixation at high level during drought, thus contributing to its improved productivity. To provide an answer to this question, in this study, we initially carried out a comprehensive comparative analysis between DT2008 and the reference cultivar W82, and whole genome was sequenced [16], thereby enabling us to identify potentially important mutations or SNPs responsible for enhanced N_2 fixation under drought in future molecular studies.

3.1. Plant Growth and Biomass Production are Less Negatively Affected in the Drought-Tolerant Cultivar DT2008. The alteration in biomass allocation is a principle strategy for coping with progressive soil-drying conditions [14]. Several groups have reported that DM partitioning is very important in the determination of soybean productivity [19, 24]. Total biomass has been used as a selection criterion for assessing drought tolerance in soybean. Understanding assimilation and allocation processes affected by water deficit is a fundamental prerequisite step in identifying and improving soybean tolerance to drought [11].

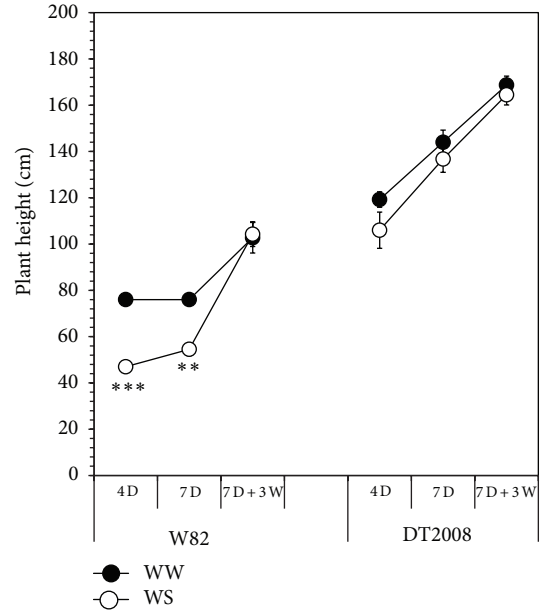

Figure 1: Comparison of plant height of the nodulated W82 and DT2008 plants after growth for 21 days in vermiculite soil and exposure to drought stress and recovery treatments. Well-watered (WW) plants were irrigated every day, whereas drought stress (WS) was imposed by withholding water for either four (4 D) or seven (7 D) days. Recovery treatment was experienced by withholding water for seven days followed by a subsequent rewatering for three days (7 D + 3 W). Error bars represent standard errors (n = 4 plants/genotype). Asterisks indicate significant differences as determined by Student's t-test ($^{**}P < 0.01$; $^{***}P < 0.001$).

In this study, soybean genotypes tested showed differential responses for growth traits examined. For example, the DT2008 plants exhibited more stable shoot growth in terms of shoot length (Figure 1), shoot fresh weight (FW), and dry weight (DW) (Table 1), when compared with W82, suggesting that DT2008 possesses a better shoot growth rate than W82 under the examined water deficit regimes (4 D, 7 D). In contrary, W82 exhibited a higher degree of susceptibility upon subjection to the equivalent drought treatments as indicated by significant decreases in the shoot length (Figure 1), shoot FW, and DW (Table 1).

Drought tolerance mechanisms in leguminous plants are closely related to the root traits of the cultivated genotypes [23, 25]. In comparison with W82, DT2008 was more tolerant to water deficit as judged by its higher root fresh and DM biomass accumulations (Table 1). Understandably, maintenance of DT2008 root growth under progressive decline in soil water content would enhance drought tolerance due to an increased capacity of water uptake. Importantly, differences between DT2008 and W82 were observed under conditions

Table 1: Comparison of biomass production of the nodulated W82 and DT2008 plants after growth for 21 days in vermiculite soil and exposure to drought stress and recovery treatments. Well-watered (WW) plants were irrigated every day, whereas drought stress (WS) was imposed by withholding water for either four (4 D) or seven (7 D) days. Recovery treatment was experienced by withholding water for seven days followed by a subsequent rewatering for three days (7 D + 3 W). Data presented are the means ± SE of four replicates. Asterisks indicate significant differences as determined by Student's t-test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

	Treatment	W82		DT2008	
		WW	WS	WW	WS
Fresh weight (g)					
Shoot	4 D	5.46 ± 0.42	3.72 ± 0.18**	6.92 ± 0.48	6.06 ± 0.53
	7 D	6.64 ± 0.54	3.89 ± 0.18**	9.94 ± 0.50	6.51 ± 0.39**
	7 D + 3 W	10.57 ± 0.61	7.10 ± 0.87*	11.03 ± 0.58	7.31 ± 0.30**
Root	4 D	2.32 ± 0.28	0.91 ± 0.09**	2.58 ± 0.19	1.78 ± 0.09*
	7 D	3.68 ± 0.13	1.64 ± 0.15***	4.11 ± 0.30	2.44 ± 0.33**
	7 D + 3 W	3.89 ± 0.22	2.31 ± 0.26**	5.62 ± 0.33	4.23 ± 0.10**
Nodules	4 D	0.44 ± 0.04	0.31 ± 0.03*	0.61 ± 0.02	0.36 ± 0.04**
	7 D	0.57 ± 0.05	0.27 ± 0.03**	0.71 ± 0.07	0.31 ± 0.03**
	7 D + 3 W	0.68 ± 0.06	0.35 ± 0.06**	0.55 ± 0.04	0.37 ± 0.02**
Dry weight (g)					
Shoot	4 D	0.74 ± 0.04	0.60 ± 0.03*	1.16 ± 0.06	1.11 ± 0.09
	7 D	1.36 ± 0.10	0.77 ± 0.04**	1.99 ± 0.10	1.30 ± 0.08**
	7 D + 3 W	2.11 ± 0.12	1.41 ± 0.18*	2.21 ± 0.12	1.46 ± 0.06**
Root	4 D	0.15 ± 0.02	0.06 ± 0.01**	0.18 ± 0.02	0.12 ± 0.01*
	7 D	0.17 ± 0.01	0.07 ± 0.01***	0.21 ± 0.02	0.12 ± 0.02**
	7 D + 3 W	0.23 ± 0.01	0.14 ± 0.02**	0.34 ± 0.02	0.25 ± 0.01**

that ensured similar amounts of soil water as indicated by VMC measurements (Figure 2). These results further support that DT2008 is more strongly tolerant to drought than W82 as indicated recently under nonnodulation conditions [14]. Collectively, our results suggest that the enhanced root systems of DT2008 may significantly contribute to its improved drought tolerance in comparison with W82.

3.2. Drought-Induced Changes in Nodulation Patterns. Most leguminous plants, including soybean, have particular features in response to water deficit, such as reduced rates of nodulation and nitrogenase activity [26]. The acute sensitivity of nodulation to water deficit has been considered a major limiting factor towards improving soybean productivity. Despite several attempts and considerable research effort during the last decades, the molecular mechanism(s) underlining this sensitivity remains largely unidentified [27]. In this work, the effect of drought on the N_2 fixation was evaluated based on nodule growth and development, specifically, the number of nodules per plant (Figure 3) and total nodule FW (Table 1), which frequently correlate well with shoot DM, providing an acceptable basis of N_2-fixing efficiency [28].

In comparison with W82, DT2008 established relatively higher number of nodules per plant (Figure 3) and accumulated more nodule FW (Table 1) under sufficient water supply which might contribute to the higher growth rate of DT2008 versus that of W82 (Table 1). Upon exposure to water deficit (4 D and 7 D), nodulation pattern, in terms of nodule number, was found to be different between DT2008 and W82. The total nodule number per plant significantly reduced in both cultivars after 7 D of water stress; however, the nodule number in DT2008 was still significantly higher than that in W82 at 7 D ($P < 0.05$ as measured by Student's t-test) (Figure 3). In case of nodule FW, although the 4 D water stress regime resulted in a significant reduction of nodule FW in both W82 and DT2008, the nodule FW in the drought-tolerant DT2008 was still slightly higher than that of the sensitive W82 cultivar (Table 1). These results indicate that water stress has a certain varied effect on nodulation patterns between the two genotypes, which might contribute to the differential drought-tolerant levels of W82 and DT2008 in addition to the different root growth rate (Table 1). Moreover, one would also expect that the DT2008 would have additionally certain internal adaptation mechanisms that might enhance the symbiotic efficiency under stressful conditions.

3.3. DT2008 Cultivar Has a Higher Reactivation Capability Than the Model W82 Cultivar under Recovery Conditions. In the field, plants often encounter unexpected cycles of progressive soil dryness. Under such conditions, plant survival and productivity rely very much on the internal acclimatization mechanisms, which reduce or even prevent cellular damage during the stress period, as well as on the potential capacity of the stressed plant to recover and maintain normal metabolic functioning [29]. Thus, plant recovery following rewatering is an essential trait for plant survival and reflects the balance between reconstruction of damaged structures and adequate metabolism restoration [23]. Obviously, much effort has been directed towards the response of N_2 fixation under drought conditions, while few investigations, if any, have considered

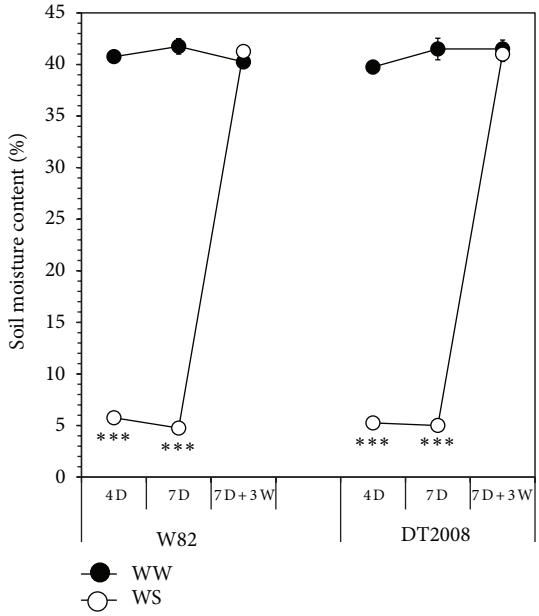

Figure 2: Monitoring of volumetric soil moisture contents during the drought stress and recovery treatments. Nodulated W82 and DT2008 plants were grown for 21 days in vermiculite soil and exposed to water deficit and rewatering treatments. Well-watered (WW) plants were irrigated every day, whereas drought stress (WS) was imposed by withholding water for either four (4 D) or seven (7 D) days. Recovery treatment was experienced by withholding water for seven days followed by a subsequent rewatering for three days (7 D + 3 W). Error bars represent standard errors. Asterisks indicate significant differences as determined by Student's t-test ($^{***}P < 0.001$).

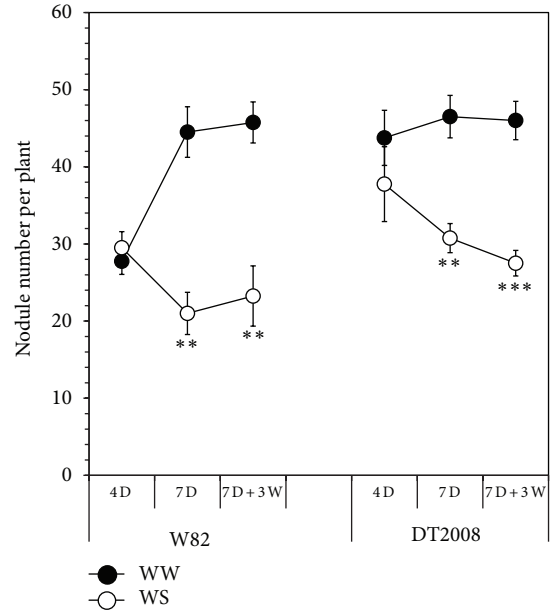

Figure 3: Comparison of nodule number per plant of the nodulated W82 and DT2008 plants after growth for 21 days in vermiculite soil and exposure to drought stress and recovery treatments. Well-watered (WW) plants were irrigated every day, whereas drought stress (WS) was imposed by withholding water for either four (4 D) or seven (7 D) days. Recovery treatment was experienced by withholding water for seven days followed by a subsequent rewatering for three days (7 D + 3 W). Error bars represent standard errors ($n = 4$ plants/genotype). Asterisks indicate significant differences as determined by Student's t-test ($^{**}P < 0.01$; $^{***}P < 0.001$).

the genotypic difference in nodulation and plant growth and development, particularly after recovery from progressive soil drying. Such investigation would be particularly useful for the analysis of the early changes occurring during the reactivation of normal nodule metabolic processes.

In the present report, the differential responses in the recovery from drought treatments were evidenced when DT2008 and W82 plants were subjected to 7 D of water withholding, followed by subsequent rewatering for 3 D. With the exception of nodule number per plant (Figure 3), plant rewatering was able to reduce or maintain the negative impact of drought on all nodulation and growth traits examined in the DT2008 genotype. Although in DT2008 the total nodule number per plant was not recovered in response to rewatering (Figure 3), the specific fixation per unit nodule mass would still have a chance to increase and compensate the observed reduction in nodulation number. Alternatively, the recovery time of 3 days (7 D + 3 W) was not sufficient for DT2008 to assume an effective recovery of nodule growth and development. Indeed, many small nodules were observed in DT2008 after 3 days of recovery. However, these nodules were too small in size to be considered in the data analysis. It should still be noticed that the total nodule number, as well as the total nodule FW, of DT2008 was still slightly higher than that of W82 following the recovery treatment (Figure 3 and Table 1). As for comparison of plant growth and development under recovery conditions, we found that rewatering drastically affected all of the parameters examined in W82 and DT2008. Although DT2008 and W82 were shown to be similarly affected at similar significant level upon being exposed to the recovery treatments, the DT2008 genotype remarkably exhibited a better performance when compared with W82 at the same equivalent treatment (Table 1).

4. Conclusions

This study aimed to characterize the newly developed soybean cultivar DT2008 when fully grown under symbiotic N_2 fixation conditions with principle objective to determine the divergent drought responses versus the reference cultivar W82 when both are subjected to drought and recovery treatments. Contrasting tolerant and sensitive symbiotic responses were identified for each genotype in association with the microsymbiont *B. japonicum* strain USDA110. The results reported here indicated that DT2008 has a superior nodule development under water deficit and recovery in comparison with W82, highlighting that it might be a heritable trait. In addition to difference in root growth rate (this work and [14]), difference in nodule development rate might contribute to differential drought-tolerant levels of DT2008

and W82 under symbiotic conditions. Thus, DT2008 and W82 genotypes can offer a genetic resource for comparative genomics, ultimately enabling soybean scientists to identify novel SNPs and genes underlining N_2 fixation under drought for development of soybean cultivars with improved drought tolerance. Strategies involving various omic approaches, such as transcriptomics, proteomics, and metabolomics, will be highly promising in this platform for determination of genes, mutations, and SNPs responsible for enhanced drought tolerance of DT2008 for genetic engineering. In fact, a combination of the conventional breeding, marker-assisted breeding, and genetic engineering strategies will be necessary in soybean improvement under increasing water limitation in the near future. As such, the generation of novel improved soybean cultivars bearing drought-tolerant trait(s) is highly expected to cope with the current and future expected water limitations.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] L. P. Manavalan, S. K. Guttikonda, L.-S. P. Tran, and H. T. Nguyen, "Physiological and molecular approaches to improve drought resistance in soybean," *Plant and Cell Physiology*, vol. 50, no. 7, pp. 1260–1276, 2009.

[2] D. F. Herridge, M. B. Peoples, and R. M. Boddey, "Global inputs of biological nitrogen fixation in agricultural systems," *Plant and Soil*, vol. 311, no. 1-2, pp. 1–18, 2008.

[3] P. D. Goldsmith, "Economics of soybean production, marketing and utilization," in *Soybeans Chemistry, Production, Processing, and Utilization*, L. P. Johnson, P. A. White, and R. Galloway, Eds., pp. 117–150, AOCS Press, Urbana, Ill, USA, 2008.

[4] F. Salvagiotti, K. G. Cassman, J. E. Specht, D. T. Walters, A. Weiss, and A. Dobermann, "Nitrogen uptake, fixation and response to fertilizer N in soybeans: a review," *Field Crops Research*, vol. 108, no. 1, pp. 1–13, 2008.

[5] M. B. Peoples, J. Brockwell, D. F. Herridge et al., "The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems," *Symbiosis*, vol. 48, no. 1–3, pp. 1–17, 2009.

[6] E. S. Jensen, M. B. Peoples, R. M. Boddey et al., "Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. A review," *Agronomy for Sustainable Development*, vol. 32, no. 2, pp. 329–364, 2012.

[7] R. Serraj, T. R. Sinclair, and L. C. Purcell, "Symbiotic N_2 fixation response to drought," *Journal of Experimental Botany*, vol. 50, no. 331, pp. 143–155, 1999.

[8] C. A. King and L. C. Purcell, "Inhibition of N_2 fixation in soybean is associated with elevated ureides and amino acids," *Plant Physiology*, vol. 137, no. 4, pp. 1389–1396, 2005.

[9] N. P. Thao, N. B. A. Thu, X. L. T. Hoang, C. V. Ha, and L.-S. P. Tran, "Differential expression analysis of a subset of drought-responsive *GmNAC* genes in two soybean cultivars differing in drought tolerance," *International Journal of Molecular Sciences*, vol. 14, no. 12, pp. 23828–23841, 2013.

[10] USDA GAIN, "Vietnam—oilseeds and products annual 2013," Global Agricultural Information Network (GAIN) Report VM3018, USDA GAIN, Hanoi, Vietnam, 2013.

[11] N. B. A. Thu, Q. T. Nguyen, X. L. T. Hoang, N. P. Thao, and L.-S. P. Tran, "Evaluation of drought tolerance of the vietnamese soybean cultivars provides potential resources for soybean production and genetic engineering," *BioMed Research International*, vol. 2014, Article ID 809736, 9 pages, 2014.

[12] A. Dai, "Increasing drought under global warming in observations and models," *Nature Climate Change*, vol. 3, no. 1, pp. 52–58, 2013.

[13] M. Q. Vinh, P. T. B. Chung, N. V. Manh, and L. T. A. Hong, "Results of research, creation, drought-tolerant soybean variety, DT2008," *Journal of Vietnamese Agricultural Science and Technology*, vol. 6, pp. 46–50, 2010.

[14] C. V. Ha, D. T. Le, R. Nishiyama et al., "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," *BioMed Research International*, vol. 2013, Article ID 759657, 8 pages, 2013.

[15] M. Q. Vinh, D. K. Thinh, D. T. Bang, D. H. At, L. H. Ham, and Q. Y. Shu, "Current status and research directions of induced mutation application to seed crops improvement in Vietnam," in *Induced Plant Mutations in the Genomics Era*, pp. 341–345, Food and Agriculture Organization of the United Nations, Rome, Italy, 2009.

[16] J. Schmutz, S. B. Cannon, J. Schlueter et al., "Genome sequence of the palaeopolyploid soybean," *Nature*, vol. 463, no. 7278, pp. 178–183, 2010.

[17] T. Kaneko, Y. Nakamura, S. Sato et al., "Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110," *DNA Research*, vol. 9, no. 6, pp. 189–197, 2002.

[18] A. C. Delves, A. Mathews, D. A. Day, A. S. Carter, B. J. Carroll, and P. M. Gresshoff, "Regulation of the soybean-*Rhizobium* nodule symbiosis by shoot and root factors," *Plant Physiology*, vol. 82, no. 2, pp. 588–590, 1986.

[19] T. R. Sinclair, L. C. Purcell, C. A. King, C. H. Sneller, P. Chen, and V. Vadez, "Drought tolerance and yield increase of soybean resulting from improved symbiotic N_2 fixation," *Field Crops Research*, vol. 101, no. 1, pp. 68–71, 2007.

[20] E. Gil-Quintana, E. Larrainzar, C. Arrese-Igor, and E. M. González, "Is N-feedback involved in the inhibition of nitrogen fixation in drought-stressed *Medicago truncatula*?" *Journal of Experimental Botany*, vol. 64, no. 1, pp. 281–292, 2013.

[21] N. P. Thao and L.-S. P. Tran, "Potentials toward genetic engineering of drought-tolerant soybean," *Critical Reviews in Biotechnology*, vol. 32, no. 4, pp. 349–362, 2012.

[22] C. V. Ha, D. T. Le, R. Nishiyama et al., "The auxin response factor transcription factor family in soybean: genome-wide

identification and expression analyses during development and water stress," *DNA Research*, vol. 20, no. 5, pp. 511–524, 2013.

[23] Y. Kang, Y. Han, I. Torres-Jerez et al., "System responses to long-term drought and re-watering of two contrasting alfalfa varieties," *Plant Journal*, vol. 68, no. 5, pp. 871–889, 2011.

[24] J. G. Streeter, "Effects of drought on nitrogen fixation in soybean root nodules," *Plant, Cell and Environment*, vol. 26, no. 8, pp. 1199–1204, 2003.

[25] A. Sarker, W. Erskine, and M. Singh, "Variation in shoot and root characteristics and their association with drought tolerance in lentil landraces," *Genetic Resources and Crop Evolution*, vol. 52, no. 1, pp. 89–97, 2005.

[26] A. J. Gordon, F. R. Minchin, L. Skøt, and C. L. James, "Stress-induced declines in soybean N_2 fixation are related to nodule sucrose synthase activity," *Plant Physiology*, vol. 114, no. 3, pp. 937–946, 1997.

[27] E. Larrainzar, J. A. Molenaar, S. Wienkoop et al., "Drought stress provokes the down-regulation of methionine and ethylene biosynthesis pathways in *Medicago truncatula* roots and nodules," *Plant, Cell and Environment*, vol. 37, no. 9, pp. 2051–2063, 2014.

[28] P. Somasegaran and H. J. Hoben, "Quantifying the growth of Rhizobia," in *Handbook for Rhizobia: Methods in Legume-Rhizobium Technology*, P. Somasegaran and H. J. Hoben, Eds., pp. 47–57, Springer, New York, NY, USA, 1994.

[29] A. Blum, "Crop responses to drought and the interpretation of adaptation," *Plant Growth Regulation*, vol. 20, no. 2, pp. 135–148, 1996.

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Research Article

tasiRNA-ARF Pathway Moderates Floral Architecture in *Arabidopsis* Plants Subjected to Drought Stress

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In plants, miRNAs and siRNAs, such as transacting siRNAs (ta-siRNAs), affect their targets through distinct regulatory mechanisms. In this study, the expression profiles of small RNAs (smRNAs) in *Arabidopsis* plants subjected to drought, cold, and high-salinity stress were analyzed using 454 DNA sequencing technology. Expression of three groups of ta-siRNAs (TAS1, TAS2, and TAS3) and their precursors was downregulated in *Arabidopsis* plants subjected to drought and high-salinity stress. Analysis of ta-siRNA synthesis mutants and mutated *ARF3*-overexpressing plants that escape the tasiRNA-ARF target indicated that self-pollination was hampered by short stamens in plants under drought and high-salinity stress. Microarray analysis of flower buds of *rdr6* and wild-type plants under drought stress and nonstressed conditions revealed that expression of floral development- and auxin response-related genes was affected by drought stress and by the *RDR6* mutation. The overall results of the present study indicated that tasiRNA-ARF is involved in maintaining the normal morphogenesis of flowers in plants under stress conditions through fine-tuning expression changes of floral development-related and auxin response-related genes.

1. Introduction

In order to adapt and survive the exposure to biotic and abiotic stress, plants have evolved various molecular responses for fine-tuning the control of adaptive responses that involve posttranscriptional regulatory mechanisms, as well as epigenetic and posttranslational modifications [1, 2]. Recent genome-wide transcriptome analyses using tiling arrays and next generation sequencing (NGS) have revealed a large number of stress-responsive noncoding RNAs (ncRNAs) [3–5].

Several small RNAs (smRNAs), such as miRNAs and siRNAs, were shown to function in development and stress responses in plants [6–9]. In plants, smRNAs exhibit a high

level of complexity in their biogenesis and function. At the moment, smRNAs are classified into microRNAs (miRNAs) and three classes of small interfering RNAs (siRNAs) [6–9]. Transacting siRNAs (ta-siRNAs) are derived from *TAS* ncRNAs that are targeted by miR173 or miR390 [10–14]. Double-stranded RNAs (dsRNAs) are generated from cleaved ncRNAs by RNA-dependent RNA polymerase 6 (RDR6) and dsRNAs are processed into 21nt ta-siRNAs. *ARF2*, *ARF3 (ETT)*, and *ARF4* were demonstrated to be targets of *TAS3* ta-siRNA (tasiRNA-ARF) [10–14].

In the present study, *Arabidopsis* deep smRNA sequencing was used to identify novel roles for smRNAs in abiotic stress response and it was discovered that ta-siRNAs and their precursors (*TAS1, TAS2,* and *TAS3*) were downregulated by drought and high-salinity stress treatments. Analysis of ta-siRNA synthesis mutants subjected to drought and high-salinity stresses revealed a short stamen phenotype and changes in the expression of floral development-related and auxin response-related genes, which was enhanced by the *RDR6* mutation. These results demonstrate that the tasiRNA-ARF pathway functions in maintaining normal flower morphogenesis under environmental stress.

2. Materials and Methods

2.1. 454 Sequencing of Small RNAs. Two-week-old wild-type plants (*Arabidopsis thaliana* ecotype Columbia), grown on MS medium [3], were transferred to drought, cold (4°C), and high-salinity (250 mM NaCl) stress as previously reported [3]. The treated plants were harvested hourly from 1 to 10 hrs after the treatment was initiated. The 1–5 hr stress-treated samples and the 6–10 hr stress-treated samples were pooled into two groups. Total RNAs were prepared using an ISOGEN kit (Nippon Gene) and precipitated with 2 M LiCl and equal volume of ethanol. RNAs were resuspended in RNase-free water at 65°C and extracted twice with an equal volume of phenol for 30 min on ice. The RNAs were then precipitated with 2 M LiCl and equal volume of ethanol. They were resuspended in RNase-free water at 65°C and extracted twice with an equal volume of phenol for 30 min on ice and precipitated again by adding 1/10 volume of 3 M sodium acetate and 3 volume of ethanol. Subsequently, 17–30 nt smRNAs were extracted using flashPAGE (Life Technologies). A cDNA library was then constructed using a small RNA Cloning Kit (Takara). First, smRNAs were ligated with a 5′ adapter F: and a 3′ adapter to generate cDNAs. The cDNAs were electrophoresed in 8 M urea and 7.5% acrylamide gel, and 60–80 nt cDNAs were recovered. The cDNAs were amplified by 12–15 cycles of PCR and subjected to 454 DNA sequencing according to the manufacturer's instructions. To eliminate RNA degradation fragments, the number of RNA sequences was normalized against the total number of miRNA sequences obtained. The normalized number of smRNAs was then subjected to data analysis. Data sets of 454 sequencing are available in DDBJ (http://www.ddbj.nig.ac.jp/index-e.html) under the accession number AB948670-AB967973.

2.2. Stress Treatments Applied to tasiRNA-ARF Pathway-Related Mutants. *rdr6-15* (SAIL_617), *ago7* (SALK_095997), *sgs3* (SALK_039005), *dcl4-2* (GK-160G05), *ARF3pro;ARF3* [15], *ARF3pro:ARF3mut* [15], *arf3* (*ett-15*) [10], *arf4-2* (SALK_070506), and wild-type *Arabidopsis* plants were grown for two weeks in pots containing 30 g of vermiculite soil. The drought stress treatment consisted of subjecting the two-week-old plants to water depletion for one week. After the one-week period, the watering of plants was then reinitiated. The high-salinity stress treatment consisted of watering three-week-old plants that started to bolt, with 100 mM NaCl-containing water for five days.

2.3. RNA Extraction. Total RNA was extracted with a Plant RNA Isolation Reagent (Life Technologies) and treated with DNase I (Life Technologies). The RNAs were then subjected to RT-quantitative PCR (RT-qPCR), microarray, and Northern analyses.

2.4. RT-qPCR Analysis. cDNAs were prepared from 1 μg of total RNA using Superscript III (Life Technologies). The target RNA concentration was obtained by measuring 1/10 of the cDNA using an ABI Prism 3100 (Life Technologies) and SYBR Premix Ex Taq II kit (Takara). For detecting small RNAs, a SYBR Advantage qPCR Kit (Takara) was used. The relative expression was calculated using the delta-delta CT method. U2 and *ACT2* for smRNA and mRNA, respectively, were used as a reference gene for normalization. Three independent biological replicates were used in all of the RT-qPCR analyses.

2.5. Microarray Analysis. Two-week-old *rdr6* mutants and wild-type plants were subjected to a drought stress treatment consisting of withholding water for one week. Microarray experiments using flower buds subjected to a drought stress or nonstressed treatment were carried out according to the manufacturer's (Agilent) preferred protocol using three biological replicates [16]. Fluorescent-labeled cRNAs were prepared from each total RNA sample using a Low Input Quick Amp Labeling Kit and were then hybridized to an Agilent *Arabidopsis* V4 microarray. The microarrays were scanned using an Agilent DNA Microarray Scanner G2539A ver. C. 75 percentile normalization was performed for the signals generated by the microarray probes according to the Agilent data analysis protocol. For microarray analysis, R program ver. 2.12.1 was used. Significant differentially expressed genes were identified by 2-way ANOVA analysis (FDR < 0.075) [17, 18]. The data set derived from the microarray analysis is available in GEO (http://www.ncbi.nlm.nih.gov/geo/info/linking.html) under the accession number GSE57174.

3. Results

3.1. RNA Sequencing of Arabidopsis Small RNAs in Plants under Abiotic Stress. Two-week-old wild type *Arabidopsis* plants were subjected to drought, cold, and high-salinity stress as described in Section 2. Six smRNA libraries were

prepared from pooled samples of stress-treated plants and one pooled sample from nonstressed control plants using 454 DNA sequencing technology as described in Section 2. A total of 480,343 reads were obtained from these seven libraries. The smRNA sequences (17–30 nt) were used for further analysis. After the smRNA sequence data were assembled to unique sequences in each library and they were mapped to the *Arabidopsis* genome, resulting in 59,284 sequences that were perfectly matched to at least one locus (Table 1). The sequences represented 12,028 unique signatures in whole 7 libraries. Approximately 39% (4,681) of the unique signatures were represented by a single sequence. The smRNAs were classified based on their mapped genomic position. The composite profiles of the types of identified smRNAs were different in each stress treatment (Supplemental Figure 1; Supplementary Material available online at http://dx.doi.org/10.1155/2014/303451). The percentile of smRNAs mapped to the sense strand of AGI code genes was higher in drought-stress and high-salinity-stress treated samples than in the nonstressed control sample, suggesting that mRNA degradation occurs preferentially under these stress conditions.

Table 1: Number of smRNA-seqs and smRNA loci in each treatment.

Treatment	Number of sequences[a]	Number of loci[b]
Drought		
1–5 h	17,758	4,574
6–10 h	10,367	3,138
Cold		
1–5 h	2,104	1,595
6–10 h	2,869	2,018
High salinity		
1–5 h	10,247	3,645
6–10 h	6,148	2,417
No treatment	9,791	4,162

[a]smRNA sequences were mapped on *Arabidopsis* genome. Sequences of tRNAs, rRNAs snoRNAs, and snRNAs were eliminated. [b]smRNAs mapped within less than 150 nt distance were grouped as the same loci.

3.2. Identification of Stress-Responsive miRNAs. Deep RNA sequencing analysis of smRNAs identified signatures of various miRNAs, including those previously reported to be stress responsive miRNAs in *Arabidopsis* (Supplemental Figure 2). Expression of miR169 was downregulated in response to drought stress (Supplemental Figure 2). Downregulation of miR169 by drought has also been previously reported and demonstrated to be required for the acquisition of drought stress tolerance [19]. Expression of miR156 and miR319 was upregulated by salinity stress and miR408 expression was upregulated by cold stress (Supplemental Figure 2). These results are also consistent with a previous report [20].

3.3. Accumulation of ta-siRNA Expression. A further analysis of smRNA sequences identified in the present study revealed that the number of ta-siRNAs was reduced in response to the stress treatments when compared to the number of ta-siRNAs identified in the nonstressed control (Figure 1(a) and Supplemental Figure 2). The precursors of tasiRNA are transcribed as ncRNAs and processed into siRNAs after a miRNA cleavage event [10–14]. An analysis of *Arabidopsis* expression profiling data obtained from a previous study utilizing a tiling array [3] indicated that the expression of the *TAS1/2/3* family was downregulated under drought and high-salinity stress (Figure 1(b)). Semiquantitative RT-PCR analysis, using primer sets that span the miRNA cleavage sites, showed that the expression of *TAS1/2/3* precursors decreased under drought and high-salinity stress (Figure 1(c)). These results are consistent with the ta-siRNA expression profiling data obtained from smRNA sequencing. Collectively, the data indicate that abiotic stress signaling regulates ta-siRNA production through transcriptional regulation of their precursors.

It is known that ta-siRNA-ARF guides the cleavage of *ARF3* and *ARF4* mRNAs [10, 11, 14, 15]. Therefore, the accumulation of *TAS3 precursor*, ta-siRNA-ARF, *MIR390, ARF3,* and *ARF4* in wild-type plants and a ta-siRNA synthesis mutant, *rdr6,* in response to a five-hour-drought stress treatment was measured using RT-qPCR in order to better understand the function of ta-siRNA in abiotic stress response (Figure 2). Expression of the *TAS3a* precursor and *RDR6* was downregulated under drought stress (Figure 2). The TAS3a expression data is consistent with the tiling array data (Figure 1(b)) [3] and results obtained by semiquantitative RT-PCR (Figure 1(c)). Expression of *ARF3* and *ARF4* was downregulated under drought stress in wild-type plants but the level of downregulation in *rdr6* mutants was much less (Figure 2). These results suggest that effective downregulation of ARF3/ARF4 mRNAs under drought stress occurs by degradation activity of tasiRNA-ARF (Figure 2) and transcriptional repression of ARF3/ARF4 mRNAs (Figure 2) under drought stress. Downregulation of tasiRNA-ARF might function in fine-tuning quantitative expression of ARF3/ARF4 under drought stress.

3.4. ta-siRNA Generation-Related Mutants Fail to Self-Pollinate due to Modifications in Flower Architecture. In order to identify the biological function of ta-siRNA under environmental stress, a moderate drought stress was applied to a variety of *Arabidopsis* mutants that are deficient in ta-siRNA biosynthesis (*rdr6, sgs3*, *dcl4,* and *ago7*). The moderate drought stress was applied to two-week-old plants grown in soil by withholding water for one week, resulting in an approximate 20% decrease in soil water content (Supplemental Figure 3(a)). Following the drought treatment, the plants were rewatered. After recovery, wild-type plants produced a number of seeds that was similar to the numbers produced in nonstressed control plants (Figures 3(a) and 3(b)). On the other hand, ta-siRNA mutants, such as *rdr6, sgs3, dcl4,* and *ago7,* produced a lower number of seeds after recovery from the drought stress compared to the numbers produced in nonstressed ta-siRNA mutants (Figures 3(a) and 3(b) and Supplemental Figure 3(b)).

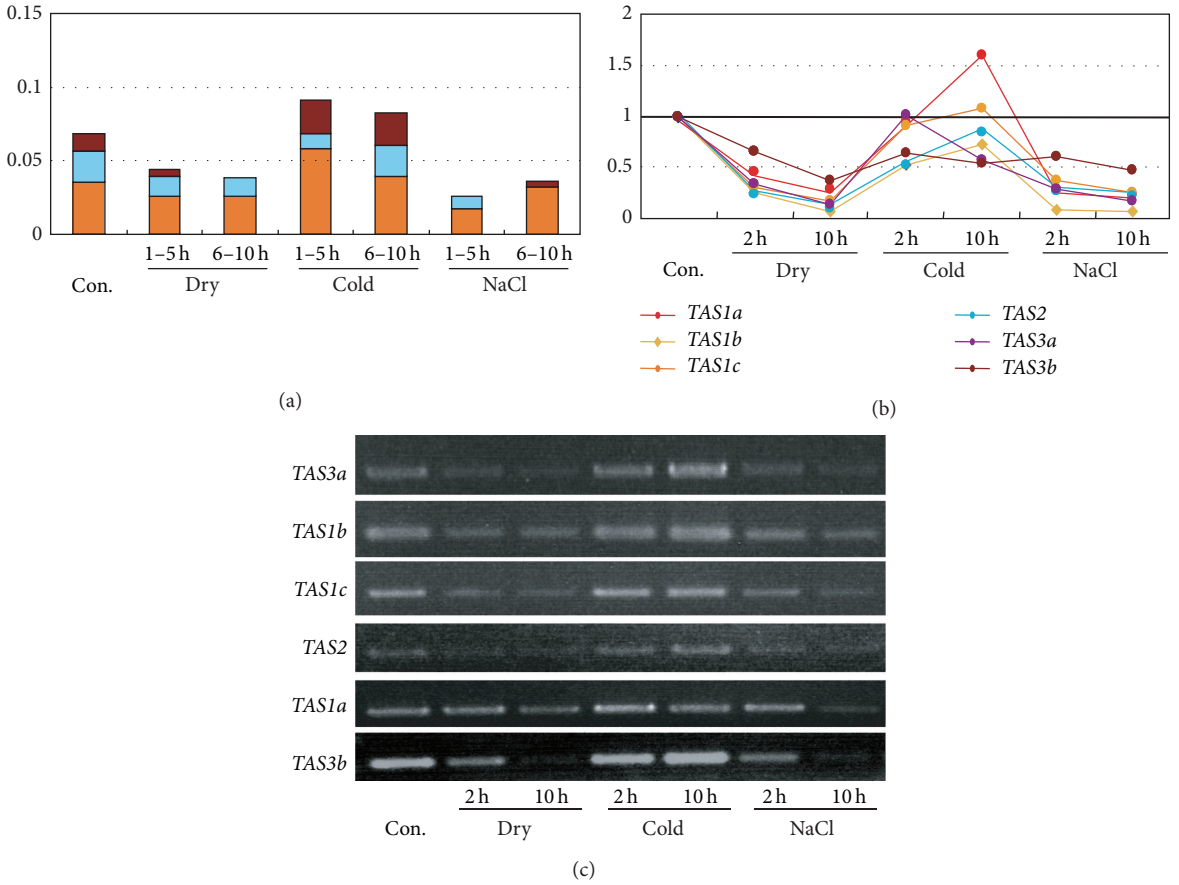

Figure 1: Downregulation of ta-siRNAs and TAS precursors in plants under drought and high-salinity stress. (a) Relative number of ta-siRNA sequences obtained by 454 DNA sequencing of cDNA libraries prepared from small RNAs. Orange, blue, and red boxes represent the relative number of *TAS1* ta-siRNA, *TAS2* ta-siRNA,and *TAS3* ta-siRNA, respectively. (b) Relative expression of *TAS* precursors was analyzed using previous data from a tiling array [3]. Two-week-old *Arabidopsis* plants were subjected to drought (2 hr, 10 hr), cold (2 hr, 10 hr), and high-salinity (2 hr, 10 hr) (see Section 2). (c) PCR primer sets (Supplemental Table 1) spanning miRNA cleavage site were designed to determine the expression profiles of *TAS* precursors. RNA samples were isolated from drought (2 hr, 10 hr), cold (2 hr, 10 hr), and high-salinity (2 hr, 10 hr) treated wild-type plants and nonstress controls. Expression of *TAS* precursors was analyzed by semiquantitative RT-PCR.

The reproduction failure phenotype was further investigated and it was found that the reproduction failure of *rdr6* plants under drought stress could be rescued by artificial pollination (Supplemental Figure 3(c)). These data suggested that the reproduction failure under drought stress was due to insufficient contact between the anther and the stigma. Abnormal floral architecture was observed in stage 13 flowers of *rdr6* plants, which had shorter stamens relative to the length of the stigma (Figure 3(c)). The length of stigmas and stamens in stage 13 flowers was examined in *rdr6* and wild-type plants under drought stress and nonstressed conditions. Stigma lengthin *rdr6* and wild-type plants was similar in plants under drought stress and nonstressed conditions. In contrast, the length of *rdr6* stamens was shorter in plants under drought stress compared to the length of stamens in wild-type plants under drought stress (Figures 3(c) and 3(d)). Although *rdr6* plants exhibited a tendency to produce slightly shorter stamens, relative to wild-type plants, even in nonstressed conditions, the difference was not statistically significant (Figures 3(c) and 3(d)). These data were consistent with a previous report [21].

It has been demonstrated that tasiRNA-ARF negatively regulates *ARF3* and *ARF4*, both of which are genes that control flower organ identity [22–24]. The ability of tasiRNA-ARF to regulate correct stamen development under drought stress was examined using *ARF3pro:ARF3mut*. This transgenic genotype has a mutated *ARF3* sequence that confers the ability of *ARF3* to avoid tasiRNA-ARF targeted degradation and still translate ARF3 protein [15]. Similar to *rdr6* plants, the *ARF3pro:ARF3mut* exhibited a short stamen phenotype in plants subjected to drought stress (Figures 3(c) and 3(d)), suggesting that the tasiRNA-ARF pathway has an important role in regulating stamen length under drought stress. Additionally, reproductive failure and the short stamen phenotype were also observed in *rdr6* mutants and *ARF3pro:ARF3mut* plants that were subjected to high-salinity stress (Supplemental Figures 3(d), 3(e), and 3(f)). These results suggest that the tasiRNA-ARF pathway plays

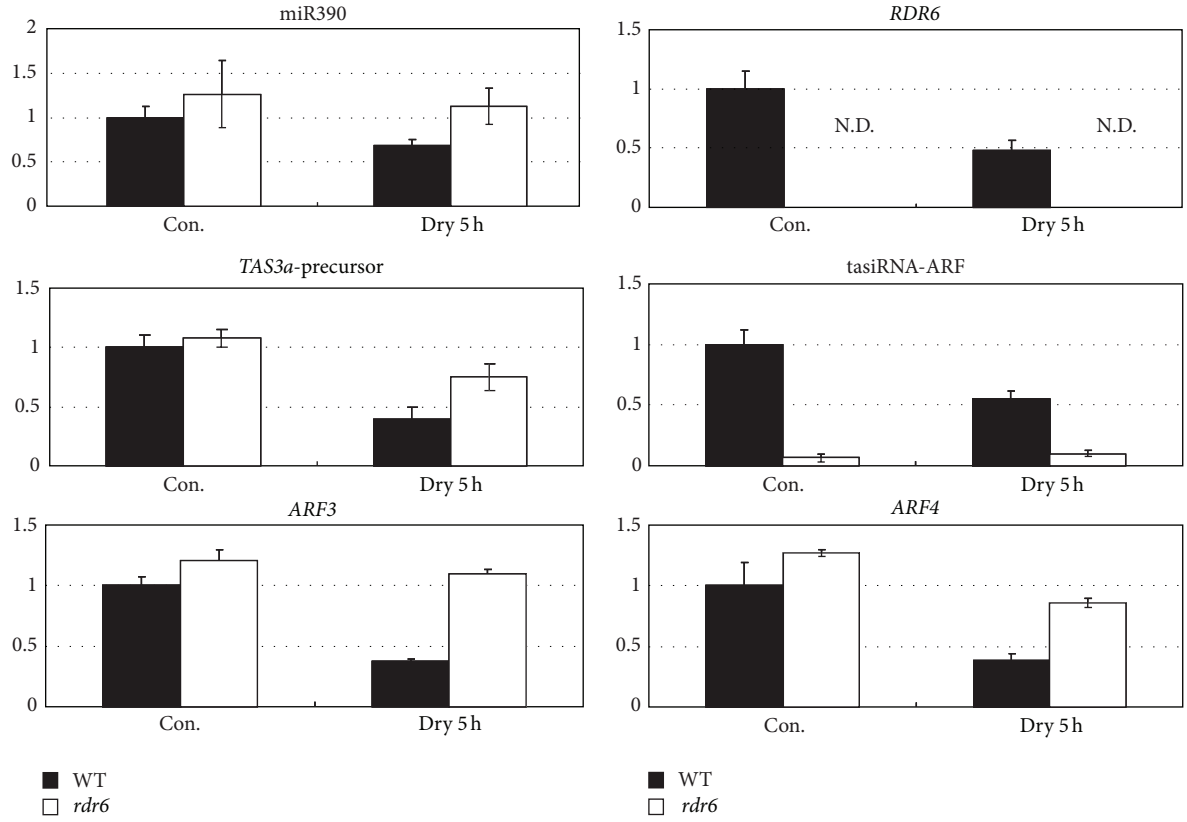

Figure 2: Expression profiles of miR390, *TAS3* precursor, *ARF3,* and *ARF4* in plants under drought stress. Expression profiles of tasiRNA-ARF pathway-related genes were analyzed in *rdr6* mutants and wild-type plants subjected to a 5 hr drought stress and nonstressed conditions by RT-qPCR. The bar graphs indicate relative expression compared to *ACT2*. Values represent the mean and standard deviation of three experiments.

a critical role in the regulation of floral organ development under both drought and high-salinity stress.

3.5. RDR6 Functions in the Stabilization of Stress-Dependent Changes in the Expression of Floral Development-Related and Auxin-Related Genes in Plants under Drought Stress. Gene expression profiles in flower buds of *Arabidopsis* plants under moderate drought stress and nonstressed conditions were analyzed using a microarray in order to better understand the regulation of the ta-siRNA-mediated network in response to drought stress. Results identified 513 (30.3%) genes whose level of expression was significantly different in *rdr6* plants compared to wild-type plants. The list of genes overlapped with drought stress-responsive genes (Figure 4(a), Supplemental Table 2). No correlation ($R^2 = 0.09$) was observed between the expression ratios of drought/control and those of *rdr6*/wild-type for the GO category of water deprivation response-related genes (GO:0009414) (Figure 4(b)). *rdr6* and wild-type plants showed similar drought stress-responsive expression in the water deprivation response-related genes (Supplemental Figure 4(a)). These results suggest that a similar level of drought stress was applied to *rdr6* and wild-type plants.

Among the differentially expressed genes in *rdr6* plants were a number of floral development-related genes (Supplemental Table 3). Expression levels of the C-class homeotic gene *AGAMOUS* [25] and *AGAMOUS* downstream genes that promote the development of stigma, style, and medial tissue of ovules, such as *SHATTERPROOF 1* and *SHATTERPROOF 2* [26], and the stigma and stamen identity gene, *SUPERMAN* [27], were upregulated under drought stress and their upregulation was affected in *rdr6* mutants (Supplemental Table 3). In contrast, expression of the E-class organ identity gene, *SEPALLATA 4* [28], and the petal identity gene *PETALLOSS* [29] was downregulated in response to drought stress in both wild-type and *rdr6* mutants; however, their expression was significantly lower in *rdr6* mutants relative to wild-type plants (Supplemental Table 3). These results indicate that the *RDR6* mutation affects organ whorl identity genes and their downstream genes. It seems that tasiRNA-ARF regulation represses central floral organ development and enhances peripheral floral development under drought stress.

To better understand the relationship between drought stress response and the *RDR6* mutation on flower development-related genes (GO:0048437), the expression ratios

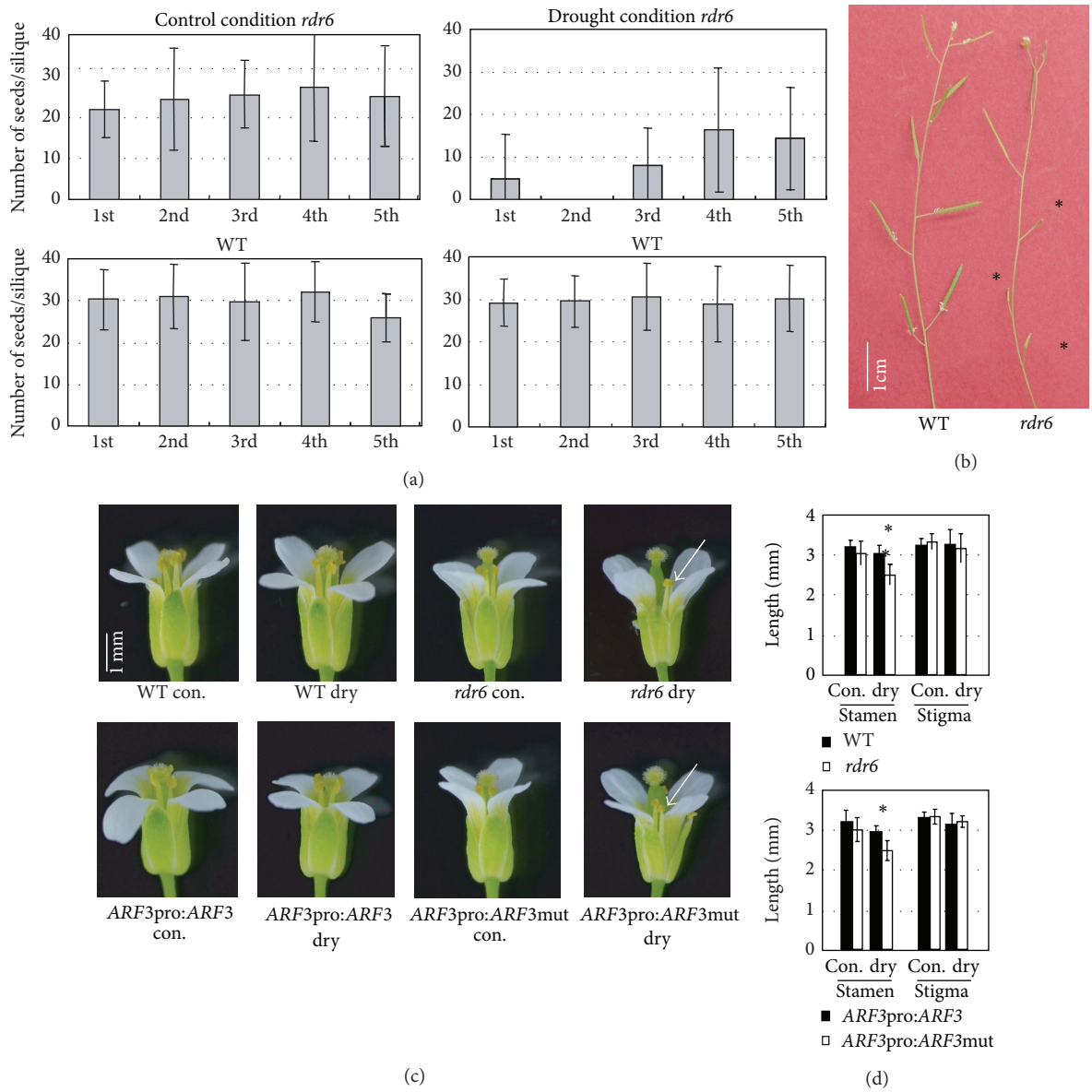

Figure 3: Modification of flower architecture and reduction in seed number in the ta-siRNA biosynthesis mutant, *rdr6*, under drought stress. (a) Two-week-old *rdr6* and wild-type plants were subjected to drought stress treatment for one week and then rewatered. Seed number in the 1st through the 5th siliques was counted ($n = 12$). Siliques were numbered starting at the basal end of the main shoot. (b) Siliques in plants subjected to drought stress followed by rewatering. (c) Floral architecture in drought-stress and nonstressed wild-type, *rdr6*, *ARF3pro:ARF3*, and *ARF3pro:ARF3mut* plants. Flowers in *rdr6* and *ARF3pro:ARF3* plants have a slightly exerting stigma phenotype under nonstressed conditions. In contrast, flowers in *rdr6* and *ARF3pro:ARF3mut* plants under drought stress exhibit short stamens, as shown as white arrows. (d) Average length of the stigmas and stamens in drought-stressed and nonstressed WT, *rdr6*, *ARF3pro:ARF3*, and *ARF3pro:ARF3mut* plants.

of drought/control and those of *rdr6*/wild-type were compared. A moderate positive correlation ($R^2 = 0.387$) was observed between the drought response and *RDR6* mutation (Figure 4(c)). These data suggest that tasiRNA-ARF is involved in fine-tuning the expression of floral development-related genes in plants subjected to drought stress.

4. Discussion

The results of the present study demonstrate that tasiRNA-ARF functions in keeping correct flower architecture which is critical to self-pollination under drought and high-salinity stress. Various abiotic stresses, such as heat, high-salinity, drought, and cold, induce reproductive failure in plants [30–35]. This failure is the result of morphological abnormalities that arise during various stages of floral development. Molecular mechanisms responsible for the abortion of pollen development have been well-characterized [30–35]. Although defects in stamen development in plants under drought, high-salinity, and heat stress have been reported [32, 34, 35], the molecular mechanisms that protect floral development from the adverse effects of abiotic stress, however, remain unclear.

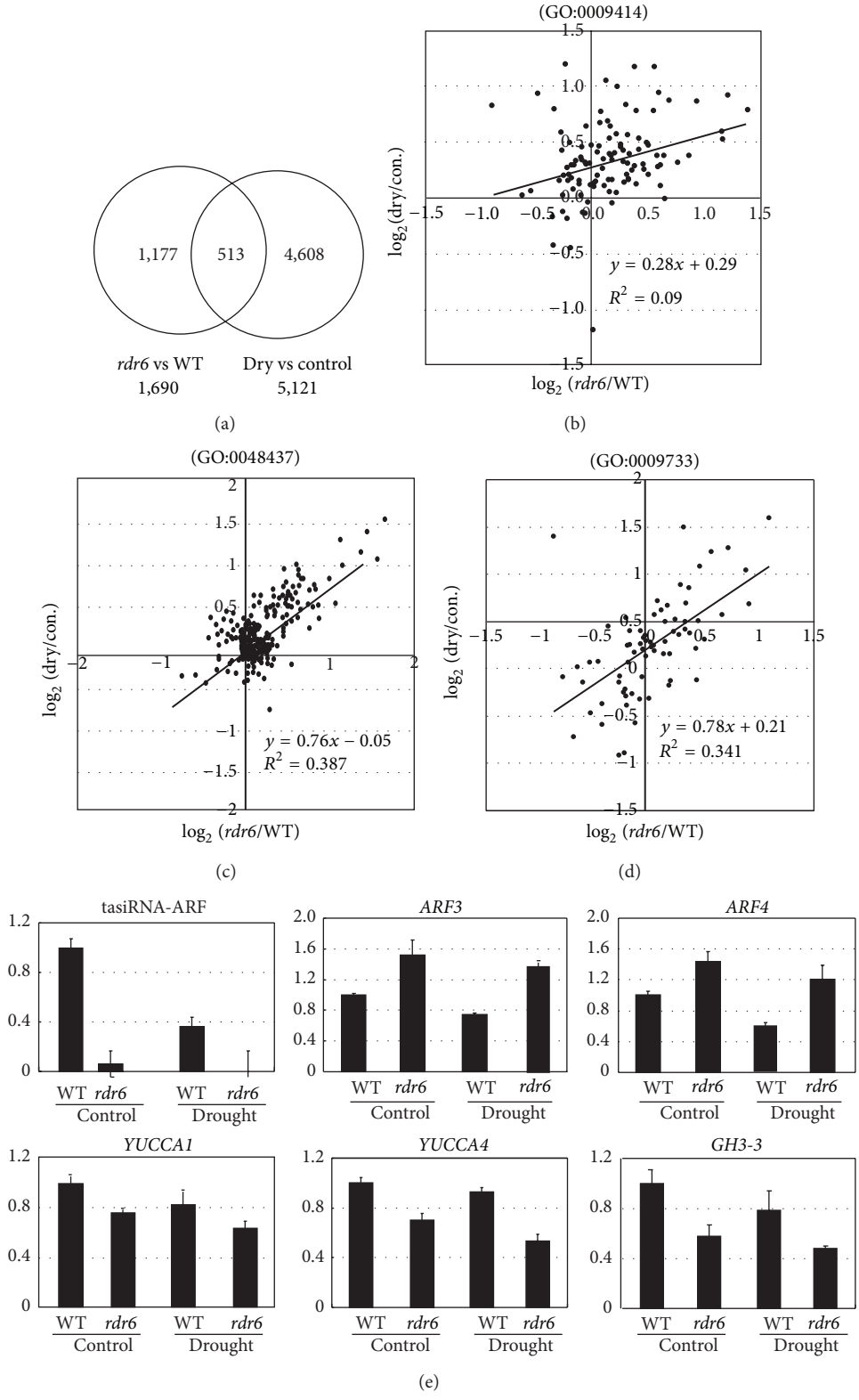

Figure 4: Microarray analysis of flower buds in plants under drought stress. (a) Venn diagram of differentially expressed genes in *RDR6* plants compared to wild-type plants and drought stress-responsive genes. Statistically significant differentially expressed genes were identified based on the following criteria: FDR of 2-way ANOVA (*rdr6* versus WT or drought-stressed versus nonstressed) < 0.075. ((b)–(d)) Scatter plot analysis of genes from different categories of GO terms. Horizontal axis represents $\log_2$ ratio of (*rdr6* non-stressed + *rdr6* drought)/(WT nonstressed + WT drought). Vertical axis represents $\log_2$ ratio of (WT drought+ *rdr6* drought)/(WT nonstressed + *rdr6* non-stressed). (b) Scatter plot analysis of water deprivation response-related genes. (c) Scatter plot analysis of floral organ development-related genes. (d) Scatter plot analysis of auxin response-related genes. (e) RT-qPCR expression profiles of tasiRNA-ARF, *ARF3*, *ARF4*, *GH3-3*, *YUCCA1*, and *YUCCA4* in flower buds of *rdr6* mutant and wild-type plants subjected to drought stress or nonstressed control.

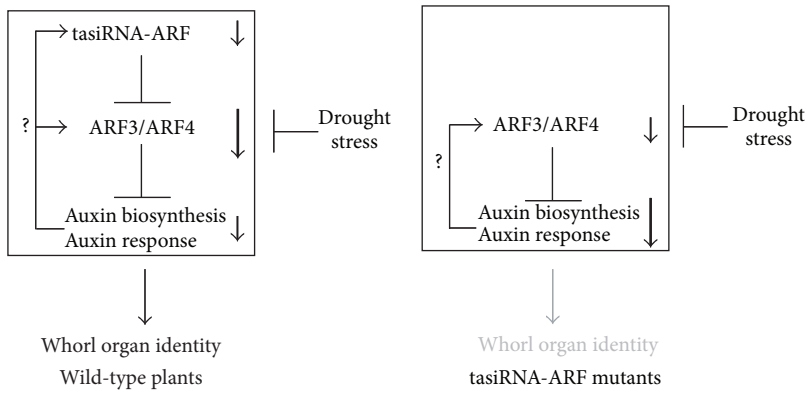

Figure 5: Proposed model for the fine-tuning of whorl architecture in flowers subjected to environmental stress via tasiRNA-ARF. tasiRNA-ARF is required for normal morphogenesis of floral whorl organs in plants subjected to drought stress. tasiRNA-ARF acts by modulating the expression of floral development-related genes in plants subjected to drought stress. Expression of *YUCCA4* is downregulated by drought stress and in the *RDR6* mutation, suggesting that auxin biosynthesis is modulated by tasiRNA-ARF.

tasiRNA-ARF is required for the normal development of lateral organs, such as leaves, lateral roots, and flowers [14, 21, 36, 37]. For example, *rdr6* mutant exhibits altered stamen and pistil elongation that results in variable seed production and supports the premise that reproduction in *rdr6* plants is sensitive to growth conditions [22]. Interestingly, a mutation of *RDR6* enhanced self-incompatibility in a transgenic, self-incompatible, *Arabidopsis thaliana* system [38]. These results suggest that tasiRNA-ARF functions as a key mediator for maintaining the correct pattern of flower architecture, as well as their development. *ARF3* and *ARF4* function in central organ identity in flowers and apical-basal patterning defects in the gynoecium [22–24].

*ARF*s regulate the expression of auxin-responsive genes by binding specifically to auxin response elements (AuxRE) [39]. Regarding auxin response-related genes (GO:0009733), a moderate positive correlation was observed between drought stress response and the effect of the *RDR6* mutation (Figure 4(d)). The genes downregulated by both the drought stress treatment and the *RDR6* mutation included the auxin-induced conjugating enzymes, *GH3-2*, *GH3-3*, *GH3-6*, *GH3-10*, and an *auxin-responsive GH3-like protein* (*AT1G48660*) (Supplemental Table 2). In addition, microarray analysis also identified that an expression of auxin biosynthesis-related gene, *YUCCA 4*, was significantly downregulated by drought stress and the *RDR6* mutation (Supplemental Table 2). After conducting a closer study of 4 *YUCCA* genes that are mainly expressed in flowers [40], *YUCCA 1* and *YUCCA 4* were downregulated by drought stress and *RDR6* mutation and *YUCCA 2* and *YUCCA 6* were downregulated by *RDR6* mutation (Supplemental Figure 4(b)). The regulation of tasiRNA-ARF and mRNAs of *ARF3, GH3-3*, *YUCCA 1*, and *YUCCA 4* in flower buds was confirmed by RT-qPCR (Figure 4(e)). The previous report showed that expression of an auxin reporter, DR5:GUS, was decreased in *ARF3pro:ARF3mut* plants [41]. These results suggest that the auxin biosynthesis and auxin response were attenuated in floral development by drought stress and loss of tasiRNA-ARF regulation. It was known that auxin signaling was important for floral development. The pin-shaped flower of *yucca1/4* was similar to the flowers produced in *ARF3*- or *ARF4*-overexpressing plants [40]. Experiments utilizing an auxin transport inhibitor indicate that *ARF3* functions as a modulator of auxin response during floral development [42]. There is a possible hypothesis that tasiRNA-ARF controls floral development by maintaining the proper level of auxin signaling under drought stress (Figure 5).

Both tasiRNA-ARF and *ARF3/4* were downregulated under drought stress, suggesting that tasiRNA-ARF are required for quantitative adjustment of *ARF3/4* expression. Positive feedback regulation of auxin signaling might also function in the regulation of these genes (Figure 5). It is known that initiation of lateral roots modulated by positive and negative feedback regulation between tasiRNA-ARF and *ARF2/3/4* through auxin signaling [38, 39]. *ARF3* expression was also induced by increased auxin biogenesis through upregulation of *YUCCA 4* in shoot initiation [43]. These previous reports invoke that drought stress affects tasiRNA-ARF regulatory network, but it remained unclear.

To search candidate genes connecting abiotic stress and tasiRNA-ARF regulatory network, microarray coexpression analysis of ARF3 ($P < 0.01$) and ARF4 ($P < 0.01$) was performed. 155 genes coexpressed with *ARF3* and *ARF4* involved twenty-five abiotic stress response-related genes, such as *DREB2C* [44], *DWD* (*DDB1-binding WD40 protein*) [45], *ascorbate peroxidase 1* [46], *glutathione S-transferase* [47], and *RD21B* [48] (Supplemental Table 4). Thegenes also included ta-siRNA pathway-related genes, such as *RDR6*, *TAS3*, other *TAS* genes, and ta-siRNA target genes [49]. Sixty-four of the 155 genes possessed an ARF binding motif (tgtctc) [50] in the promoter region within 1kb upstream of the start codon (Supplemental Table 4). These genes may represent candidates that connect tasiRNA-ARF regulatory network and drought stress signaling pathway.

In conclusion, this study demonstrates that tasiRNA-ARF acts as a central modifier, negatively regulating changes

in the expression of floral development-related genes in plants under drought and assists in maintaining normal floral morphogenesis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] E. Mazzucotelli, A. M. Mastrangelo, C. Crosatti, D. Guerra, A. M. Stanca, and L. Cattivelli, "Abiotic stress response in plants: when post-transcriptional and post-translational regulations control transcription," *Plant Science*, vol. 174, no. 4, pp. 420–431, 2008.

[2] T. Hirayama and K. Shinozaki, "Research on plant abiotic stress responses in the post-genome era: past, present and future," *Plant Journal*, vol. 61, no. 6, pp. 1041–1052, 2010.

[3] A. Matsui, J. Ishida, T. Morosawa et al., "*Arabidopsis* transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array," *Plant and Cell Physiology*, vol. 49, no. 8, pp. 1135–1149, 2008.

[4] L. A. Rymarquis, J. P. Kastenmayer, A. G. Hüttenhofer, and P. J. Green, "Diamonds in the rough: mRNA-like non-coding RNAs," *Trends in Plant Science*, vol. 13, no. 7, pp. 329–334, 2008.

[5] P. Laporte, F. Merchan, B. B. Amor, S. Wirth, and M. Crespi, "Riboregulators in plant development," *Biochemical Society Transactions*, vol. 35, no. 6, pp. 1638–1642, 2007.

[6] A. Matsui, A. H. Nguyen, K. Nakaminami, and M. Seki, "*Arabidopsis* non-coding RNA regulation in abiotic stress responses," *International Journal of Molecular Sciences*, vol. 14, no. 11, pp. 2642–2654, 2013.

[7] B. Khraiwesh and J. Zhu, "Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants," *Biochimica et Biophysica Acta—Gene Regulatory Mechanisms*, vol. 1819, no. 2, pp. 137–148, 2012.

[8] J. C. de Lima, G. Loss-Morais, and R. Margis, "MicroRNAs play critical roles during plant development and in response to abiotic stresses," *Genetics and Molecular Biology*, vol. 35, no. 4, pp. 1069–1077, 2012.

[9] R. Sunkar, V. Chinnusamy, and J.-K. Zhu, "Small RNAs as big players in plant abiotic stress responses and nutrient deprivation," *Trends in Plant Science*, vol. 12, no. 7, pp. 301–309, 2007.

[10] C. Hunter, M. R. Willmann, G. Wu, M. Yoshikawa, M. de la Luz Gutierrez-Nava, and S. Poethig, "Trans-acting siRNA-mediated repression of ETTIN and ARF4 regulates heteroblasty in *Arabidopsis*," *Development*, vol. 133, no. 15, pp. 2973–2981, 2006.

[11] E. Allen, Z. Xie, A. M. Gustafson, and J. C. Carrington, "microRNA-directed phasing during trans-acting siRNA biogenesis in plants," *Cell*, vol. 121, no. 2, pp. 207–221, 2005.

[12] M. Yoshikawa, A. Peragine, Y. P. Mee, and R. S. Poethig, "A pathway for the biogenesis of trans-acting siRNAs in Arabidopsis," *Genes and Development*, vol. 19, no. 18, pp. 2164–2175, 2005.

[13] Z. Xie, E. Allen, A. Wilken, and J. C. Carrington, "DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in *Arabidopsis thaliana*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 36, pp. 12984–12989, 2005.

[14] X. Adenot, T. Elmayan, D. Lauressergues et al., "DRB4-dependent *TAS3* trans-acting siRNAs control leaf morphology through *AGO7*," *Current Biology*, vol. 16, no. 9, pp. 927–932, 2006.

[15] N. Fahlgren, T. A. Montgomery, M. D. Howell et al., "Regulation of *AUXIN RESPONSE FACTOR3* by *TAS3* ta- siRNA affects developmental timing and patterning in *Arabidopsis*," *Current Biology*, vol. 16, no. 9, pp. 939–944, 2006.

[16] T. K. To, K. Nakaminami, J. Kim et al., "Arabidopsis HDA6 is required for freezing tolerance," *Biochemical and Biophysical Research Communications*, vol. 406, no. 3, pp. 414–419, 2011.

[17] Y. Benjamini and Y. Hochberg, "Controlling the false discovery rate: a practical and powerful approach to multiple testing," *Journal of the Royal Statistical Society. Series B. Methodological*, vol. 57, no. 1, pp. 289–300, 1995.

[18] J. M. Chambers, A. Freeny, and R. M. Heiberger, "Analysis of variance; designed experiments," in *Statistical Models in S*, chapter 5, pp. 145–193, 1992.

[19] W. Li, Y. Oono, J. Zhu et al., "The Arabidopsis NFYA5 transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance," *Plant Cell*, vol. 20, no. 8, pp. 2238–2251, 2008.

[20] H. H. Liu, X. Tian, Y. J. Li, C. A. Wu, and C. C. Zheng, "Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*," *RNA*, vol. 14, no. 5, pp. 836–843, 2008.

[21] A. Peragine, M. Yoshikawa, G. Wu, H. L. Albrecht, and R. S. Poethig, "SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in *Arabidopsis*," *Genes and Development*, vol. 18, no. 19, pp. 2368–2379, 2004.

[22] A. Sessions, J. L. Nemhauser, A. McColl, J. L. Roe, K. A. Feldmann, and P. C. Zambryski, "ETTIN patterns the *Arabidopsis* floral meristem and reproductive organs," *Development*, vol. 124, no. 22, pp. 4481–4491, 1997.

[23] R. A. Sessions and P. C. Zambryski, "Arabidopsis gynoecium structure in the wild type and in ettin mutants," *Development*, vol. 121, no. 5, pp. 1519–1532, 1995.

[24] I. Pekker, J. P. Alvarez, and Y. Eshed, "Auxin response factors mediate Arabidopsis organ asymmetry via modulation of KANADI activity," *Plant Cell*, vol. 17, no. 11, pp. 2899–2910, 2005.

[25] J. L. Bowman, D. R. Smyth, and E. M. Meyerowitz, "Genes directing flower development in Arabidopsis," *The Plant cell*, vol. 1, no. 1, pp. 37–52, 1989.

[26] M. Colombo, V. Brambilla, R. Marcheselli, E. Caporali, M. M. Kater, and L. Colombo, "A new role for the SHATTERPROOF

genes during *Arabidopsis* gynoecium development," *Developmental Biology*, vol. 337, no. 2, pp. 294–302, 2010.

[27] H. Sakai, L. J. Medrano, and E. M. Meyerowitz, "Role of SUPERMAN in maintaining Arabidopsis floral whorl boundaries," *Nature*, vol. 378, no. 6553, pp. 199–203, 1995.

[28] G. Ditta, A. Pinyopich, P. Robles, S. Pelaz, and M. F. Yanofsky, "The SEP4 gene of Arabidopsis thaliana functions in floral organ and meristem identity," *Current Biology*, vol. 14, no. 21, pp. 1935–1940, 2004.

[29] M. E. Griffith, A. da Silva Conceiçao, and D. R. Smyth, "PETAL LOSS gene regulates initiation and orientation of second whorl organs in the Arabidopsis flower," *Development*, vol. 126, no. 24, pp. 5635–5644, 1999.

[30] P. K. Koonjul, J. S. Minhas, C. Nunes, I. S. Sheoran, and H. S. Saini, "Selective transcriptional down-regulation of anther invertases precedes the failure of pollen development in water-stressed wheat," *Journal of Experimental Botany*, vol. 56, no. 409, pp. 179–190, 2005.

[31] P. O. Cheptou, A. Berger, A. Blanchard, C. Collin, and J. Escarre, "The effect of drought stress on inbreeding depression in four populations of the mediterranean outcrossing plant Crepis sancta (Asteraceae)," *Heredity*, vol. 85, part 3, pp. 294–302, 2000.

[32] R. Sulpice, H. Tsukaya, H. Nonaka, L. Mustardy, T. H. H. Chen, and N. Murata, "Enhanced formation of flowers in salt-stressed *Arabidopsis* after genetic engineering of the synthesis of glycine betaine," *Plant Journal*, vol. 36, no. 2, pp. 165–176, 2003.

[33] T. Satake and H. Hayase, "Male sterility caused by cooling treatment at the young microspore stage in rice plants. V. Estimation of pollen developmental stage and the most sensitive stage to coolness," *Proceedings of the Crop Science Society of Japan*, vol. 39, pp. 468–473, 1970.

[34] T. Sakata, T. Oshino, S. Miura et al., "Auxins reverse plant male sterility caused by high temperatures," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 19, pp. 8569–8574, 2010.

[35] Z. Su, X. Ma, H. Guo et al., "Flower development under drought stress: morphological and transcriptomic analyses reveal acute responses and long-term acclimation in Arabidopsis," *Plant Cell*, vol. 25, no. 10, pp. 3785–3807, 2013.

[36] E. Marin, V. Jouannet, A. Herz et al., "miR390, *Arabidopsis TAS3* tasiRNAs, and their *AUXIN RESPONSE FACTOR* targets define an autoregulatory network quantitatively regulating lateral root growth," *Plant Cell*, vol. 22, no. 4, pp. 1104–1117, 2010.

[37] E. K. Yoon, J. H. Yang, J. Lim, S. H. Kim, and W. S. Lee, "Auxin regulation of the microRNA390-dependent transacting small interfering RNA pathway in Arabidopsis lateral root development," *Nucleic Acids Research*, vol. 38, no. 4, pp. 1382–1391, 2009.

[38] T. Tantikanjana, N. Rizvi, M. E. Nasrallah, and J. B. Nasrallah, "A dual role for the s-locus receptor kinase in self-incompatibility and pistil development revealed by an arabidopsis rdr6 mutation," *Plant Cell*, vol. 21, no. 9, pp. 2642–2654, 2009.

[39] S. B. Tiwari, G. Hagen, and T. Guilfoyle, "The roles of auxin response factor domains in auxin-responsive transcription," *Plant Cell*, vol. 15, no. 2, pp. 533–543, 2003.

[40] Y. Cheng, X. Dai, and Y. Zhao, "Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in *Arabidopsis*," *Genes & Development*, vol. 20, no. 13, pp. 1790–1799, 2006.

[41] T. Tantikanjana and J. B. Nasrallah, "Non-cell-autonomous regulation of crucifer self-incompatibility by Auxin Response Factor ARF3," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 47, pp. 19468–19473, 2012.

[42] J. L. Nemhauser, L. J. Feldman, and P. C. Zambryski, "Auxin and ETTIN in Arabidopsis gynoecium morphogenesis," *Development*, vol. 127, no. 18, pp. 3877–3888, 2000.

[43] Z. J. Cheng, L. Wang, W. Sun et al., "Pattern of auxin and cytokinin responses for shoot meristem induction results from the regulation of cytokinin biosynthesis by AUXIN RESPONSE FACTOR3," *Plant Physiology*, vol. 161, no. 1, pp. 240–251, 2013.

[44] S. J. Lee, J. Y. Kang, H. J. Park et al., "DREB2C interacts with ABF2, a bZIP protein regulating abscisic acid-responsive gene expression, and its overexpression affects abscisic acid sensitivity," *Plant Physiology*, vol. 153, no. 2, pp. 716–727, 2010.

[45] J. H. Lee, H. J. Yoon, W. Terzaghi et al., "DWA1 and DWA2, two Arabidopsis DWD protein components of CUL4-based E3 ligases, act together as negative regulators in ABA signal transduction," *Plant Cell*, vol. 22, no. 6, pp. 1716–1732, 2010.

[46] G. Miller, N. Suzuki, L. Rizhsky, A. Hegie, S. Koussevitzky, and R. Mittler, "Double mutants deficient in cytosolic and thylakoid ascorbate peroxidase reveal a complex mode of interaction between reactive oxygen species, plant development, and response to abiotic stresses," *Plant Physiology*, vol. 144, no. 4, pp. 1777–1785, 2007.

[47] P. G. Sappl, A. J. Carroll, R. Clifton et al., "The *Arabidopsis* glutathione transferase gene family displays complex stress regulation and co-silencing multiple genes results in altered metabolic sensitivity to oxidative stress," *The Plant Journal*, vol. 58, no. 1, pp. 53–68, 2009.

[48] K. Yamaguchi-Shinozaki and K. Shinozaki, "The plant hormone abscisic acid mediates the drought-induced expression but not the seed-specific expression of rd22, a gene responsive to dehydration stress in *Arabidopsis thaliana*," *Molecular & General Genetics*, vol. 238, no. 1-2, pp. 17–25, 1993.

[49] C. Meng, J. Chen, S. Ding, J. Peng, and S. Wong, "Hibiscus chlorotic ringspot virus coat protein inhibits trans-acting small interfering RNA biogenesis in Arabidopsis," *Journal of General Virology*, vol. 89, no. 9, pp. 2349–2358, 2008.

[50] T. Ulmasov, G. Hagen, and T. J. Guilfoyle, "Dimerization and DNA binding of auxin response factors," *Plant Journal*, vol. 19, no. 3, pp. 309–319, 1999.

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Review Article

Recent Advances in Polyamine Metabolism and Abiotic Stress Tolerance

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Global warming is an alarming problem in agriculture and its effect on yield loss has been estimated to be five per cent for every degree centigrade rise in temperature. Plants exhibit multiple mechanisms like optimizing signaling pathway, involvement of secondary messengers, production of biomolecules specifically in response to stress, modulation of various metabolic networks in accordance with stress, and so forth, in order to overcome abiotic stress factors. Many structural genes and networks of pathway were identified and reported in plant systems for abiotic stress tolerance. One such crucial metabolic pathway that is involved in normal physiological function and also gets modulated during stress to impart tolerance is polyamine metabolic pathway. Besides the role of structural genes, it is also important to know the mechanism by which these structural genes are regulated during stress. Present review highlights polyamine biosynthesis, catabolism, and its role in abiotic stress tolerance with special reference to plant systems. Additionally, a system based approach is discussed as a potential strategy to dissect the existing variation in crop species in unraveling the interacting regulatory components/genetic determinants related to PAs mediated abiotic stress tolerance.

1. Introduction

Global warming has emerged as major environmental challenge that modulates diverse environmental factors like temperature extremities, altered oxygen levels, and salt and mineral deficiency including toxicity. In bird's eye view, global warming is being visualized as climate change that transmits cautious signals for living organisms to modulate themselves so as to withstand the environmental effects. Impact of climate change on plant system, expressed in multitude of forms like drought, heat, oxidative burst, salinity, and so forth, has shifted the focus of plant biotechnology more towards dissection of genetic elements involved in stress tolerance [1]. It has been estimated that there is a potential yield loss of up to five per cent for rise in temperature by every degree centigrade [2]. Environmental changes are the signals, perceived by plant sensors and transmitted through secondary messengers to kinases which lead to altered expression of genes and metabolites through the respective transcription factors in a cascade of processes to respond and acquaint itself [3].

Bhatnagar-Mathur and coworkers [4] had classified the genes expressed under stress into three groups, *namely*, genes encoding for known enzymatic or metabolic or structural functions; proteins with unknown functions; and regulatory proteins. Irrespective of the three groups of genes that are classified based on their role during stress, it can further be grouped into two based on its applicability, *namely*, novel genes from the model organisms whose ecological habitat was at extremity and novel allelic variants of known genes that get modulated during stress period. Novel genes from model organisms will broaden our knowledge and understanding on the network of genes and metabolic pathways required to keep the organism physiologically normal even under environmental extremity. Novel allelic forms of same gene from different species resulting in differential modulation pattern and thereby effecting varied levels of stress tolerance will help to understand the biochemical and physiological mechanism that gets altered to overcome stressed conditions. Of the above said two types, in case of the former one (novel genes), it may not be just enough to transfer the genes in stress-sensitive organism to impart tolerance; rather it may require

other interacting genes as well for imparting stress tolerance due to the fact that the gene itself is novel, whereas latter one (novel allelic form) will probably gain practical utility to impart stress tolerance (if the said gene is the key regulator in its metabolic pathway) in sensitive organism due to its functional expression under normal ambient conditions being known. Polyamine metabolic pathway is one such pathway in plants [5] existing under normal developmental phases (organogenesis, embryogenesis, flower and fruit development, and senescence) with a prime function to stabilize macromolecular structures [6] and gets modulated in response to various environmental stimuli of both abiotic and biotic nature [7]. This review is focused on the importance of polyamines and its biosynthetic pathway in imparting abiotic stress tolerance with special reference to crop plants.

2. Polyamine Biosynthesis in Plants

Polyamines, cationic compounds having two or more amine groups, are low molecular weight organic molecules present in most of the organisms with diverse functions due to the diversity in number and position of amino groups [8]. Due to the cationic nature of polyamines, they bind easily with DNA, RNA, and proteins through electrostatic linkages resulting either in stabilization or destabilization [9, 10]. Polyamines are known to be involved in various developmental processes, *namely*, survival of plant embryos and translation in eukaryotes [11]; cell signaling and membrane stabilization [12]; cell proliferation and modulated gene expression [13]; and apoptosis and cell death [14, 15]. Putrescine (Put), spermidine (Spd), and spermine (Spm) are the major polyamines found in higher plants either in free or soluble conjugated (mainly in the form of hydroxycinnamic acid amides) or insoluble bound forms [5, 16]. Besides some unique polyamines like caldopentamine and caldohexamine that are specific to certain organisms with special reference to thermophiles including thermospermine, a structural isomer of spermine [17]. Numerous reviews on metabolic pathway with genes and enzymes involved in polyamine biosynthesis were available [18–20] and an overview was provided in Figure 1.

Occurrence of putrescine was detected in ergot as early as 1908 [21] and in potassium deficient barley plants [22]. Formation of putrescine from arginine through ornithine in the presence of ARGINASE (EC 3.5.3.1) was reported [23]. Within few years, Nakamura in 1944 had identified an alternate pathway for putrescine from arginine through agmatine which shows that in bacteria arginine is the primary precursor [24]. The difference between the two pathways is that agmatine is formed through decarboxylation reaction whereas, in the former pathway, after ornithine formation decarboxylation reaction takes place. With special reference to plants, Coleman and Hegartv in 1957 had used radioactive ornithine (^{14}C) in barley for formation of putrescine [25], whereas Smith and Richards in 1962 had reported the formation of radioactive putrescine through radioactive arginine (^{14}C) feeding experiments in barley [26]. These two experiments clarify the possibility of two independent pathways one from arginine and another from ornithine for putrescine biosynthesis. Greene in 1957 had showed in *Neurospora crassa* that methionine and ATP are required for the formation of spermidine and spermine by using 2-C^{14}-DL-methionine [27]. Experiments by Tabor and coworkers in *Escherichia coli* had confirmed that putrescine is the precursor for spermidine and spermine biosynthesis by using C^{14}-N^{15}-putrescine [28]. With these studies, overall picture on biosynthetic pathway of standard polyamines, *namely*, putrescine, spermine, and spermidine, was elucidated. Ornithine or arginine acts as a primary precursor for putrescine in polyamine biosynthesis through ornithine decarboxylase (ODC; EC 4.1.1.17) or arginine decarboxylase (ADC; EC 4.1.1.9), agmatine iminohydrolase (AIH; EC 3.5.3.12), and N-carbamoylputrescine amidohydrolase (CPA; EC 3.5.1.53), respectively, with special reference to plants [10]. Decarboxylated *S*-adenosylmethionine (dcSAM) is the key aminopropyl group donor for synthesis of spermidine from putrescine through spermidine synthase (SPDS; EC 2.5.1.16); spermine from spermidine through spermine synthase (SPMS; EC 2.5.1.22); and thermospermine (tSpm), a structural isomer of spermine, from spermidine through thermospermine synthase (ACL5 or TSPMS; EC 2.5.1.79). dcSAM is synthesized by action of *S*-adenosylmethionine decarboxylase (AdoMetDC; EC 4.1.4.50) on *S*-adenosyl-methionine which in turn is synthesized through *S*-adenosylmethionine synthetase or methionine adenosyltransferase (MAT; EC 2.5.1.6) from methionine [29]. Of all these genes involved in polyamine biosynthesis, key regulators (with reference to plant system) in the polyamine biosynthesis, *namely*, *Odc*, *Adc*, and *AdoMetDC* genes, were known to act as a key regulator in modulating the endogenous levels of polyamines (Put, Spd, and Spm) during various developmental stages and including stressed conditions [30].

3. Polyamine Catabolism in Plants

Endogenous titers of polyamines (especially Put, Spd, and Spm) are modulated not only through regulated gene expression patterns of polyamine biosynthetic genes but also through the regulated expression of genes involved in catabolism of polyamines (generally known as amine oxidases) that catalyze the oxidative deamination of polyamines having its own functionalities. In other ways, we can even call it biosynthetic pathway of H_2O_2 because, in plants, compartmentalized production of H_2O_2 through oxidation of polyamines was reported to have functional input in cell wall maturation and lignifications during ontogeny [31–33] and also help in combating biotic [29, 34–36] and abiotic stresses [29, 37–39].

Amine oxidases, which catalyze oxidation of polyamines through deamination, are of two types, *namely*, copper dependent amine oxidase (CuAO; EC 1.4.3.6) and the flavin dependent polyamine oxidase (PAO; EC 1.5.3.11) that help in polyamine homeostasis spatially and temporally [31]. CuAO oxidizes diamines, *namely*, putrescine (Put) and cadaverine (Cad) at primary amino group, and PAO oxidizes Spd and Spm along with their acetylated derivatives at the secondary amino group [40]. Oxidation of putrescine by CuAO in

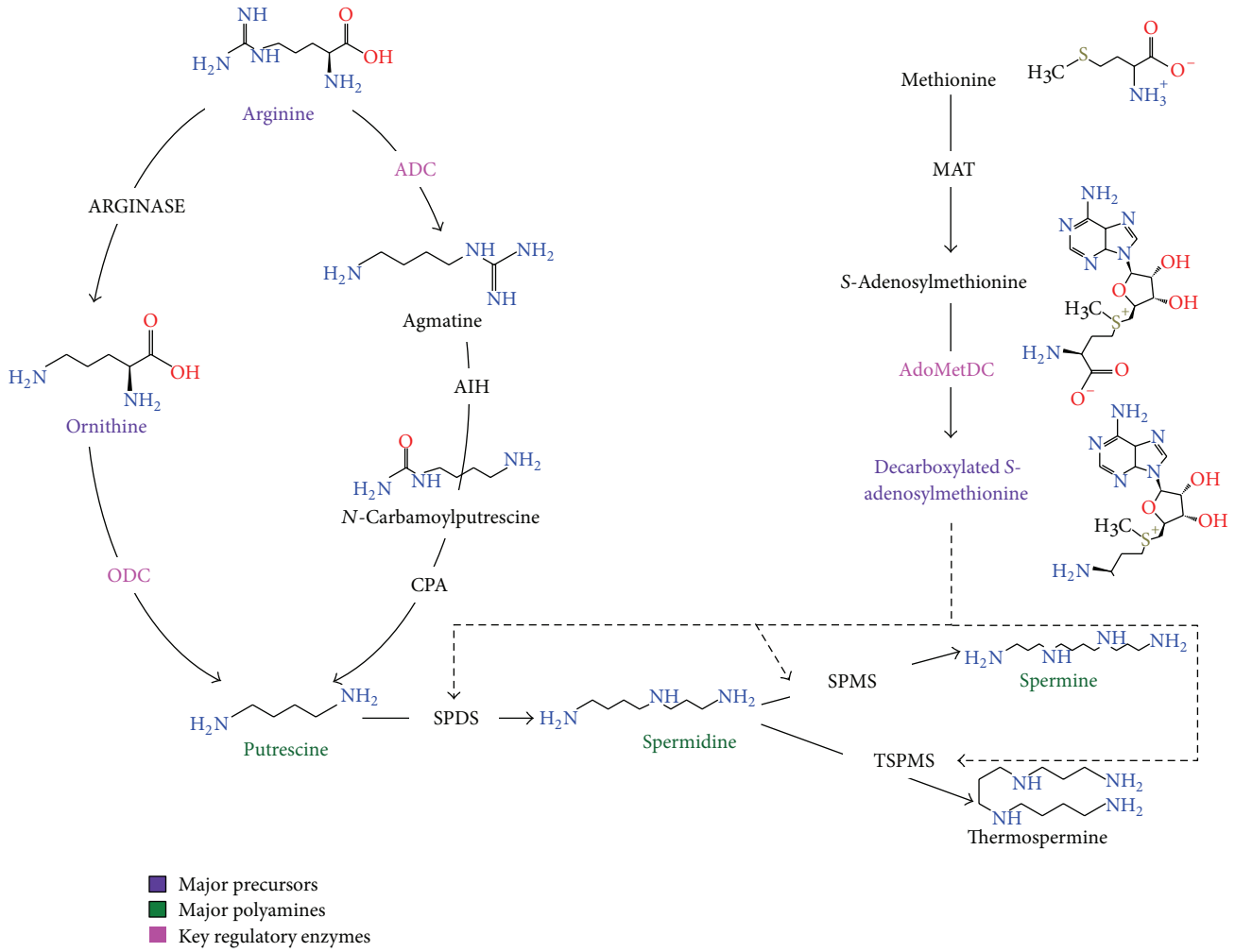

Figure 1: Polyamine biosynthetic pathway with special reference to plants. *ADC*: arginine decarboxylase (EC 4.1.1.9); *AdoMetDC*: *S*-adenosylmethionine decarboxylase (EC 4.1.4.50); *AIH*: agmatine iminohydrolase (EC 3.5.3.12); *CPA*: *N*-carbamoylputrescine amidohydrolase (EC 3.5.1.53); *MAT*: *S*-adenosylmethionine synthetase or methionine adenosyltransferase (EC 2.5.1.6); *ODC*: ornithine decarboxylase (EC 4.1.1.17); *SPDS*: spermidine synthase (EC 2.5.1.16); *SPMS*: spermine synthase (EC 2.5.1.22); and *TSPMS*: thermospermine synthase (EC 2.5.1.79).

plants results in formation of ammonia, H_2O_2, and 4-aminobutanal. 4-Aminobutanal and Δ^1-pyrroline are interconverted spontaneously and by the catalytic action of aminobutyraldehyde dehydrogenase (ABALDH; EC 1.2.1.19; *synonym*: 1-pyrroline dehydrogenase) on Δ^1-pyrroline, γ-aminobutyric acid (GABA) is produced [31] and upon its transamination and oxidation yields succinic acid which enters Krebs cycle [41]. PAOs in plant system oxidize the carbon at the *endo*-side of the N^4-nitrogen of Spd and Spm resulting in the production of 4-aminobutanal and *N*-(3-aminopropyl)-4-aminobutanal, respectively, with H_2O_2 and 1,3-diaminopropane in common thereby leading to the terminal catabolism of polyamines [42]. Many uncommon polyamines like caldopentamine, caldohexamine, homocaldopentamine, homocaldohexamine, norspermidine, and norspermine were produced through the involvement of PAO and known to be involved in ontogeny of various organisms under environmental extremities [43].

Compartmentalization of substrates (polyamines) might play a major role in regulating its catabolic pathway for specific destined physiological processes like lignification, oxidative burst, suberization, and defense mechanisms, as PAO and CuAO are present extracellularly [37, 41]. Tavladoraki and coworkers in 2006 had reported for the first time in plant system the polyamine back-conversion pathway by AtPAO1 through heterologous expression [44], which recent reports also authenticate [45–47]. In case of back-conversion pathway in animal system, spermidine and spermine were acetylated through SSAT (spermidine/spermine N^1-acetyltransferase; EC 2.3.1.57) by using acetyl-CoA and this step was reported to be the rate limiting step of catabolic pathway [48]. So far in plant system, back-conversion of spermine by AtPAO1 was reported to be specific for spermine and occur independently of SSAT, the acetylation step, as in case of animal system [44], though there were reports on the presence of glutamyl and acetyl derivatives of polyamines

in chloroplasts [49] and on the presence of back-conversion pathway (irrespective of SSAT-dependent or -independent) using ^{14}C studies [50, 51]. Rather, spermidine acts as a competitive inhibitor for the conversion of spermine in the presence of AtPAO1 [44]. Activity of AtPAO1 was much lower in conversion efficiency while using N^1-acetylspermine as substrate [44]. Though back-conversion of polyamines in both plant and animal system has been reported, question still remains about the importance of acetylation in back-conversion process with special reference to animal system (in evolutionary view point in comparison with plant system), since acetylation is a prerequisite for back conversion only in animal system [40, 44]. Also, comparison of different plant PAOs (monocot and dicot) with its corresponding counterparts from animal system may help in identifying the actual genetic variation or causal factor for the specificity of back-conversion process only for spermine and not spermidine in plant system.

4. Polyamines and Abiotic Stress

Polyamines modulate the plant's response to much broader range of abiotic stresses than expected, *namely*, drought, salinity, heavy metal toxicity, oxidative stress, chilling injury, high temperature, osmotic stress, water logging, and flooding tolerance as proved either by exogenous application of polyamines or by development of transgenic plants overexpressing the genes involved in polyamine biosynthesis [52]. Modulated levels of polyamines may act either as a signal or as a messenger (to transmit the perceived signals from the sensors) to articulate the plants' behavioral response spatially and temporally in order to avoid or overcome stress. Altered endogenous polyamine (free or conjugated or bound) levels are known to be involved in formation of polyamine-RNA complexes, thereby generating structural changes in RNA (m-, r-, and t-) at physiological concentrations of potassium and magnesium ions [13]. Covalent linkage of polyamines to various enzymes or proteins (posttranslational modification) involved in physiological processes under normal or stressed conditions was catalyzed by transglutaminase (TGase; EC 2.3.2.13) class of enzymes [53, 54].

Of various abiotic environmental stimuli under which polyamines get modulated and thereby its cellular functions were mineral nutrient deficiency [22, 25], metal toxicity [55, 56], salinity [16, 30], high [17] and low temperature [30], drought [4, 6], hypoxia [38], osmotic [16], and oxidative factors [38, 52, 57]. Polyamines, besides responding to external stimuli by their modulated titers, also alter ion channels [11]; stimulate special kind of protein synthesis; stimulate assembly of 30S ribosomal subunits; and stimulate Ile-tRNA formation [13]. Also, modulated titers of polyamines in combination with epibrassinolides, active form of brassinosteroids, were reported to regulate abscisic acid (ABA) and indole-3-acetic acid (IAA) pathways which in turn enhances tolerance to metal toxicity [55]. Besides heavy metals themselves being toxic to plants, they also stimulate oxidative stress due to the fact that heavy metals are basically ionic in nature. Polyamines in combination with brassinosteroids besides modulating ABA and IAA pathways with their cascading effects for heavy metal tolerance also modulate levels of antioxidants like glutathione, ascorbic acid, proline, glycine-betaine, and so forth and antioxidant enzymes like glutathione reductase, superoxide dismutase, catalase, peroxidase, and so forth to impart stress tolerance [56]. Brief overview of polyamines on abiotic stress tolerance was provided in Figure 2. Enhanced levels of polyamines either through exogenous feeding [52] or through heterologous expression of polyamine biosynthetic genes in transgenic plants [58] had been shown to enhance abiotic stress tolerance. However, use of constitutively expressed promoters like CaMV35S, ubiquitin, and actin with polyamine biosynthetic genes towards stress tolerance may produce modulated polyamine levels even under normal conditions resulting in deleterious effects and thereby reducing yield which is a special concern towards agricultural crops [59].

5. Molecular-Genetic Regulation of Polyamines (PAs)

In plants greater accumulation of PAs (Put, Spm, and Spd) during abiotic stress is well documented and is implicated in increased tolerance to abiotic stress [60]. Induction of majority of genes associated with PAs biosynthesis during one or another type of abiotic stresses suggests the plausible functional relation between PAs metabolism and abiotic stress factors [39, 61]. Moreover induction of PAs biosynthetic genes by ABA, a known regulator of stress which acts upstream of PAs biosynthetic pathway [62]; changes in cellular level of PAs during abiotic stress condition [63]; and enhancement of abiotic stress tolerance in many plant species upon exogenous application of PAs [64] all support the strong connection between PAs and stress.

Though a clear picture of roles of PAs in abiotic stress is beginning to emerge, their unequivocal role in imparting abiotic stress tolerance is puzzled/complicated by the fact that substrate of PAs is also shared by other important metabolites such as ethylene, proline, NO, and metabolites associated with N-metabolism [61] as well. The silencing of ethylene biosynthetic genes ACC synthase and ACC oxidase with concomitant increase in PAs (Put and Spd) leads to improved abiotic tolerance in tobacco plant [65]. Also, modulation of arginase (an enzyme of N-metabolism) resulted in alteration of PAs (Put and Spm), thus effecting enhancement of abiotic stress tolerance [66]. Additionally, modulation of PAO, the enzyme associated with PAs catabolism, resulted in salt stress tolerance in tobacco [67].

In addition to the above, the combinatorial network of action among the ABA, NO, and PAs vis-a-vis abiotic stress has also started to unfold the interactomes associated with regulation of abiotic stress tolerance. The observation of ABA induced accumulation of PAs [68] and induction of NO synthesis in different plant species through PAs (Spd and Spm) catabolism by CuAO and PAO [29, 69] during abiotic stress reveals the complex interaction among these three

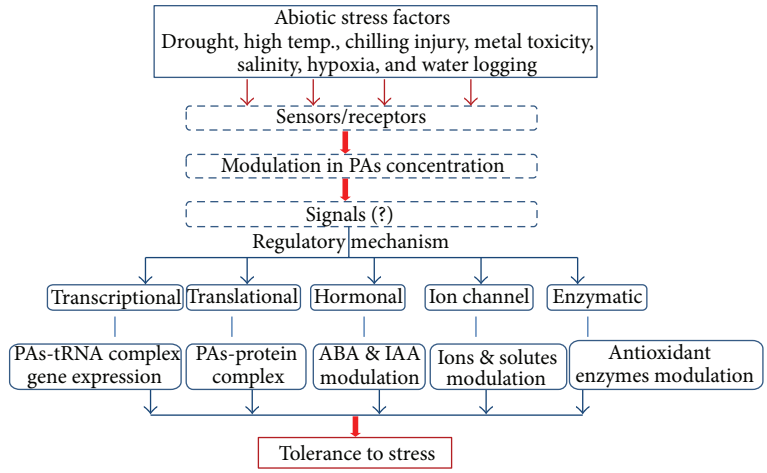

Figure 2: Overview of polyamines (PAs) mediated abiotic stress tolerance in plants.

metabolites, namely, ABA, Pas, and NO. Moreover the cellular balance of PAs in cell is mainly determined through the regulation of its biosynthesis and catabolism. In plants the PAs concentrations are much higher than those of phytohormones; however plant PAs are also regarded as growth regulators due to their diverse role during the course of plant growth and development [70].

Among the factors which modulate cellular concentration of PAs, it is the species nature and their degree of tolerance and sensitivity towards stress are of paramount importance for gaining knowledge and translating such idea into agriculturally important crop species [71]. In this regard, there is lack of sufficient information as how the levels of PAs are being modulated among the allelic variants of PAs and interacting metabolites biosynthetic genes found among natural population of economically important crop species. There are numerous reports on differential expression of genes due to allelic variation within a species [72]. Also, there are reports on altered levels of expression of the same gene in different species [73], thereby exhibiting enormous diversity in expression efficiency. These reports indicate variability at nucleotide level within or between species resulting in altered gene expression levels due to various environmental factors or spatial and temporal expressions. The heterologous expression of *DsAdoMetDC* [74] and *DsAdc* [75] gene from *Datura stramonium* in rice resulted in robust recovery from drought in transgenic rice plants despite having endogenous *AdoMetDC* and *Adc* gene. This indicates that possible sequence variation in genic region regulates expression levels, thereby modulating the efficiency and activity of the enzymes involved in polyamine biosynthesis.

In spite of the huge genetic variation existing in plants, identification and utilization of allelic variation within the species to enhance stress tolerance are still a major area of research that needs a conscious effort. The knowledge gained from genomics assisted discovery of biosynthetic genes of PAs and its signaling pathway in model plant Arabidopsis [68] can well be translated into agriculturally important crops to generate stress tolerant plants [60]. Analyses of variants under natural rather than subjected to controlled condition have proved quite useful in dissecting the novel allele in Arabidopsis as subtle variations in gene expression between individuals are largely unmasked and thus are quite useful approach to uncover functional links between genes and unravel regulatory influences [76]. The above aspect assumes significance as finding a suitable variant tolerant to stress can act as donor through either molecular breeding or genetic engineering approaches for genetic enhancement of stress tolerant traits in targeted crop species.

With the present state of knowledge of PAs mediated enhancement of stress tolerance as demonstrated from molecular-genetic studies (loss or gain of function mutants) and overexpression studies involving biotechnological tools in different plant species, it has become relatively feasible to screen and identify the natural variant within existing collection of plants of a particular crop species. The screening of collection of putative stress tolerant plants for PAs and interacting metabolite profiling using GC-MS, LC-MS, and other appropriate tools can be quite useful for gaining knowledge of overall status of metabolites. Such "targeted" metabolomic studies are quite useful in deciphering function of genes in system based approach [77]. Simultaneously, the genomics approach using expression profiling and SNPs assay of potential genes can further elaborate the functional correlation between metabolite and genes associated with PAs mediated stress tolerance. A brief overview of schematic for such system based analyses is presented (Figure 3). Moreover, integration of both metabolomics and genomics data not only reveals the complex molecular regulation of PAs mediated stress tolerance but also demonstrates how such physiological phenomena are being regulated by metabolites and their fluxes.

6. Conclusion and Future Perspectives

It is possible to assign the functionality to genes/sequences, but, it is much difficult to understand its behavior spatially and temporally in a systems approach manner under different

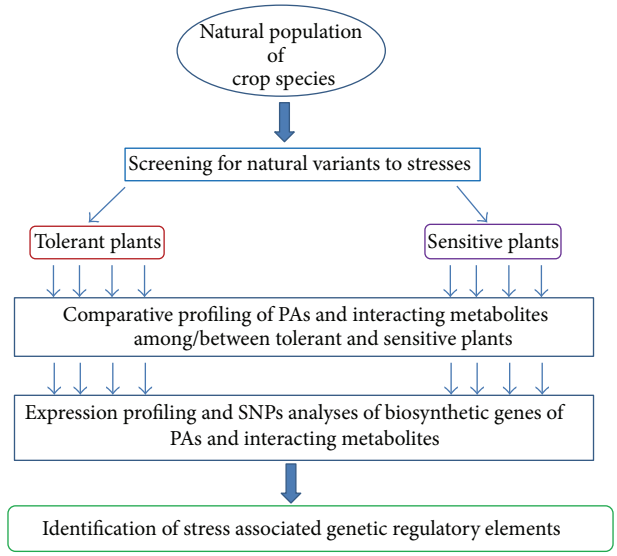

Figure 3: Schematic representation of system based analyses for identification of abiotic stress tolerance regulatory genetic elements.

environmental conditions at species level or even at genotype level. It is mainly due to the altered expression levels for same gene in different species [73] or even due to allelic variation existing between different genotypes within a species or even within a genotype [72]. Recently, understanding about polyamines in response to stress condition in plants had increased significantly. However, to understand the interconnections of PAs biosynthesis, its catabolism, and conjugation along with PAs signaling aspect a detailed metabolite profiling among allelic variants will provide a framework for unraveling genetic determinants (structural/regulatory) with genomic assisted analyses. Additionally, the novel regulatory mechanisms based on small regulatory RNA [78] and uORF [79] can further accelerate our effort to provide clues in reasoning the mysteries involved in basic (back-conversion specificity for spermine in plant system) and applied (modulating polyamines to impart stress tolerance) sciences towards better understanding of polyamine metabolism. Emphasis on the above approaches will provide us with appropriate genomic tools to manage abiotic stress, the immediate effect of climate change, in plant systems with special focus on crop plants. Therefore, with the existence of natural variation, it will be an excellent opportunity to dissect the existing genetic diversity for identifying genotypes possessing compatible allelic variants for imparting stress tolerance in an efficient manner.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] Y. Higashi, N. Ohama, T. Ishikawa et al., "HsfA1d, a protein identified via FOX hunting using *Thellungiella salsuginea* cDNAs improves heat tolerance by regulating heat-stress-responsive gene expression," *Molecular Plant*, vol. 6, no. 2, pp. 411–422, 2013.

[2] D. B. Lobell, W. Schlenker, and J. Costa-Roberts, "Climate trends and global crop production since 1980," *Science*, vol. 333, no. 6042, pp. 616–620, 2011.

[3] T. Ohnishi, M. Nakazono, and N. Tsutsumi, "Abiotic stress," in *Rice Biology in the Genomics Era*, H.-Y. Hirano, A. Hirai, Y. Sano, and T. Sasaki, Eds., vol. 62 of *Biotechnology in Agriculture and Forestry*, pp. 337–355, Springer, Berlin, Germany, 2008.

[4] P. Bhatnagar-Mathur, V. Vadez, and K. K. Sharma, "Transgenic approaches for abiotic stress tolerance in plants: retrospect and prospects," *Plant Cell Reports*, vol. 27, no. 3, pp. 411–424, 2008.

[5] P. T. Evans and R. L. Malmberg, "Do polyamines have roles in plant development?" *Annual Review of Plant Physiology and Plant Molecular Biology*, vol. 40, pp. 235–269, 1989.

[6] R. Alcázar, T. Altabella, F. Marco et al., "Polyamines: molecules with regulatory functions in plant abiotic stress tolerance," *Planta*, vol. 231, no. 6, pp. 1237–1249, 2010.

[7] T. Kusano, K. Yamaguchi, T. Berberich, and Y. Takahashi, "Advances in polyamine research in 2007," *Journal of Plant Research*, vol. 120, no. 3, pp. 345–350, 2007.

[8] D. M. L. Morgan, "Polyamines: an overview," *Molecular Biotechnology*, vol. 11, no. 3, pp. 229–250, 1999.

[9] I. Flink and D. E. Pettijohn, "Polyamines stabilise DNA folds," *Nature*, vol. 253, no. 5486, pp. 62–63, 1975.

[10] T. Kusano, T. Berberich, C. Tateda, and Y. Takahashi, "Polyamines: essential factors for growth and survival," *Planta*, vol. 228, no. 3, pp. 367–381, 2008.

[11] T. Takahashi and J.-I. Kakehi, "Polyamines: ubiquitous polycations with unique roles in growth and stress responses," *Annals of Botany*, vol. 105, no. 1, pp. 1–6, 2010.

[12] C. W. Tabor and H. Tabor, "Polyamines," *Annual Review of Biochemistry*, vol. 53, pp. 749–790, 1984.

[13] K. Igarashi and K. Kashiwagi, "Polyamines: mysterious modulators of cellular functions," *Biochemical and Biophysical Research Communications*, vol. 271, no. 3, pp. 559–564, 2000.

[14] T. Thomas and T. J. Thomas, "Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications," *Cellular and Molecular Life Sciences*, vol. 58, no. 2, pp. 244–258, 2001.

[15] N. Seiler and F. Raul, "Polyamines and apoptosis," *Journal of Cellular and Molecular Medicine*, vol. 9, no. 3, pp. 623–642, 2005.

[16] I. Lefèvre, E. Gratia, and S. Lutts, "Discrimination between the ionic and osmotic components of salt stress in relation to free polyamine level in rice (*Oryza sativa*)," *Plant Science*, vol. 161, no. 5, pp. 943–952, 2001.

[17] T. Oshima, "Unique polyamines produced by an extreme thermophile, *Thermus thermophilus*," *Amino Acids*, vol. 33, no. 2, pp. 367–372, 2007.

[18] N. Bagni and A. Tassoni, "Biosynthesis, oxidation and conjugation of aliphatic polyamines in higher plants," *Amino Acids*, vol. 20, no. 3, pp. 301–317, 2001.

[19] H. P. Bais and G. A. Ravishankar, "Role of polyamines in the ontogeny of plants and their biotechnological applications," *Plant Cell, Tissue and Organ Culture*, vol. 69, no. 1, pp. 1–34, 2002.

[20] R. Kaur-Sawhney, A. F. Tiburcio, T. Altabella, and A. W. Galston, "Polyamines in plants: an overview," *Journal of Cellular and Molecular Biology*, vol. 2, no. 1, pp. 1–12, 2003.

[21] A. Rielander, "Einige neue Bestandteile des Extractum secalis cornuti," *Sitzungsberichte der Gesellschaft zur Beforderung der gesammten Naturwissenschaften zu Marburg*, vol. 7, p. 173, 1908.

[22] F. J. Richards and R. G. Coleman, "Occurrence of putrescine in potassium-deficient barley," *Nature*, vol. 170, no. 4324, article 460, 1952.

[23] E. F. Gale, "The production of amines by bacteria. 3. The production of putrescine from *l*(+)-Arginine by *Bacterium coli* in symbiosis with *Streptococcus faecalis*," *Biochemical Journal*, vol. 34, no. 6, pp. 853–857, 1940.

[24] S. Nakamura, "Uber die spezifität der Arginase (About the specificity of Arginase)," *The Journal of Biochemistry*, vol. 36, no. 1, pp. 243–261, 1944.

[25] R. G. Coleman and M. P. Hegartv, "Metabolism of dl-ornithine-2-14C in normal and potassium-deficient barley," *Nature*, vol. 179, no. 4555, pp. 376–377, 1957.

[26] T. A. Smith and F. J. Richards, "The biosynthesis of putrescine in higher plants and its relation to potassium nutrition," *The Biochemical Journal*, vol. 84, pp. 292–294, 1962.

[27] R. C. Greene, "Incorporation of the carbon chain of methionine into spermidine 1, 2," *Journal of the American Chemical Society*, vol. 79, no. 14, p. 3929, 1957.

[28] H. Tabor, S. M. Rosenthal, and C. W. Tabor, "The biosynthesis of spermidine and spermine from putrescine and methionine.," *The Journal of Biological Chemistry*, vol. 233, no. 4, pp. 907–914, 1958.

[29] R. Wimalasekera, F. Tebartz, and G. F. E. Scherer, "Polyamines, polyamine oxidases and nitric oxide in development, abiotic and biotic stresses," *Plant Science*, vol. 181, no. 5, pp. 593–603, 2011.

[30] I. Hummel, G. Gouesbet, A. El Amrani, A. Aïnouche, and I. Couée, "Characterization of the two arginine decarboxylase (polyamine biosynthesis) paralogues of the endemic subantarctic cruciferous species Pringlea antiscorbutica and analysis of their differential expression during development and response to environmental stress," *Gene*, vol. 342, no. 2, pp. 199–209, 2004.

[31] A. Cona, G. Rea, R. Angelini, R. Federico, and P. Tavladoraki, "Functions of amine oxidases in plant development and defence," *Trends in Plant Science*, vol. 11, no. 2, pp. 80–88, 2006.

[32] K. A. Paschalidis and K. A. Roubelakis-Angelakis, "Sites and regulation of polyamine catabolism in the tobacco plant. Correlations with cell division/expansion, cell cycle progression, and vascular development," *Plant Physiology*, vol. 138, no. 4, pp. 2174–2184, 2005.

[33] G. Su, W. Zhang, and Y. Liu, "Involvement of hydrogen peroxide generated by polyamine oxidative degradation in the development of lateral roots in soybean," *Journal of Integrative Plant Biology*, vol. 48, no. 4, pp. 426–432, 2006.

[34] R. Angelini, M. Bragaloni, R. Federico, A. Infantino, and A. Porta-Puglia, "Involvement of polyamines, diamine oxidase and peroxidase in resistance of chickpea to *Ascochyta rabiei*," *Journal of Plant Physiology*, vol. 142, no. 6, pp. 704–709, 1993.

[35] A. Tisi, R. Angelini, and A. Cona, "Wound healing in plants: cooperation of copper amine oxidase and flavin-containing polyamine oxidase," *Plant Signaling and Behavior*, vol. 3, no. 3, pp. 204–206, 2008.

[36] D. R. Walters, "Polyamines and plant disease," *Phytochemistry*, vol. 64, no. 1, pp. 97–107, 2003.

[37] Z. An, W. Jing, Y. Liu, and W. Zhang, "Hydrogen peroxide generated by copper amine oxidase is involved in abscisic acid-induced stomatal closure in *Vicia faba*," *Journal of Experimental Botany*, vol. 59, no. 4, pp. 815–825, 2008.

[38] P. N. Moschou, K. A. Paschalidis, I. D. Delis et al., "Spermidine exodus and oxidation in the apoplast induced by abiotic stress is responsible for H_2O_2 signatures that direct tolerance responses in tobacco," *Plant Cell*, vol. 20, no. 6, pp. 1708–1724, 2008.

[39] H. Shi and Z. Chan, "Improvement of plant abiotic stress tolerance through modulation of the polyamine pathway," *Journal of Integrative Plant Biology*, vol. 56, no. 2, pp. 114–121, 2014.

[40] S. S. Cohen, *A Guide to the Polyamines*, Oxford University Press, New York, NY, USA, 1998.

[41] G. Rea, M. C. de Pinto, R. Tavazza et al., "Ectopic expression of maize polyamine oxidase and pea copper amine oxidase in the cell wall of tobacco plants," *Plant Physiology*, vol. 134, no. 4, pp. 1414–1426, 2004.

[42] R. Federico and R. Angelini, "Polyamine catabolism in plants," in *Biochemistry and Physiology of Polyamines in Plants*, R. D. Slocum and H. E. Flores, Eds., pp. 41–56, CRC Press, Boca Raton, Fla, USA, 1991.

[43] G. C. Phillips and G. D. Kuehn, "Uncommon polyamines in plants and other organisms," in *Biochemistry and Physiology of Polyamines in Plants*, R. D. Slocum and H. E. Flores, Eds., pp. 121–136, CRC Press, Boca Raton, Fla, USA, 1991.

[44] P. Tavladoraki, M. N. Rossi, G. Saccuti et al., "Heterologous expression and biochemical characterization of a polyamine oxidase from Arabidopsis involved in polyamine back conversion," *Plant Physiology*, vol. 141, no. 4, pp. 1519–1532, 2006.

[45] P. Fincato, P. N. Moschou, V. Spedaletti et al., "Functional diversity inside the Arabidopsis polyamine oxidase gene family," *Journal of Experimental Botany*, vol. 62, no. 3, pp. 1155–1168, 2011.

[46] T. Liu, D. W. Kim, M. Niitsu, T. Berberich, and T. Kusano, "*Oryza sativa* polyamine oxidase 1 back-converts tetraamines, spermine and thermospermine, to spermidine," *Plant Cell Reports*, vol. 33, no. 1, pp. 143–151, 2014.

[47] Y. Ono, D. W. Kim, K. Watanabe et al., "Constitutively and highly expressed *Oryza sativa* polyamine oxidases localize in peroxisomes and catalyze polyamine back conversion," *Amino Acids*, vol. 42, no. 2-3, pp. 867–876, 2012.

[48] H. M. Wallace, A. V. Fraser, and A. Hughes, "A perspective of polyamine metabolism," *The Biochemical Journal*, vol. 376, no. part 1, pp. 1–14, 2003.

[49] S. Del Duca, S. Beninati, and D. Serafini-Fracassini, "Polyamines in chloroplasts: identification of their glutamyl and acetyl derivatives," *Biochemical Journal*, vol. 305, part 1, pp. 233–237, 1995.

[50] M. de Agazio, M. Zacchini, R. Federico, and S. Grego, "Putrescine accumulation in maize roots treated with spermidine: evidence for spermidine to putrescine conversion," *Plant Science*, vol. 111, no. 2, pp. 181–185, 1995.

[51] M. De Agazio, S. Grego, M. Zacchini et al., "1-*N*-acetylspermidine in roots of maize seedlings," *Plant Science*, vol. 121, no. 2, pp. 143–149, 1996.

[52] S. S. Gill and N. Tuteja, "Polyamines and abiotic stress tolerance in plants," *Plant Signaling & Behavior*, vol. 5, no. 1, pp. 26–33, 2010.

[53] S. Beninati, M. Piacentini, M. P. Argento-Cerù, S. Russo-Caia, and F. Autuori, "Presence of di- and polyamines covalently bound to protein in rat liver," *Biochimica et Biophysica Acta*, vol. 841, no. 1, pp. 120–126, 1985.

[54] J. E. Folk, "Transglutaminases," *Annual Review of Biochemistry*, vol. 49, pp. 517–531, 1980.

[55] S. P. Choudhary, H. V. Oral, R. Bhardwaj, J. Yu, and L. P. Tran, "Interaction of brassinosteroids and polyamines enhances copper stress tolerance in raphanus sativus," *Journal of Experimental Botany*, vol. 63, no. 15, pp. 5659–5675, 2012.

[56] S. P. Choudhary, M. Kanwar, R. Bhardwaj, J. Q. Yu, and L. P. Tran, "Chromium stress mitigation by polyamine-brassinosteroid application involves phytohormonal and physiological strategies in raphanus sativus L," *PLoS ONE*, vol. 7, no. 3, Article ID e33210, 2012.

[57] A. Bouchereau, A. Aziz, F. Larher, and J. Martin-Tanguy, "Polyamines and environmental challenges: recent development," *Plant Science*, vol. 140, no. 2, pp. 103–125, 1999.

[58] J.-H. Liu, H. Kitashiba, J. Wang, Y. Ban, and T. Moriguchi, "Polyamines and their ability to provide environmental stress tolerance to plants," *Plant Biotechnology*, vol. 24, no. 1, pp. 117–126, 2007.

[59] S. Katiyar-Agarwal, M. Agarwal, and A. Grover, "Emerging trends in agricultural biotechnology research: use of abiotic stress-induced promoter to drive expression of a stress resistance gene in the transgenic system leads to high level stress tolerance associated with minimal negative effects on growth," *Current Science*, vol. 77, no. 12, pp. 1577–1579, 1999.

[60] P. Ahmad, A. Kumar, A. Gupta, X. Hu, M. M. Azooz, and S. Sharma, "Polyamines: role in plants under abiotic stress," in *Crop Production for Agricultural Improvement*, pp. 491–512, Springer, Amsterdam, The Netherlands, 2012.

[61] H. Shi, T. Ye, and Z. Chan, "Comparative proteomic and physiological analyses reveal the protective effect of exogenous polyamines in the bermudagrass (*Cynodon dactylon*) response to salt and drought stresses," *Journal of Proteome Research*, vol. 12, no. 11, pp. 4807–4864, 2013.

[62] R. Alcázar, J. C. Cuevas, M. Patron, T. Altabella, and A. F. Tiburcio, "Abscisic acid modulates polyamine metabolism under water stress in *Arabidopsis thaliana*," *Physiologia Plantarum*, vol. 128, no. 3, pp. 448–455, 2006.

[63] A. I. Alet, D. H. Sánchez, J. C. Cuevas et al., "New insights into the role of spermine in *Arabidopsis thaliana* under long-term salt stress," *Plant Science*, vol. 182, no. 1, pp. 94–100, 2012.

[64] G. H. M. Sagor, T. Berberich, Y. Takahashi, M. Niitsu, and T. Kusano, "The polyamine spermine protects *Arabidopsis* from heat stress-induced damage by increasing expression of heat shock-related genes," *Transgenic Research*, vol. 22, no. 3, pp. 595–605, 2013.

[65] P. Tavladoraki, A. Cona, R. Federico et al., "Polyamine catabolism: target for antiproliferative therapies in animals and stress tolerance strategies in plants," *Amino Acids*, vol. 42, no. 2-3, pp. 411–426, 2012.

[66] H. Shi, T. Ye, F. Chen et al., "Manipulation of arginase expression modulates abiotic stress tolerance in Arabidopsis: effect on arginine metabolism and ROS accumulation," *Journal of Experimental Botany*, vol. 64, no. 5, pp. 1367–1379, 2013.

[67] P. N. Moschou, J. Wu, A. Cona, P. Tavladoraki, R. Angelini, and K. A. Roubelakis-Angelakis, "The polyamines and their catabolic products are significant players in the turnover of nitrogenous molecules in plants," *Journal of Experimental Botany*, vol. 63, no. 14, pp. 5003–5015, 2012.

[68] R. Alcázar, J. Planas, T. Saxena et al., "Putrescine accumulation confers drought tolerance in transgenic Arabidopsis plants over-expressing the homologous Arginine decarboxylase 2 gene," *Plant Physiology and Biochemistry*, vol. 48, no. 7, pp. 547–552, 2010.

[69] R. Wimalasekera, C. Villar, T. Begum, and G. F. E. Scherer, "COPPER AMINE OXIDASE1 (CuAO1) of arabidopsis thaliana contributes to abscisic acid-and polyamine-induced nitric oxide biosynthesis and abscisic acid signal transduction," *Molecular Plant*, vol. 4, no. 4, pp. 663–678, 2011.

[70] A. B. Menéndez, A. A. Rodriguez, S. J. Maiale, K. M. Rodriguez, B. J. F. Jimenez, and O. A. Ruiz, "Polyamines contribution to the improvement of crop plants tolerance to abiotic stress," in *Crop Improvement Under Adverse Conditions*, pp. 113–136, Springer, New York, NY, USA, 2013.

[71] Q. Fariduddin, P. Varshney, M. Yusuf, and A. Ahmad, "Polyamines: potent modulators of plant responses to stress," *Journal of Plant Interactions*, vol. 8, no. 1, pp. 1–16, 2013.

[72] M. Guo, M. A. Rupe, C. Zinselmeier, J. Habben, B. A. Bowen, and O. S. Smith, "Allelic variation of gene expression in maize hybrids," *The Plant Cell*, vol. 16, no. 7, pp. 1707–1716, 2004.

[73] C. Wang, X. Fan, G. Wang, J. Niu, and B. Zhou, "Differential expression of Rubisco in sporophytes and gametophytes of some marine microalgae," *PLoS ONE*, vol. 6, no. 1, Article ID e16351, 2011.

[74] A. Peremarti, L. Bassie, P. Christou, and T. Capell, "Spermine facilitates recovery from drought but does not confer drought tolerance in transgenic rice plants expressing Datura stramonium S-adenosylmethionine decarboxylase," *Plant Molecular Biology*, vol. 70, no. 3, pp. 253–264, 2009.

[75] T. Capell, L. Bassie, and P. Christou, "Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 26, pp. 9909–9914, 2004.

[76] R. Bhosale, J. B. Jewell, J. Hollunder et al., "Predicting gene function from uncontrolled expression variation among individual

wild-type Arabidopsis plants," *The Plant Cell*, vol. 25, no. 8, pp. 2865–2877, 2013.

[77] T. Obata and A. R. Fernie, "The use of metabolomics to dissect plant responses to abiotic stresses," *Cellular and Molecular Life Sciences*, vol. 69, no. 19, pp. 3225–3243, 2012.

[78] V. Ambros and X. Chen, "The regulation of genes and genomes by small RNAs," *Development*, vol. 134, no. 9, pp. 1635–1641, 2007.

[79] M. L. Guerrero-Gonzalez, M. Rodriguez-Kessler, and J. F. Jimenez-Bremont, "uORF, a regulatory mechanism of the Arabidopsis polyamine oxidase 2," *Molecular Biology Reports*, vol. 41, no. 4, pp. 2427–2443, 2014.

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Research Article

Phosphate/Zinc Interaction Analysis in Two Lettuce Varieties Reveals Contrasting Effects on Biomass, Photosynthesis, and Dynamics of Pi Transport

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Inorganic phosphate (Pi) and Zinc (Zn) are essential nutrients for normal plant growth. Interaction between these elements has been observed in many crop plants. Despite its agronomic importance, the biological significance and genetic basis of this interaction remain largely unknown. Here we examined the Pi/Zn interaction in two lettuce (*Lactuca sativa*) varieties, namely, "Paris Island Cos" and "Kordaat." The effects of variation in Pi and Zn supply were assessed on biomass and photosynthesis for each variety. Paris Island Cos displayed better growth and photosynthesis compared to Kordaat under all the conditions tested. Correlation analysis was performed to determine the interconnectivity between Pi and Zn intracellular contents in both varieties. Paris Island Cos showed a strong negative correlation between the accumulation levels of Pi and Zn in shoots and roots. However, no relation was observed for Kordaat. The increase of Zn concentration in the medium causes a decrease in dynamics of Pi transport in Paris Island Cos, but not in Kordaat plants. Taken together, results revealed a contrasting behavior between the two lettuce varieties in terms of the coregulation of Pi and Zn homeostasis and provided evidence in favor of a genetic basis for the interconnection of these two elements.

1. Introduction

Zinc (Zn) and phosphorous (P) are important micro- and macronutrients required for optimal plants growth [1–4]. Plants absorb these elements from the soil solution using root system. Often the concentration of these elements in agriculture soil is very low, thus causing Zn and Pi deficiency in plants which negatively affects plants metabolism and photosynthesis [5]. Worldwide agriculture has become dependent on external sources of Zn and Pi fertilizers in order to address the issue of sustainable food resources for the growing world population. Nevertheless, this strategy has adverse economic and ecological impacts, particularly for Pi. It is predicted that high-grade and easily-extractable Pi from rocks will be exhausted [6]. Therefore, substantial efforts have been made to improve Zn and Pi nutrition in plants based on our current understanding on how plants respond to the deficiency of each individual element. However, in practice, application of such knowledge is hindered by complex cross-talks, which are emerging in the face of evidences overwhelmingly showing that Zn and Pi nutrition are interrelated, which likely to sustain plants growth and development. Lines of evidences support the fact that Pi-Zn interaction occurs within the plant [7–12]. Such interconnections have

consequences on comprehending the regulation of Zn and Pi homeostasis and can account for shortcomings of current agronomic models that are typically focused to improve the assimilation of individual elements.

Zn availability or its absence in the medium can either increase or decrease the accumulation of Pi in plants, respectively [10, 12, 13]. The positive effects of Zn deficiency on the Pi uptake by roots and its overaccumulation in leaves have been observed in numerous plant species such as tomato [14], okra [15] and cotton [7], and barley [9]. Lately such effect was reported in Arabidopsis [12]. The specificity of the Zn-Pi relationship has been further demonstrated by the fact that in barley only Zn deficiency could induce Pi uptake and not nitrogen, sulfur, nor manganese deficiency [9]. Similarly, cotton or tomato plants do not show an overaccumulation of Pi under iron or copper deficiency [7, 16]. It seems that plants lose the capacity to regulate Pi homeostasis under Zn deficiency and can overaccumulate Pi in shoots under high Pi concentrations leading to phytotoxic symptoms [7]. Excessive Zn application has been shown to decrease Pi concentration in plants [17, 18]. Nevertheless, the underlying mechanisms for the Pi-Zn homeostasis interaction *in planta* remain to be deciphered, which is of primary importance to improve the Pi and Zn nutrition in vegetable crops using agronomical/biotechnological programs together with an appropriate fertilizer management schemes.

Among cultivated plant species, lettuce *Lactuca sativa* (family *Asteraceae*) is a major vegetable in western countries [19]. Lettuce is the second most consumed fresh vegetable in the USA at 28.0 pounds per capita in 2008, behind potato at 36.7 pounds [19]. Lettuce has limited roots and rapid top growth requires high levels of P supply for maintaining proper growth. As aforementioned, the excessive use of Pi fertilizers contributes in lowering Zn concentrations in soil [20] and more adversely can favor the uptake of other heavy metals [21–23]. Thus improving the Pi use efficiency, while maintaining an appropriate level of Zn in lettuce, is of primary importance for sustainable agriculture. Lettuce varieties with contrasting features for essential nutrients accumulation may constitute a good plant material to study the Pi and Zn nutrition.

Two lettuce varieties, namely, Paris Island Cos and Kordaat, exhibiting contrasting features for the characters of heavy metal accumulation [23] were considered to investigate Zn and Pi interaction. The effects of the Pi and/or Zn treatments on the growth capacity and accumulation of these ions in the shoots and roots were determined. Photosynthesis and stomatal conductance was assessed under each stress condition. Zn deficiency influence on Pi uptake and translocation in the lettuce varieties was studied using ^{33}P isotope. Results revealed differential regulation of Zn-Pi homeostasis interaction in the two lettuce varieties.

2. Materials and Methods

2.1. Plant Material and Growth Conditions. Two varieties of *L. sativa* (lettuce) considered for this work were Paris Island Cos and Kordaat. Lettuce seeds were germinated on top of humidified paper (Whatman) with distilled water for 3 days and then with modified Hoagland nutrient (2.5 mM KNO_3, 0.5 mM NaH_2PO_4, 2.5 mM$Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.1 mM FeIIINaEDTA, 0.05 mM H_3BO_3, 0.05 mM $MnSO_4$, 15 μM $ZnSO_4$, 3 μM Na_2MoO_4, 2.5 μM KI, 0.05 μM $CuSO_4$, and 0.044 μM $CoCl_2$) for 7 additional days. Seedlings were carefully transferred to 9-L tanks containing the same nutrient solution. 10 days later, plants were treated with different Zn (0, 15, 90, 360, 1440, and 2880 μM) and Pi (0 and 500 μM) concentrations for eight additional days. Plants were grown in a growth chamber under the following environmental conditions: light/dark cycle of 8 h/16 h with light intensity being 250 μmol·m^{-2}·s^{-1}, temperature of 20°C, and relative humidity of 65%. Nutritive solutions were renewed every 4 days during the whole experiment. Analyses were performed on separated shoots and roots of individual plants.

2.2. Zinc and Phosphate Contents Measurement. Zn concentration was determined using dried plant samples. The digestion and extraction were done using hydrogen peroxide and nitric acid as described in [23]. Concentrations of Zn in the extracts were determined by atomic absorption spectrophotometry (SpectrAA 220, Varian, Australia). Pi measurements were performed as described by [24]. Briefly, the extraction was performed on fresh shoots and roots samples by incubating in ultrapure water at 70°C for 30 min. Pi content was evaluated by colorimetry at 820 nm using the molybdate assay, according to the procedure of [25].

2.3. Phosphate Uptake and Transfer Measurements. Phosphate uptake and root-to-shoot transfer measurements were performed using whole lettuce plants grown hydroponically, after germination stage for ten days, and for additional ten days in different Zn concentrations (0, 15, 90, and 180 μM) containing 500 μM PO_4^{2-}. For root influx and root-to-shoot translocation, roots of whole plants were placed in Na_2PO_4 solution at pH 5.0 in the presence of 10 μCi/mL of the radiotracer ^{33}P-Orthophosphoric Acid (PerkinElmer) for 5 min and 2:30 h, respectively. Lettuce plants were then washed in an ice-cold 5 mM Na_2PO_4 solution and then shoots and roots were harvested separately, dried, and the radioactivity was measured using scintillation counting [24]. Root-to-shoot Pi transport was expressed as the percentage of radioactivity located in the shoot over the total amount of radioactivity in the whole lettuce plant.

2.4. Measurements of Photosynthesis and Stomatal Conductance. Leaf net photosynthetic rate (A, μmol fixed CO_2 m^{-2}·s^{-1}) and stomatal conductance (B, mol·m^{-2}·s^{-1}) rates were measured on the third fully expanded leaves using a portable photosynthesis system with a light-emitting diode light source (LI-6400, LI-COR, Inc.; Lincoln, NE) according to the manufacturer's protocol. Experiments were performed under controlled conditions (20°C, 65% relative humidity, and controlled CO_2 supply of 400 μmol mol^{-1}) with photon flux density 225 μmol·m^{-2} s^{-1}.

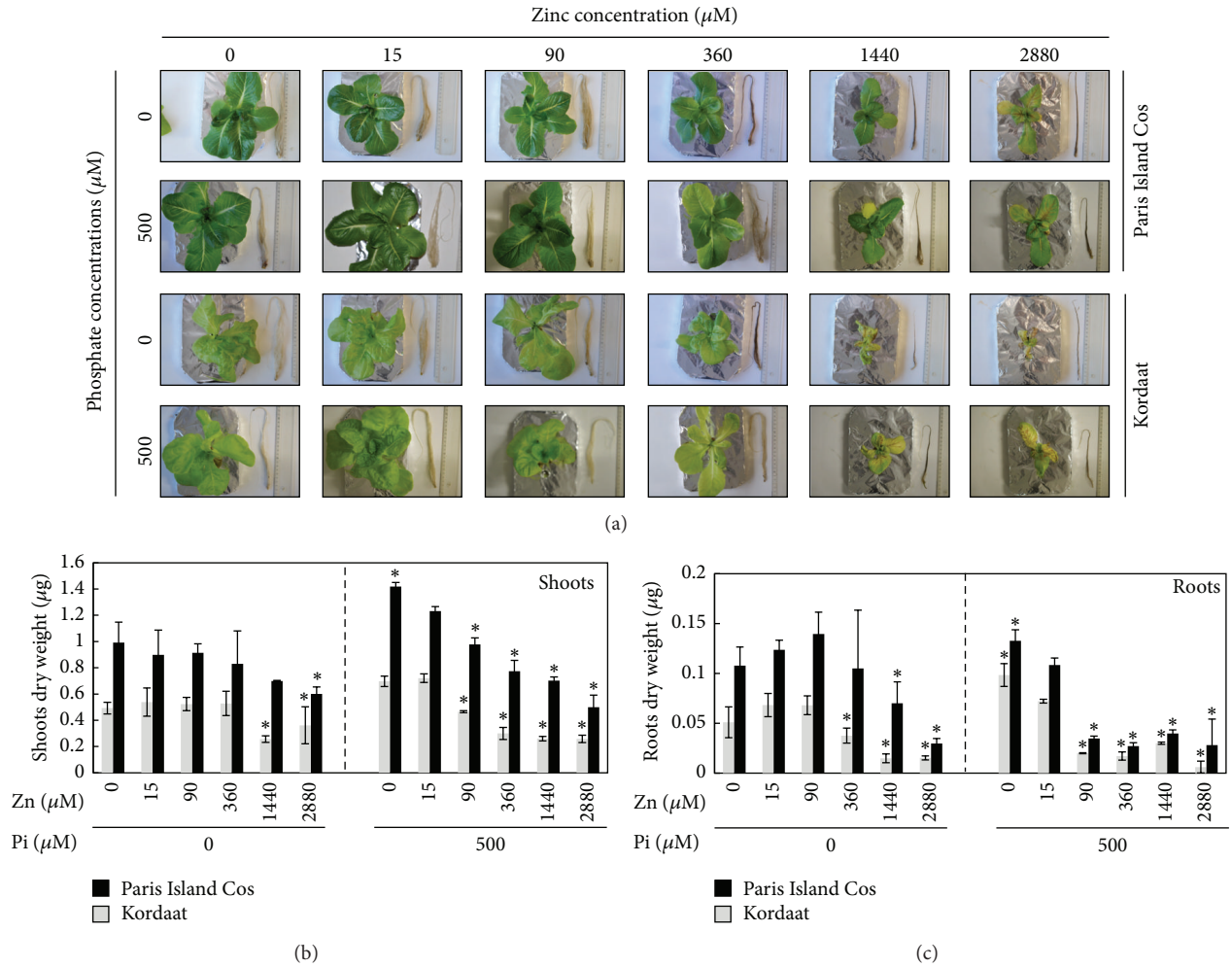

Figure 1: Zinc and phosphate treatment significantly alters Paris Island Cos and Kordaat growth capacity. 30-day-old Paris Island Cos and Kordaat lettuce varieties were grown and exposed to various zinc and phosphate concentrations in the culture medium (a). Shoot (b) and root (c) dry weight measured in different growth conditions for the Paris Island Cos and Kordaat lettuce varieties. Results are averages of three replicates ± SE. Asterisks indicate statistically significant differences compared to either 0 μM of Pi, 15 μM Zn (left parts of (b) and (c)) or 500 μM of Pi, 15 μM Zn (right parts of (b) and (c)) treatments of each lettuce variety ($P \leq 0.05$).

2.5. Statistical Analysis. Data are presented as means of at least three independent experiments and mean values were taken for statistical analyses using one-way ANOVA. Significant differences were further analyzed using Tukey's parametric or nonparametric tests to identify differences between treatments and/or varieties. The differences were considered significant if $P \leq 0.05$.

3. Results and Discussion

3.1. Paris Island Cos and Kordaat Varieties Displayed Differential Biomass Production and Photosynthesis. The Zn availability can adversely alter plant growth in situations where it is present in either too low (deficiency) or too high (toxicity) concentration [16]. The plant biomass production is also altered in case of low Pi supply [2]. Such symptoms are often described without taking into account the bioavailability of one or the other element. Herein, we investigated the effects of varying external concentrations of Zn and/or Pi on the growth capacity of two lettuce varieties, namely, Paris Island Cos and Kordaat. Lettuce plants were grown hydroponically under control condition (500 μM Pi and 15 μM Zn) for one week then transferred on 12 different mediums resulting from the combination of two Pi (0 and 500 μM) and six Zn (0, 15, 90, 360, 1440, and 2880 μM) concentrations. The dry weight of 4-week-old plants were determined (Figure 1). Results show that the increase of Zn concentration in the medium leads to the reduction of shoot and root dry weight in both varieties (Figures 1(b) and 1(c)). The most visible Zn toxicity symptoms included decrease in leaf size and appearance of necrosis (Figure 1(a)). Under high Zn concentration (1,440 mM and 2,880 mM), no shoots growth pattern was observed for both varieties (Figure 1(a)). The presence or absence of Pi from the medium mitigates or aggravates the Zn toxicity (Figure 1(a)). Nevertheless, overall Paris Island Cos displayed a better growth capacity compared to Kordaat under all the conditions tested (Figure 1). A part of the explanation of the observed effects of Pi and Zn nutrition

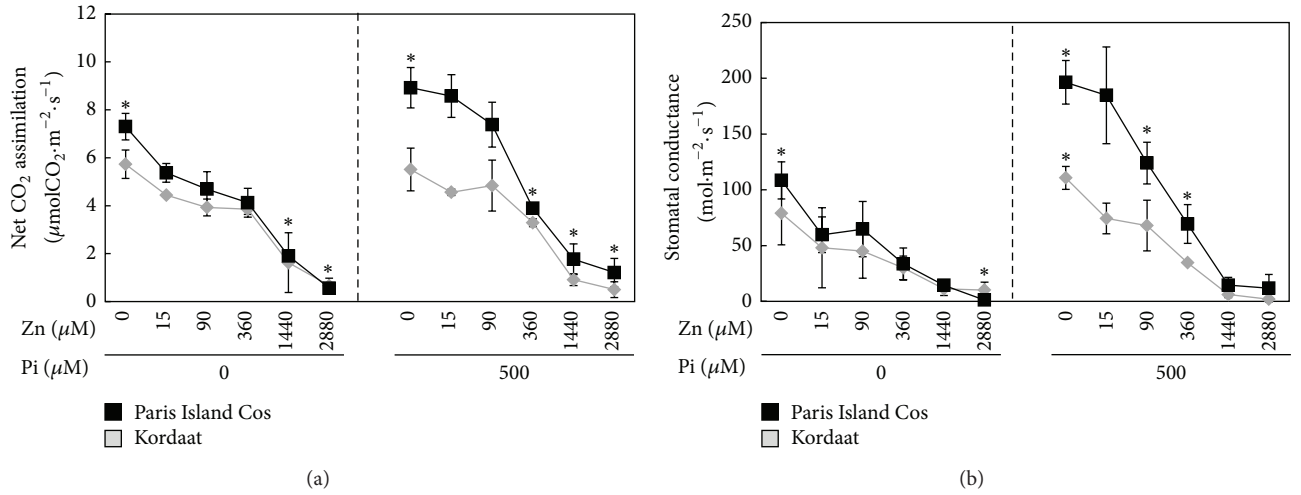

Figure 2: Varying zinc and phosphate concentrations in the culture medium alter the photosynthetic potential of the two lettuce varieties. Photosynthesis (a) and stomatal conductance (b) were measured for the Paris Island Cos and Kordaat lettuce varieties on third fully expanded leaves of each plant. Individual measurements were obtained from a pool of "n" plants ($n \geq 3$). Error bars indicate SD.

on the growth capacity of both lettuce varieties could be an alteration in photosynthesis. Indeed, both elements are known for their influences on this vital process. On one hand, Pi participates in plant photosynthesis in the form of ATP, which supplies energy for the CO_2 fixation [26]. Zn role as an essential constituent of enzymes important to photosynthesis, such as the carbonic anhydrase, is well documented [27]. Zn excess affects the photochemical reactions of the photosystems [27]. To test this hypothesis, the net CO_2 assimilation and stomatal conductance were assessed in Paris Island Cos and Kordaat grown on the aforementioned 12 growth conditions. Results revealed that in contrast to Kordaat plants, the photosynthesis was severely affected in Paris Island Cos in response to Pi deficiency and showed a strong reduction when grown under 0 μM Pi and 15 μM Zn compared to control condition (500 μM Pi and 15 μM Zn) (Figure 2). This result is in agreement with previous one in maize (*Z. mays*) or with bean (*P. vulgaris*) plants, in which plants grown in low Pi condition resulted in decline of the photosynthetic rate by 68% and 50% compared to the control plants, respectively [28, 29]. Results presented in Figure 2(a) shows that net CO_2 assimilation decreased with increasing Zn concentration in the medium for both lettuce varieties. These observations suggest that the registered growth reduction for both lettuce varieties is the overall effect of Zn toxicity which may be related to the major effect of Zn toxicity on inhibition of photosynthesis. Interestingly, the presence of Pi contributes to the alleviating effect of Zn excess on this process. While both varieties behave similarly regardless of Zn concentration in the absence of Pi (Figures 2(a) and 2(b)), they exhibited contrasting behavior in presence of Pi. Paris Island Cos grown in presence of 500 μM of Pi and 0, 15, or 90 μM of Zn showed significantly higher CO_2 assimilation and stomatal conductance than Kordaat. Although high Zn concentration strongly reduced the above parameters in both varieties, overall Paris Island Cos was more superior than Kordaat which can be credited to its improved growth performance and likely to a better Pi use efficiency (Figures 1(a), 1(b), and 1(c)).

3.2. Effect of Zn and Pi Supply on Their Endogenous Content in Shoots and Roots. As already mentioned, earlier studies have reported that Zn deficiency leads to the overaccumulation of Pi in the shoot, and inversely [12, 13]. In different plant species, a negative correlation between tissue Pi and Zn treatments has been observed. However, the nature of this correlation in conditions where Zn and/or Pi treatments vary from the depletion to the excess in the medium is poorly documented. We have determined the accumulation level of intracellular Zn and Pi contents in the two lettuce varieties. The observed typical Zn toxicity symptoms described above (Figure 1(a)) correspond to the highest shoot Zn accumulation in both of the varieties. The Zn overaccumulation can be ascribed to an impaired control mechanism of Zn uptake and release from root cells to xylem due to the altered morphology of roots under Zn excess in the medium [30, 31]. Such dysfunctional roots can also explain the high Pi accumulation in roots exposed to high Zn concentration (Table 1). Under control conditions, (15 μM Zn; 500 μM Pi) the Paris Island Cos and Kordaat accumulated 9.79 ± 0.15 μmol·g^{-1} FW and 6.11 ± 0.9 μmol·g^{-1} FW of Pi, respectively. Both varieties accumulate similar Zn concentrations in shoots (≈0.01 μg·g^{-1} DW). Interestingly, Zn treatment was found to significantly affect the shoots Pi content in Paris Island Cos which got decreased as the Zn concentration increased in the medium (Table 1). Pi content was at maximum under Zn deficiency (11.02 ± 2.18 μmol·g^{-1} FW) and dropped significantly (4.31 ± 0.17 μmol·g^{-1} FW) in presence of 1,440 mM of Zn (Table 1). This result is in agreement with previous studies that have shown that Zn deficient plants can overaccumulate Pi, such as cotton [7] and barley [9]. Results revealed that variation in Zn supply affected differentially the accumulation of Pi in the shoot and roots of the two lettuce

Table 1: Zinc and phosphate contents in Paris Island Cos and Kordaat submitted to zinc and phosphate treatments.

Tissues	Pi in shoots		Pi in roots	
	Kordaat	Paris Island Cos	Kordaat	Paris Island Cos
Treatments				
0 Zn, 0 Pi	2.13 ± 0.28	2.04 ± 0.11	14.77 ± 5.62	6.18 ± 0.68
15 Zn, 0 Pi	2.61 ± 0.69	2.65 ± 1.25	8.84 ± 3.34	10.25 ± 5.53
90 Zn, 0 Pi	1.79 ± 0.21	1.80 ± 0.26	11.16 ± 1.41	9.65 ± 2.49
360 Zn, 0 Pi	2.49 ± 0.29	3.10 ± 0.97	8.14 ± 4.00	16.32 ± 2.28
1440 Zn, 0 Pi	7.24 ± 2.34	4.16 ± 1.11	32.65 ± 6.02	18.18 ± 0.45
2880 Zn, 0 Pi	15.63 ± 2.80	6.47 ± 1.74	18.39 ± 13.02	8.48 ± 4.02
0 Zn, 500 Pi	6.16 ± 0.79	11.02 ± 2.18	136.17 ± 8.19	146.78 ± 7.03
15 Zn, 500 Pi	6.11 ± 0.91	9.79 ± 0.15	144.04 ± 4.40	206.07 ± 54.35
90 Zn, 500 Pi	5.58 ± 2.49	5.81 ± 4.18	40.53 ± 10.04	48.78 ± 22.37
360 Zn, 500 Pi	8.68 ± 2.76	4.39 ± 0.81	86.48 ± 11.74	46.27 ± 7.65
1440 Zn, 500 Pi	4.73 ± 0.74	4.31 ± 0.17	90.33 ± 10.09	113.49 ± 30.62
2880 Zn, 500 Pi	18.72 ± 2.39	8.62 ± 1.57	67.59 ± 25.04	109.63 ± 23.85
	Zn in shoots		**Zn in roots**	
	Kordaat	Paris Island Cos	Kordaat	Paris Island Cos
0 Zn, 0 Pi	0.02 ± 0.00	0.01 ± 0.00	0.03 ± 0.02	0.02 ± 0.00
15 Zn, 0 Pi	0.03 ± 0.00	0.03 ± 0.01	0.07 ± 0.02	0.07 ± 0.01
90 Zn, 0 Pi	0.22 ± 0.04	0.11 ± 0.05	1.16 ± 0.54	0.46 ± 0.23
360 Zn, 0 Pi	0.24 ± 0.03	0.13 ± 0.07	5.74 ± 5.80	5.01 ± 1.28
1440 Zn, 0 Pi	4.60 ± 2.46	2.58 ± 0.87	200.75 ± 165.78	2642.38 ± 1942.29
2880 Zn, 0 Pi	2.99 ± 0.38	3.00 ± 0.46	512.96 ± 121.85	480.58 ± 92.58
0 Zn, 500 Pi	0.03 ± 0.04	0.01 ± 0.00	0.05 ± 0.03	0.03 ± 0.01
15 Zn, 500 Pi	0.01 ± 0.00	0.01 ± 0.00	0.08 ± 0.03	0.05 ± 0.01
90 Zn, 500 Pi	0.28 ± 0.14	0.45 ± 0.43	0.22 ± 0.07	0.15 ± 0.02
360 Zn, 500 Pi	1.30 ± 0.97	0.43 ± 0.43	0.31 ± 0.03	0.17 ± 0.02
1440 Zn, 500 Pi	0.72 ± 0.62	0.24 ± 0.12	8.32 ± 1.92	7.15 ± 246.13
2880 Zn, 500 Pi	0.38 ± 0.04	0.14 ± 0.04	263.92 ± 316.46	288.12 ± 4.39

Individual measurements were obtained from the analysis of shoots or roots collected from a pool of "n" plants ($n \geq 3$). ± indicate SD.

varieties. A correlation analysis was performed to determine the interconnectivity of Zn and Pi in the plants (Figure 3). Interestingly, a strong correlation was found between the Zn concentration in the shoot and the Pi concentration in either the root or the shoot of Paris Island Cos (Figures 3(c) and 3(d)), but not in Kordaat variety. Our data thus supported the role of shoot Zn content on the regulation of Pi content in Paris Island Cos, but not for the Kordaat variety. The contrasting behavior between Paris Island Cos and Kordaat may be explained by a difference in regulating Pi uptake and translocation in response to Zn availability. These results revealed that Pi-Zn homeostasis interaction may vary even within the same plant species, which pave the way for a genetic study for cloning quantitative trait loci (QTL)/gene(s) governing these traits. This approach has been successfully used to identify QTL for Pi and Zn in wheat, which appeared to be colocalized [32].

3.3. Differential Effect of Zn Supply on Pi Transport Dynamic in Paris Island Cos and Kordaat. It has been proposed that Zn deficiency may depress root Pi uptake and may also be involved in a high rate of Pi transfer to the shoot, leading to its overaccumulation in shoots [10, 14, 15]. In wheat, Zn deficiency increases the roots membrane permeability for Pi [33]. In this study, the dynamics of Pi transport was examined for lettuce plants grown in the presence of constant concentration of Pi (500 μM) and changed concentration of Zn from 0 to 180 μM using radiolabeled ^{33}Pi. Our results also showed that increasing the Zn concentration had limited effect on the Pi uptake and translocation capacity of Kordaat (Figures 4(a) and 4(b)). However, increased Zn concentration reduced both Pi uptake and transfer of Pi to the shoots in Paris Island Cos (Figures 4(a) and 4(b)). This result is in line with previous studies in many plants species such as cotton [7], barley [9], and wheat [10] showing that the feedback control mechanism from the shoots was impaired thusly suppressing the uptake and translocation rate of Pi at high P concentration in the shoots under Zn deficiency. The fact of whether the low Zn content may also limit the redistribution of Pi from shoot to root needs further investigations (Figure 5). At the molecular level, genes and precise mechanisms underlying this process remain to be identified. Huang et al., 2000 have provided evidence for the involvement of the high

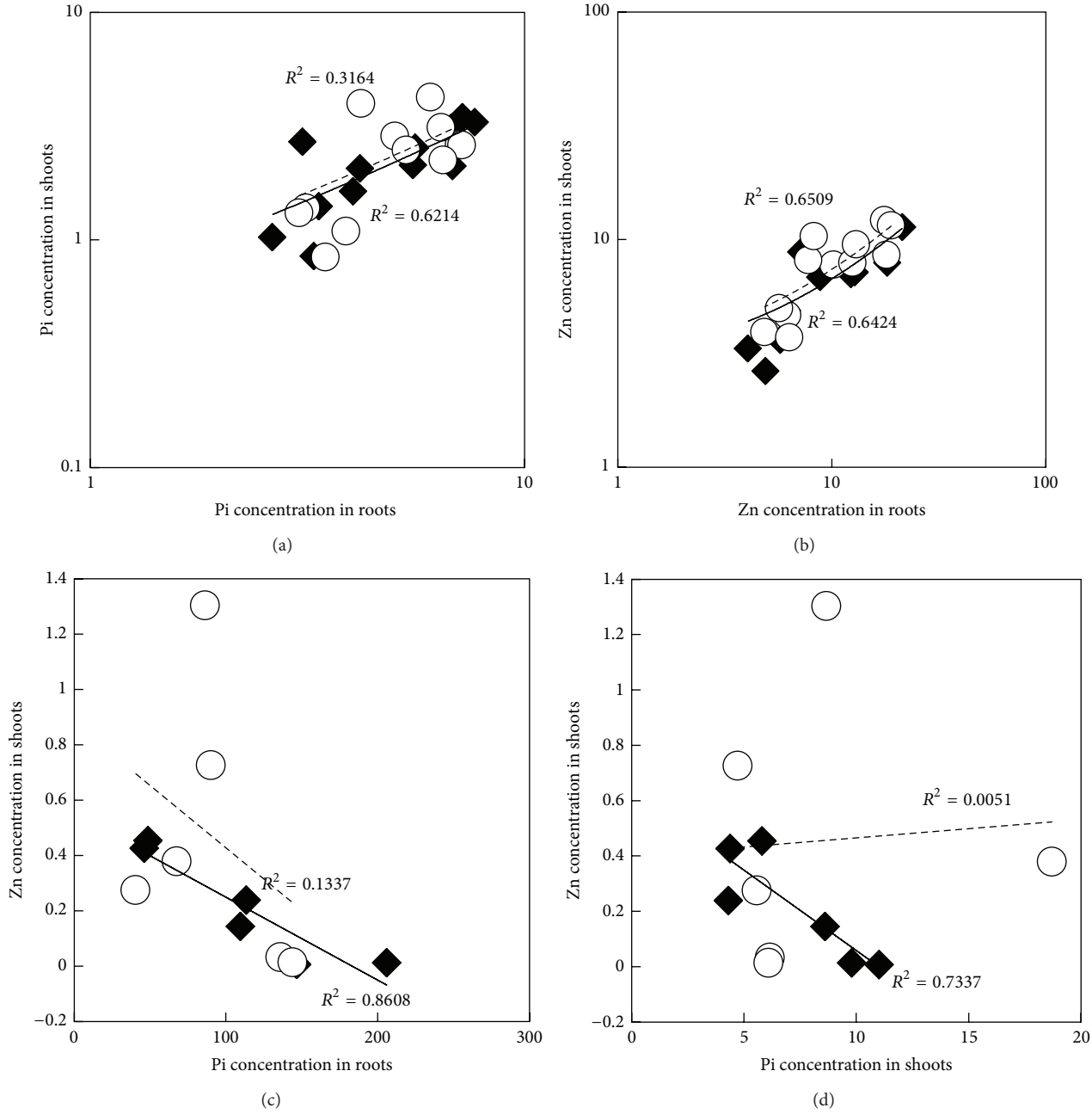

Figure 3: Correlation between zinc and inorganic phosphate in Paris Island Cos and Kordaat. Zinc and inorganic phosphate contents were determined in shoots and roots of the two lettuce varieties (◯, Kordaat; ◆ Paris Island Cos) grown in the presence of 500 μM of Pi and changing Zn concentrations (0, 15, 90, 360, 1440, or 2880 μM). Correlation between Pi content in shoots and Pi content in roots (a). Correlation between Zn content in shoots and roots (b) Correlation between Zn content in shoots and Pi content in roots (c). Correlation between Zn content in shoots and Pi content in shoots (d). Lines correspond to linear regression. For each regression, the square of Pearson's correlation coefficient (R^2) is reported.

affinity Pi transporter (PHT) in the increase of Pi uptake in barley Pi-deficient plants. These results [11, 12] showed that Zn deficiency could induce the expression of the PHT1;1 in Arabidopsis. Recently, genes that are necessary for the increase in Pi overaccumulation in response to Zn deficiency in Arabidopsis have been identified, namely, the Pi exporter *PHO1* and its homologue *PHO1;H3* [12]. In Arabidopsis, *PHO1* gene is predominantly expressed in the root vascular system and it is involved in Pi loading into root xylem. *PHO1;H3* is involved in the control of Pi accumulation in response to Zn deficiency in Arabidopsis. The fact of whether the homologue of these Arabidopsis genes (*PHO1s* and *PHTs*) in lettuce is also involved in the regulation of Pi uptake and its transfer from root to shoot under Zn deficiency

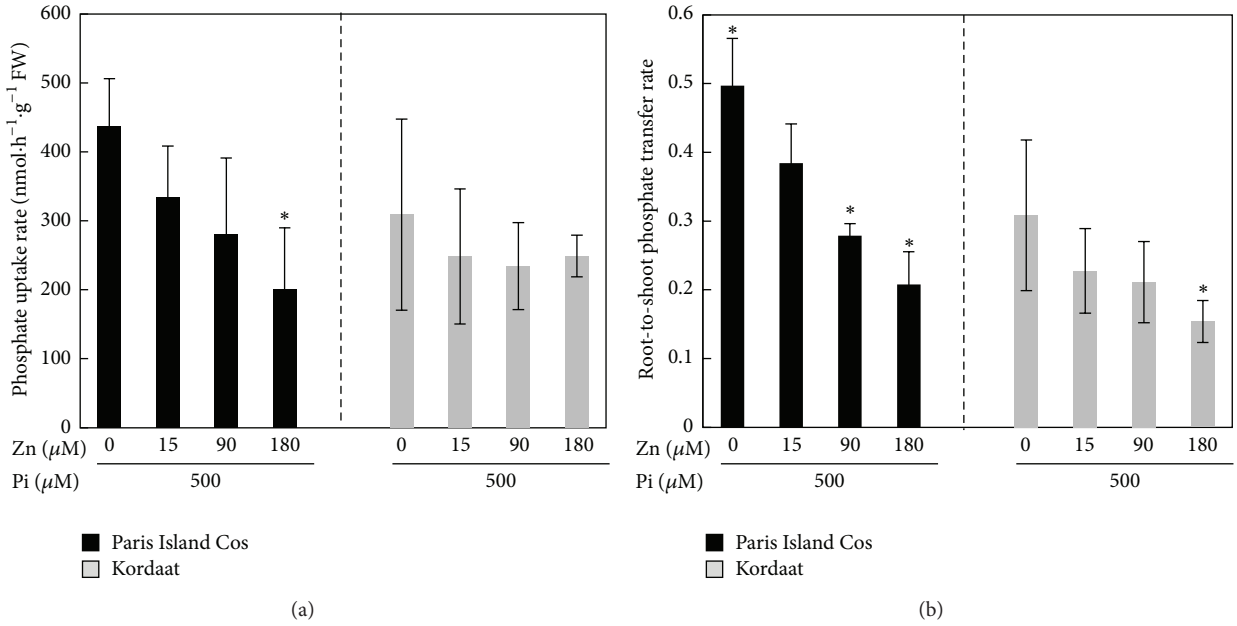

FIGURE 4: Zinc deficiency affects phosphate transport dynamics. Paris Island Cos and Kordaat lettuce varieties were grown hydroponically in media with various concentrations of zinc and inorganic phosphate (Pi). Plant uptake is defined as μmol of Pi acquired by the whole plant per g of root fresh weight per hour. Pi root-to-shoot transfer is defined as the ratio of radioactive Pi in the shoot over the total radioactive Pi in the plant. Individual measurements were obtained from the analysis of shoots or roots collected from a pool of "n" plants ($n \geq 3$). Error bars indicate SD.

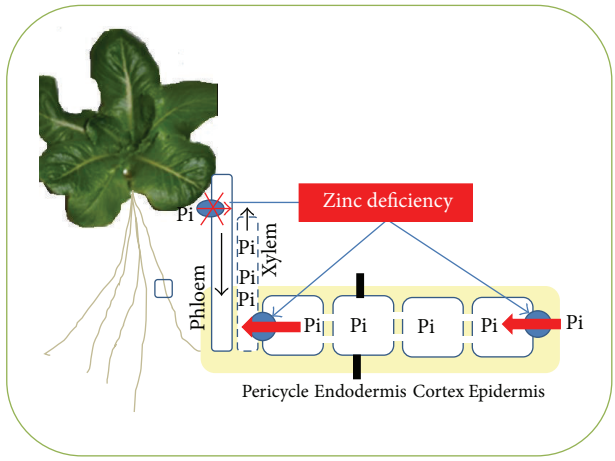

FIGURE 5: Schematic representation of the regulation of Pi transport within lettuce plant; case of Paris Island Cos. Pi is acquired into root by PHT1s. Transport into the xylem, likely through the Pi exporter PHO1s. The Zn deficiency leads to the increase of Pi uptake and is loading into root xylem. Zn deficiency might lead also to the inhibition of Pi shoot-to-root transfer via unknown protein.

is under investigation (Figure 5). This understanding on Pi metabolism is possible; thanks are due to the availability of the lettuce genome sequence, which has proved to be a great asset for the identification and characterization of the genes involved in the regulation of the Pi and Zn transport systems.

4. Conclusion

In conclusion, work presented is an extensive comparison of the effects of a wide set of combinatory stress conditions (+/−Zn and/or Pi) on the accumulation of Pi and Zn in two lettuce varieties, Paris Island Cos and Kordaat. This study revealed the difference between the effects of Pi and Zn supply on biomass and photosynthesis and the Pi transport in both lettuce varieties, which constitutes an opportunity towards decoding genetic basis of the Pi/Zn interaction. These observations indicate that the regulation of the Pi/Zn interaction in plants is more complex than previously thought. Further forward genetic work has to be undertaken using population obtained from the crossing between Paris Island Cos and Kordaat to identify additional key genes that regulate the Pi accumulation in shoot of lettuce varieties under Zn deficiency. This knowledge is required to fully appreciate the coregulation of Zn/Pi interaction in lettuce.

Conflict of Interests

The authors declare that there is no conflict of interests.

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References

[1] H. Marschner, *Mineral Nutrition of Higher Plants*, Academic Press, London, UK, 1995.

[2] Y. Poirier and M. Bucher, "Phosphate transport and homeostasis in Arabidopsis," *The Arabidopsis Book, American Society of Plant Biologists*, vol. 1, article e0024, 2002.

[3] S. A. Sinclair and U. Krämer, "The Zinc homeostasis network of land plants," *Biochimica et Biophysica Acta—Molecular Cell Research*, vol. 1823, no. 9, pp. 1553–1567, 2012.

[4] Z. Shahzad, H. Rouached, and A. Rakha, "Combating mineral malnutrition through Iron and Zinc biofortification of cereals," *Comprehensive Reviews in Food Science and Food Safety*, vol. 13, no. 3, pp. 329–346, 2014.

[5] G. Gianquinto, A. Abu-Rayyan, L. Di Tola, D. Piccotino, and B. Pezzarossa, "Interaction effects of phosphorus and Zinc on photosynthesis, growth and yield of dwarf bean grown in two environments," *Plant and Soil*, vol. 220, no. 1-2, pp. 219–228, 2000.

[6] P. H. Abelson, "A potential phosphate crisis," *Science*, vol. 283, no. 5410, p. 2015, 1999.

[7] I. Cakmak and H. Marschner, "Mechanism of phosphorus induced Zinc deficiency in cotton. I. Zinc deficiency-enhanced uptake rate of phosphorus," *Plant Physiology*, vol. 68, pp. 483–490, 1986.

[8] M. J. Webb and J. F. Loneragan, "Effect of Zinc deficiency on growth, phosphorus concentration, and phosphorus toxicity of wheat plants," *Soil Science Society of America Journal*, vol. 52, no. 6, pp. 1676–1680, 1988.

[9] C. Huang, S. J. Barker, P. Langridge, F. W. Smith, and R. D. Graham, "Zinc deficiency up-regulates expression of high-affinity phosphate transporter genes in both phosphate-sufficient and -deficient barley roots," *Plant Physiology*, vol. 124, no. 1, pp. 415–422, 2000.

[10] Y.-G. Zhu, S. E. Smith, and F. A. Smith, "Zinc (Zn)-phosphorus (P) interactions in two cultivars of spring wheat (*Triticum aestivum* L.) differing in p uptake efficiency," *Annals of Botany*, vol. 88, no. 5, pp. 941–945, 2001.

[11] A. Jain, B. Sinilal, G. Dhandapani, R. B. Meagher, and S. V. Sahi, "Effects of deficiency and excess of Zinc on morpho-physiological traits and spatiotemporal regulation of Zinc-responsive genes reveal incidence of cross talk between micro- and macronutrients," *Environmental Science & Technology*, vol. 47, no. 10, pp. 5327–5335, 2013.

[12] G. A. Khan, S. Bouraine, S. Wege et al., "Coordination between Zinc and phosphate homeostasis involves the transcription factor PHR1, the phosphate exporter PHO1, and its homologue PHO1,H3 in Arabidopsis," *Journal of Experimental Botany*, vol. 65, pp. 871–884, 2014.

[13] J. Misson, K. G. Raghothama, A. Jain et al., "A genome-wide transcriptional analysis using Arabidopsis thaliana Affymetrix gene chips determined plant responses to phosphate deprivation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 33, pp. 11934–11939, 2005.

[14] H. S. Reed, "Effects of Zinc deficiency on phosphate metabolism of the tomato plant," *American Journal of Botany*, vol. 33, no. 10, pp. 778–784, 1946.

[15] J. F. Loneragan, "Phosphorus accumulation and toxicity in leaves in relation to Zinc supply (okra Abelmoschus esculentus)," *Soil Science Society of America Journal*, vol. 46, no. 2, pp. 345–352, 1982.

[16] C. Liu, U. S. Muchhal, M. Uthappa, A. K. Kononowicz, and K. G. Raghothama, "Tomato phosphate transporter genes are differentially regulated in plant tissues by phosphorus," *Plant Physiology*, vol. 116, no. 1, pp. 91–99, 1998.

[17] A. D. Robson and M. G. Pitman, "Interactions between nutrients in higher plants," in *Inorganic Plant Nutrition*, A. Lauchli and R. L. Bieleski, Eds., vol. 15 of *Encyclopedia of Plant Physiology*, pp. 147–180, Springer, Berlin, Germany, 1983.

[18] T. S. Verma and R. S. Minhas, "Zinc and phosphorus interaction in a wheat-maize cropping system," *Fertilizer Research*, vol. 13, no. 1, pp. 77–86, 1987.

[19] B. Mou, "Mutations in lettuce improvement," *International Journal of Plant Genomics*, vol. 2011, Article ID 723518, 7 pages, 2011.

[20] R. Lambert, C. Grant, and S. Sauvé, "Cadmium and Zinc in soil solution extracts following the application of phosphate fertilizers," *Science of the Total Environment*, vol. 378, no. 3, pp. 293–305, 2007.

[21] M. S. Monteiro, C. Santos, A. M. V. M. Soares, and R. M. Mann, "Assessment of biomarkers of cadmium stress in lettuce," *Ecotoxicology and Environmental Safety*, vol. 72, no. 3, pp. 811–818, 2009.

[22] I. Ramos, E. Esteban, J. J. Lucena, and A. Gárate, "Cadmium uptake and subcellular distribution in plants of Lactuca sp. Cd-Mn interaction," *Plant Science*, vol. 162, no. 5, pp. 761–767, 2002.

[23] W. Zorrig, A. Rouached, Z. Shahzad, C. Abdelly, J.-C. Davidian, and P. Berthomieu, "Identification of three relationships linking cadmium accumulation to cadmium tolerance and Zinc and citrate accumulation in lettuce," *Journal of Plant Physiology*, vol. 167, no. 15, pp. 1239–1247, 2010.

[24] H. Rouached, A. Stefanovic, D. Secco et al., "Uncoupling phosphate deficiency from its major effects on growth and transcriptome via PHO1 expression in Arabidopsis," *Plant Journal*, vol. 65, no. 4, pp. 557–570, 2011.

[25] B. N. Ames, "Assay of inorganic phosphate, total phosphate and phosphatases," *Methods in Enzymology*, vol. 8, pp. 115–118, 1966.

[26] J. D. Lewis, K. L. Griffin, R. B. Thomas, and B. R. Strain, "Phosphorus supply affects the photosynthetic capacity of loblolly pine grown in elevated carbon dioxide," *Tree Physiology*, vol. 14, no. 11, pp. 1229–1244, 1994.

[27] F. Assche and H. Clijsters, "Multiple effects of heavy metal toxicity on photosynthesis," in *Effects of Stress on Photosynthesis*, R. Marcelle, H. Clijsters, and M. Poucke, Eds., pp. 371–382, Springer, Amsterdam, The Netherlands, 1983.

[28] H. Usuda and K. Shimogawara, "Phosphate deficiency in maize. I. Leaf phosphate status, growth, photosynthesis and carbon partitioning," *Plant and Cell Physiology*, vol. 32, no. 4, pp. 497–504, 1991.

[29] M. Mikulska, J.-L. Bomsel, and A. M. Rychter, "The influence of phosphate deficiency on photosynthesis, respiration and adenine nucleotide pool in bean leaves," *Photosynthetica*, vol. 35, no. 1, pp. 79–88, 1998.

[30] G. R. Rout and P. Das, "Effect of metal toxicity on plant growth and metabolism: I. Zinc," *Agronomie*, vol. 23, no. 1, pp. 3–11, 2003.

[31] G. Rout and P. Das, "Effect of metal toxicity on plant growth and netabolism: I. Zinc," in *Sustainable Agriculture*, E. Lichtfouse,

M. Navarrete, P. Debaeke, S. Véronique, and C. Alberola, Eds., pp. 873–884, Springer, Amsterdam, The Netherlands, 2009.

[32] R. Shi, H. Li, Y. Tong, R. Jing, F. Zhang, and C. Zou, "Identification of quantitative trait locus of Zinc and phosphorus density in wheat (*Triticum aestivum* L.) grain," *Plant and Soil*, vol. 306, no. 1-2, pp. 95–104, 2008.

[33] W. A. Norvell and R. M. Welch, "Growth and nutrient uptake by Barley (Hordeum vulgare L. cv Herta): studies using an N-(2-hydroxyethyl)ethylenedinitrilotriacetic acid-buffered nutrient solution technique: I. Zinc ion requirements," *Plant Physiology*, vol. 101, no. 2, pp. 619–625, 1993.

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Review Article

The Carbon-Nitrogen Balance of the Nodule and Its Regulation under Elevated Carbon Dioxide Concentration

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Legumes have developed a unique way to interact with bacteria: in addition to preventing infection from pathogenic bacteria like any other plant, legumes also developed a mutualistic symbiotic relationship with one gender of soil bacteria: rhizobium. This interaction leads to the development of a new root organ, the nodule, where the differentiated bacteria fix for the plant the atmospheric dinitrogen ($atmN_2$). In exchange, the symbiont will benefit from a permanent source of carbon compounds, products of the photosynthesis. The substantial amounts of fixed carbon dioxide dedicated to the symbiont imposed to the plant a tight regulation of the nodulation process to balance carbon and nitrogen incomes and outcomes. Climate change including the increase of the concentration of the atmospheric carbon dioxide is going to modify the rates of plant photosynthesis, the balance between nitrogen and carbon, and, as a consequence, the regulatory mechanisms of the nodulation process. This review focuses on the regulatory mechanisms controlling carbon/nitrogen balances in the context of legume nodulation and discusses how the change in atmospheric carbon dioxide concentration could affect nodulation efficiency.

1. Introduction

Plant-bacteria interactions are diverse in nature. While bacterial infections of plant cells are mostly perceived as pathogenic and lead to the activation of the plant defense system, some could lead to commensalism or, as described mostly in legumes, to mutualistic symbiotic interactions. Nodulation, with mycorrhization, is one of the best studied mutualistic symbiotic interactions between plant and microorganisms. Nodulation is the product of a controlled infection process of the legume root system by soil bacteria of genus *Rhizobia* and results in the development of a new plant root organ, the nodule, where differentiated bacteria named bacteroides fix and assimilate for the plant the atmospheric dinitrogen.

Because bacteria invest a lot of energy in fixing $atmN_2$ ($atmN_2 + 8e^- + 8H^+ + 16ATP = 2NH_3 + H_2 + 16ADP + 16P_i$) and because legumes provide to the bacteroides a significant amount of photosynthates (5 to 10 grams of carbons per one gram of fixed nitrogen [1]), the nodulation process is a high cost biological process for both partners. Hence, one critical aspect of the nodulation process is the establishment of well-balanced interactions between the two partners to lead to beneficial outcomes for both organisms. This interaction is highly dependent on communication between the two partners before, during, and after the initial infection process.

The molecular mechanisms controlling the recognition of the two partners, the initial infection of legume root hair cells by mutualistic symbiotic bacteria (i.e., root hair curling, invasion of the root hair cell by symbiotic nitrogen-fixing bacteria through the development, and elongation of the infection thread), legume nodule organogenesis, and the role of plant hormones in controlling nodulation have all been investigated during the past 15 years using forward and reverse genetic tools. These studies have been extensively documented and reviewed [2–7]. Similar strategies combined with elegant grafting and split-root experiments have be utilized to characterize the legumes genes controlling the autoregulation of legume nodulation (e.g., signal exchanges between the shoot and the root systems) [8–11]. For example, in the context of mutant shoot, where the shoot-root communication is jeopardized, nodulation is strongly enhanced leading to a hypernodulation phenotype [12–16]. Interestingly, plants showing a hypernodulation phenotype

do not have an enhanced uptake of $atmN_2$ compared to wild type plants [17, 18]. This latter result strongly supports that additional molecular mechanisms are controlling $atmN_2$ fixation independently of the infection level of the legume plant by symbiotic bacteria and nodule development. This review summarizes the cellular and molecular mechanisms regulating the interactions between infected plant cells and rhizobia and discusses the potential effects of the increase of the concentration of atmospheric carbon dioxide on these interactions.

2. The Secret to This Long-Term Relationship: The Selection of the Right Symbiotic Partners

Nodules and more specifically the bacteroides are considered as an important sink of plant photosynthates. Consequently, a successful nodulation relies on controlling the exchange of nutrients between the plant and the bacteria. This clearly delimits plant-microbe symbiotic to pathogenic relationships. Therefore, the coevolution between legumes and symbiotic bacteria is highly dependent on photosynthetic and symbiotic performances of the two partners (i.e., $atmN_2$ fixation efficiency; photosynthesis activity). Based on this concept, and to face the disparity of the photosynthesis activity among various legume cultivars, many studies identify symbiotic strains characterized by various $atmN_2$ fixation efficiencies [19–22].

The diversity of the microbial community in soil is leading to the presence of multiple bacterial lineages which can simultaneously infect the same legume plant. This competition for nodulation leads to the development of a heterogeneous pool of low- and high-efficiency $atmN_2$-fixation nodules [23–25]. Not surprisingly, the infection of the plant by less effective rhizobia strains which are characterized by low efficiency in fixing $atmN_2$ and high uptake of plant photosynthates is major limitation to plant development. To select their energy preferred symbionts and maximize nitrogen uptake without drastically affecting plant carbon resources, the plant developed various cellular and molecular mechanisms such as the promotion of the infection of the root hair cells and nodule by one single bacteria strain. In addition, ecological and physiological approaches have clearly demonstrated that legumes can monitor and respond to the nitrogen-fixing performance of symbiotic bacteria [26], punishing their low-efficiency hosts by reducing rhizobial viability and, ultimately, promoting nodule degeneration [26–29].

These are contributing factors to better discriminate and "punish" the low versus highly efficient $atmN_2$-fixing bacteria. Ultimately, the repetitive selection of the favorite symbionts by the host will affect the microbial ecosystem: after plant death and nodule degeneration, a significant population of the most successful symbiotic rhizobia will be released in the rhizosphere increasing their representation compared to low efficient rhizobia, bacteria strains slowly growing in the soil due to limited nutrient availability. Hence, by preventing infection by nonfixing rhizobia and increasing the overall population of highly efficient strains in the rhizosphere, one long-term outcome of the legume-rhizobia symbiosis is the preferential selection of the most beneficial bacteria strains by the plant to maximize nitrogen fixation. This concept of sanctions by the plant hosts against low efficient bacteria to maximize $atmN_2$ fixation is supported by mathematical model [30].

The preferential selection of specific rhizobia strains is likely a major reason supporting the conservation of the plant and bacteria genes required for mutualism [31]. One visible result of the coevolution of host plant and bacterial strains is the preferential concentration of *R. etli* carrying the *nodC* allele type-α and type-δ in the Mesoamerican and Andean soils, respectively [32]. Recently, the characterization of the molecular mechanisms controlling the preferential colonization of legume roots by highly efficient rhizobia strains has been initiated. Zanetti et al. [33] identified *P. vulgaris NF-YC1*, a gene encoding a C subunit of the heterotrimeric nuclear factor NF-Y transcription factor [34] as a key regulator of the infection of the plant by the most efficient strains of rhizobia. In their analysis, Zanetti et al. [33] characterized the putative orthologs of Pv*NF-YC1* in various plant species including *Arabidopsis thaliana* (At1g08970, At*NF-YC9*) and *Glycine max* (Glyma19g42460). In *M. truncatula*, two genes (Medtr1g082660; Medtr7g113680) are syntenic to Glyma19g42460. Upon mining of the soybean and medicago transcriptome atlases [35–39] and similarly to Pv*NF-YC1* [33], Glyma19g42460, Medtr1g082660 and Medtr7g113680 are ubiquitously expressed and not transcriptionally regulated in response to rhizobia (see Supplementary Figure 1 available online at http://dx.doi.org/10.1155/2014/507946). The ubiquitous expression pattern of these *NF-YC* legume genes suggests they control biological functions other than legume nodulation (e.g., At*NF-YC9*, Pv*NF-YC1* orthologous gene, controls *A. thaliana* floral transition [40]). It is likely possible that the regulation of legume nodulation by NF-YC proteins is dependent on their interaction with nodulation-specific A and B NF-Y subunits of the heterotrimeric CAATT transcription factor.

3. Cellular Communication between the Infected Plant Cells and the Bacteroides

While legume nodulation is initiated by the infection of the plant host by selected rhizobia, the long-term outcome the legume nodulation is the establishment of the symbiosis between the two partners. To reach this goal, a permanent communication between the plant cells (i.e., root hair cells and infected cells of the nodule) and the symbiosome is required. This organelle-like results of the endocytosis of bacteroides in the infected plant cell. It is delimited by the symbiosome membrane (SymM) and contains a limited number of bacteroides separated one another by the symbiosome space (SymS) [41] (usually two to four in determinate infected cells, only one in indeterminate infected cells [42–44]).

Transporters located in the SymM are playing essential roles in balancing the fluxes of metabolites between

the plant cell and the bacteroides (see below). Very interestingly, the proteome of the symbiosome very nicely reflects the complexity of the interaction existing between the plant host and the symbiotic bacteria [45]. In fact, several molecular studies clearly highlight the complex molecular organization of the symbiosome based on the translocation of plant proteins into the symbiosome membrane or cytoplasm or both. Plant protein translocation to the SymS depends on the presence of peptidic symbiosome-localization sequences [46, 47]. Lending even more support to the impact of plant proteins in regulating symbiont biology, nodule-specific cysteine-rich (NCR) peptides synthetized by the galegoid legume cells were demonstrated to be essential to bacterial endoreplication, a cellular process characteristic of fully differentiated bacteroides [48–51]. In *M. truncatula*, NCR peptides are directed to the symbiosomes by the defective in nitrogen fixation 1 (DNF1) protein, a component of the signal peptidase complex [52]. Ultimately, the NCR peptides will traffic to the bacterial periplasm and/or cytoplasm.

The role of these various plant proteins in regulating bacterial endoreplication clearly highlights the influence of the plant host on the symbiont. This likely allows the plant to exhibit better control of bacteroid differentiation and, as a result, exhibit better control of nitrogen fixation. To better support the relationship existing between the plant, the bacterium, and nitrogen fixation, transcriptomic and physiological experiments on *M. truncatula* mature nodules clearly show a decrease of the expression of more than 120 NCR genes and the decrease of nitrogenase activity upon nitrate treatment [53]. In addition to their role in bacteroid endoreplication, plant NCR proteins also act as antimicrobial chemical [54]. Adapting to the presence of these peptides, bacteroides synthetize the BacA protein, an ATP-binding cassette superfamily (ABC) [54–56]. Together, these studies support a complex interaction between plant and bacteria during the latter stage of nodulation where both partners developed specific sets of genes allowing controlling the nodulation process.

4. Transport, Conversion, and Storage of the Products of Plant Photosynthesis during Legume Nodulation

Plant cell-rhizobia symbiosis is primarily based on the exchange of metabolites especially sucrose, asparagine, and glutamine, allowing the balanced exchange of nitrogen and carbon. Numerous studies focused on the role of transporters and receptors located in symbiotic biological membranes [57–59]. Balanced carbon and nitrogen exchanges are also dependent on the transport of plant photosynthates to the nodule. There, the products of the photosynthesis are converted into malate in the plant cell through the glycolysis pathway, the Krebs cycle, the phosphoenolpyruvate carboxylase glycolysis, and the intensive nodule CO_2 dark fixation [60, 61]. Malate is assimilated and used by the symbiont. Ultimately, after its transportation through the SymM [57, 58], carbons will be stored in the bacteroid in the form of poly-3-hydroxybutyrate (PHB) particles. This carbon source can be remobilized by the bacteria in response to a stress (e.g., release of bacteria into the rhizosphere consecutively to nodule degeneration) and is also used as a redox potential to allow bacteroides survival in the anaerobic conditions existing in the nodule, which is the environment necessary for the optimal fixation of $atmN_2$ [62].

To better control the uptake of plant photosynthates by the bacteroides, legumes develop molecular strategies to control carbon sequestration in the bacteroides. For example, in addition to regulating nodulation efficiency the soybean gene *Nucleolar/Mitochondrial protein involved in Nodulation a* (Gm*NMNa*), initially characterized based on its strong and specific expression in root hair cell and nodules in response to *B. japonicum* inoculation [63, 64], controls bacteroid number in the nodule infected cell as well as the density of PHB granules in bacteroides. The mitochondrial localization of GmNMNa protein and the limited accumulation of PHB in bacteroides upon silencing of Gm*NMNa* support that Gm*NMNa* influences carbon metabolism in infected soybean nodule cells. Previous reports clearly demonstrated the essential role of mitochondria in legume infected cells to presumably maintain plant cell function under microaerobic conditions [65–67].

Interestingly, it seems that the *NMN* gene family is specialized in regulating mutualistic symbiotic plant microbe interactions since the *M. truncatula* gene ortholog to Gm*NMNa* is not only overexpressed during nodulation but also during mycorrhization (Supplementary Figure 2). This latter result suggests the functional redundancy between nodulation and mycorrhization in addition to the conservation of the initial signaling cascade activated in response to the Nod and Myc factors [6].

The essential role of PHB during the nodulation process is also demonstrated by two independent studies. First, the *Sinorhizobium meliloti phbC* mutant, mutant defective in PHB biosynthesis, does not fix $atmN_2$ [68]. Second, *Mimosa pudica* nodules infected by a modified strain of the pathogenic *Ralstonia solanacearum* accumulate PHB. This bacterial strain carries a mutation in the *HRPG* gene, gene previously described as a key regulator of bacterial virulence via the type III secretion system (T3SS; [69]), and is expressing the symbiosis genes of *Cupriavidus taiwanensis*, a *M. pudica* symbiotic bacterium [70]. This latter result suggests at least some similarities between rhizobia and pathogenic bacteria in uptaking and storing plant photosynthates. These studies lend even more support that nodulation is the result of a highly controlled interaction between legumes and soil bacteria.

5. Evolutionary Perspective of Legume Nodulation in the Context of the Environmental Changes

Legume nodulation is a complex biological process involving multiple levels of coevolution existing between the plant and symbiotic bacteria (i.e., recognition of plant flavonoids by the free-living bacteria, recognition of the bacterial Nod factor

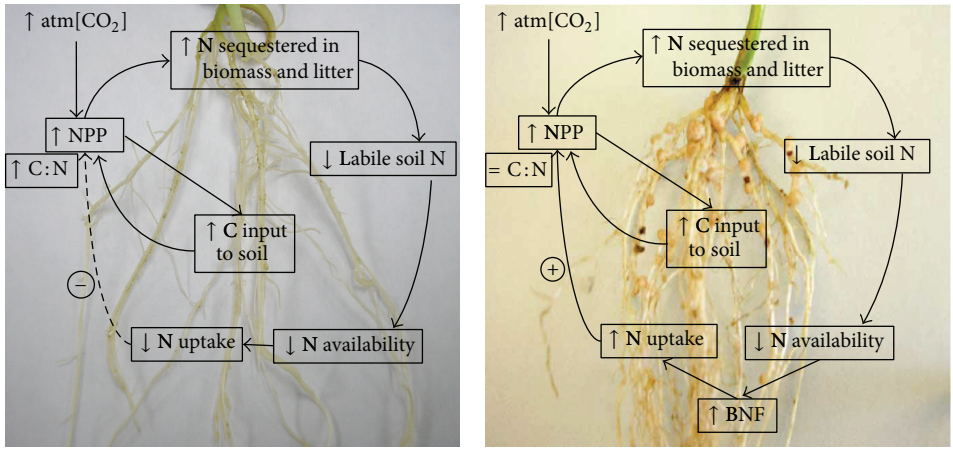

Figure 1: The increase of the concentration of atmospheric carbon dioxide will impact nitrogen uptake by the plants to balance ion balances. On the short term, this massive uptake of nitrogen will lead to the depletion of usable nitrogen resources in soil and the enrichment of the rhizosphere in carbon (upper cycles in both (a) and (b)). As a consequence, the limited access to nitrogen will lead to the unbalance between carbon and nitrogen and, as a consequence, to the limited growth of plants. In the case of legumes (b), limited nitrogen availability will enhance nodulation. The biological fixation of the atmospheric dinitrogen associated with the uptake of carbon dioxide will positively impact the net primary production of the plant (NPP). This figure was adapted from [78].

by plant receptors lysine kinases, control of bacteria differentiation and endoreplication by plant cells, and balanced exchanges of nutrients between the two organisms). The nodulation process is restricted not only to our knowledge of the infection of the root hair cell by the symbiont but also, mostly, to the establishment of a controlled interaction between the plant and the bacteria. This interaction might be jeopardized due to rapid environmental changes.

The most pessimistic predictions of climate change suggest an increase by 4°C of the current temperature as reported by the Intergovernmental Panel on Climate Change [71] and an atmospheric carbon dioxide (atm[CO_2]) concentration rising above 800 parts per million by the year 2100 in contrast to 390 ppm today (http://www.ipcc-data.org/observ/ddc_co2.html). Associated with unpredictable rainfall patterns and poor soil management, these environmental changes will affect not only plant growth but also the composition of soil microbial communities including rhizobial communities. Together, the modification of various climatic factors will require an adaptation of the legume nodulation process.

Among these factors, and based on the tight interactions between the nitrogen and carbon cycles, an increase of the concentration of atm[CO_2] is going to directly affect the crop's nitrogen/carbon balance and nodulation. To respect the balances between ions, it is predicted that plants may become nutrient limited, including nitrogen-limited, in the context of a greater carbon input [72, 73]. Models support that legumes will overcome the problem of nitrogen limitation by promoting physiological adaptations. These adaptations include the increase in nodule size, the increase in nodule number per plant (potentially correlated with an increase of the number of successful root hair cell infection), and the increase of nitrogenase activity [74–76] (Figure 1). The latter also support the idea of an increase in the allocation of plant photosynthates to the bacteroides and, more globally, of various ions to respect balances. Hence, it is assumed that legumes represent a potential solution to increase carbon sequestration and help to mitigate the impact of atm[CO_2]. While scientists have clearly demonstrated the impact of atm[CO_2] on nodulation [60, 77], the molecular mechanisms regulating nodulation under high atm[CO_2] remain unknown. In addition, it is unclear how the *rhizobia* community will adapt to higher atm[CO_2] in particular and climate change in general. The study of the adaptation of the microbe and plant as well as their interaction in response to environmental changes represents new avenues of research which could impact food production and sustainability on the long term.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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References

[1] D. A. Phillips, "Efficiency of symbiotic nitrogen fixation in legumes," *Annual Review of Plant Physiology*, vol. 31, pp. 29–49, 1980.

[2] G. Stacey, M. Libault, L. Brechenmacher, J. Wan, and G. D. May, "Genetics and functional genomics of legume nodulation," *Current Opinion in Plant Biology*, vol. 9, no. 2, pp. 110–121, 2006.

[3] G. E. D. Oldroyd and J. A. Downie, "Coordinating nodule morphogenesis with rhizobial infection in legumes," *Annual Review of Plant Biology*, vol. 59, pp. 519–546, 2008.

[4] J. D. Murray, "Invasion by invitation: rhizobial infection in legumes," *Molecular Plant-Microbe Interactions*, vol. 24, no. 6, pp. 631–639, 2011.

[5] G. E. D. Oldroyd, J. D. Murray, P. S. Poole, and J. A. Downie, "The rules of engagement in the legume-rhizobial symbiosis," *Annual Review of Genetics*, vol. 45, pp. 119–144, 2011.

[6] G. E. D. Oldroyd, "Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants," *Nature Reviews Microbiology*, vol. 11, no. 4, pp. 252–263, 2013.

[7] M. Venkateshwaran, J. D. Volkening, M. R. Sussman, and J.-M. Ané, "Symbiosis and the social network of higher plants," *Current Opinion in Plant Biology*, vol. 16, no. 1, pp. 118–127, 2013.

[8] R. M. Kosslak and B. B. Bohlool, "Suppression of nodule development of one side of a split-root system of soybeans caused by prior inoculation of the other side," *Plant Physiology*, vol. 75, pp. 125–130, 1984.

[9] A. C. Delves, A. Mathews, D. A. Day, A. S. Carter, B. J. Carroll, and P. M. Gresshoff, "Regulation of the soybean-Rhizobium nodule symbiosis by shoot and root factors," *Plant Physiology*, vol. 82, pp. 588–590, 1986.

[10] G. Caetano-Anollés and P. M. Gresshoff, "Plant genetic control of nodulation," *Annual Review of Microbiology*, vol. 45, pp. 345–382, 1991.

[11] B. J. Ferguson, A. Indrasumunar, S. Hayashi et al., "Molecular analysis of legume nodule development and autoregulation," *Journal of Integrative Plant Biology*, vol. 52, no. 1, pp. 61–76, 2010.

[12] R. Nishimura, M. Hayashit, G.-J. Wu et al., "*HAR1* mediates systemic regulation of symbiotic organ development," *Nature*, vol. 420, no. 6914, pp. 426–429, 2002.

[13] D. P. Lohar and K. A. VandenBosch, "Grafting between model legumes demonstrates roles for roots and shoots in determining nodule type and host/rhizobia specificity," *Journal of Experimental Botany*, vol. 56, no. 416, pp. 1643–1650, 2005.

[14] E. Schnabel, E.-P. Journet, F. de Carvalho-Niebel, G. Duc, and J. Frugoli, "The *Medicago truncatula SUNN* gene encodes a CLV1-like leucine-rich repeat receptor kinase that regulates nodule number and root length," *Plant Molecular Biology*, vol. 58, no. 6, pp. 809–822, 2005.

[15] S. Magori, E. Oka-Kira, S. Shibata et al., "Too much love, a root regulator associated with the long-distance control of nodulation in *Lotus japonicus*," *Molecular Plant-Microbe Interactions*, vol. 22, no. 3, pp. 259–268, 2009.

[16] C. Yoshida, S. Funayama-Noguchi, and M. Kawaguchi, "Plenty, a novel hypernodulation mutant in *Lotus japonicus*," *Plant and Cell Physiology*, vol. 51, no. 9, pp. 1425–1435, 2010.

[17] V. Bourion, G. Laguerre, G. Depret, A.-S. Voisin, C. Salon, and G. Duc, "Genetic variability in nodulation and root growth affects nitrogen fixation and accumulation in pea," *Annals of Botany*, vol. 100, no. 3, pp. 589–598, 2007.

[18] R. A. Cabeza, A. Lingner, R. Liese et al., "The activity of nodules of the supernodulating mutant Mt*sunn* is not limited by photosynthesis under optimal growth conditions," *International Journal of Molecular Sciences*, vol. 15, pp. 6031–6045, 2014.

[19] J. J. Burdon, A. H. Gibson, S. D. Searle, M. J. Woods, and J. Brockwell, "Variation in the effectiveness of symbiotic associations between native rhizobia and temperate *Australian Acacia* within-species interactions," *Journal of Applied Ecology*, vol. 36, no. 3, pp. 398–408, 1999.

[20] M. D. Denton, D. R. Coventry, W. D. Bellotti, and J. G. Howieson, "Distribution, abundance and symbiotic effectiveness of *Rhizobium leguminosarum bv. trifolii* from alkaline pasture soils in South Australia," *Australian Journal of Experimental Agriculture*, vol. 40, no. 1, pp. 25–35, 2000.

[21] G. Garau, W. G. Reeve, L. Brau et al., "The symbiotic requirements of different Medicago spp. suggest the evolution of *Sinorhizobium meliloti* and *S. medicae* with hosts differentially adapted to soil pH," *Plant and Soil*, vol. 276, no. 1-2, pp. 263–277, 2005.

[22] J. J. Terpolilli, G. W. O'Hara, R. P. Tiwari, M. J. Dilworth, and J. G. Howieson, "The model legume *Medicago truncatula* A17 is poorly matched for N_2 fixation with the sequenced microsymbiont *Sinorhizobium meliloti* 1021," *New Phytologist*, vol. 179, no. 1, pp. 62–66, 2008.

[23] N. Amarger, "Selection of *Rhizobium* strains on their competitive ability for nodulation," *Soil Biology and Biochemistry*, vol. 13, no. 6, pp. 481–486, 1981.

[24] P. W. Singleton and K. R. Stockinger, "Compensation against ineffective nodulation in soybean," *Crop Science*, vol. 23, pp. 69–72, 1983.

[25] M. Hahn and D. Studer, "Competitiveness of *anif- Bradyrhizobium japonicum* mutant against the wild-type strain," *FEMS Microbiology Letters*, vol. 33, no. 1, pp. 143–148, 1986.

[26] E. T. Kiers, R. A. Rousseau, S. A. West, and R. F. Denlson, "Host sanctions and the legume-*rhizobium* mutualism," *Nature*, vol. 425, no. 6953, pp. 78–81, 2003.

[27] D. J. Gage, "Analysis of infection thread development using Gfp- and DsRed-expressing *Sinorhizobium meliloti*," *Journal of Bacteriology*, vol. 184, no. 24, pp. 7042–7046, 2002.

[28] R. F. Denison and E. T. Kiers, "Life histories of symbiotic rhizobia and mycorrhizal fungi," *Current Biology*, vol. 21, no. 18, pp. R775–R785, 2011.

[29] R. Oono, C. G. Anderson, and R. F. Denison, "Failure to fix nitrogen by non-reproductive symbiotic rhizobia triggers host sanctions that reduce fitness of their reproductive clonemates," *Proceedings of the Royal Society B: Biological Sciences*, vol. 278, no. 1718, pp. 2698–2703, 2011.

[30] S. A. West, E. T. Kiers, E. L. Simms, and R. F. Denison, "Sanctions and mutualism stability: why do rhizobia fix nitrogen?" *Proceedings of the Royal Society B: Biological Sciences*, vol. 269, no. 1492, pp. 685–694, 2002.

[31] R. F. Denison and E. T. Kiers, "Lifestyle alternatives for rhizobia: mutualism, parasitism, and forgoing symbiosis," *FEMS Microbiology Letters*, vol. 237, no. 2, pp. 187–193, 2004.

[32] O. M. Aguilar, O. Riva, and E. Peltzer, "Analysis of *Rhizobium etli* and of its symbiosis with wild *Phaseolus vulgaris* supports coevolution in centers of host diversification," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 37, pp. 13548–13553, 2004.

[33] M. E. Zanetti, F. A. Blanco, M. P. Beker, M. Battaglia, and O. M. Aguilar, "A C subunit of the plant nuclear factor NF-Y required for rhizobial infection and nodule development affects partner selection in the common bean-*Rhizobium etli* symbiosis," *Plant Cell*, vol. 22, no. 12, pp. 4142–4157, 2010.

[34] K. Petroni, R. W. Kumimoto, N. Gnesutta et al., "The promiscuous life of plant NUCLEAR FACTOR Y transcription factors," *Plant Cell*, vol. 24, no. 12, pp. 4777–4792, 2012.

[35] V. A. Benedito, I. Torres-Jerez, J. D. Murray et al., "A gene expression atlas of the model legume *Medicago truncatula*," *Plant Journal*, vol. 55, no. 3, pp. 504–513, 2008.

[36] J. He, V. A. Benedito, M. Wang et al., "The *Medicago truncatula* gene expression atlas web server," *BMC Bioinformatics*, vol. 10, article 441, 2009.

[37] M. Libault, A. Farmer, T. Joshi et al., "An integrated transcriptome atlas of the crop model *Glycine max*, and its use in comparative analyses in plants," *Plant Journal*, vol. 63, no. 1, pp. 86–99, 2010.

[38] A. J. Severin, J. L. Woody, Y.-T. Bolon et al., "RNA-Seq Atlas of *Glycine max*: a guide to the soybean transcriptome," *BMC Plant Biology*, vol. 10, article 160, 2010.

[39] T. Joshi, K. Patil, M. R. Fitzpatrick et al., "Soybean Knowledge Base (SoyKB): a web resource for soybean translational genomics," *BMC Genomics*, vol. 13, supplement 1, article S15, 2012.

[40] D. Hackenberg, U. Keetman, and B. Grimm, "Homologous NF-YC2 subunit from arabidopsis and tobacco is activated by photooxidative stress and induces flowering," *International Journal of Molecular Sciences*, vol. 13, no. 3, pp. 3458–3477, 2012.

[41] L. E. Roth and G. Stacey, "Bacterium release into host cells of nitrogen-fixing soybean nodules: the symbiosome membrane comes from three sources," *European Journal of Cell Biology*, vol. 49, no. 1, pp. 13–23, 1989.

[42] E. M. Lodwig, M. Leonard, S. Marroqui et al., "Role of polyhydroxybutyrate and glycogen as carbon storage compounds in pea and bean bacteroids," *Molecular Plant-Microbe Interactions*, vol. 18, no. 1, pp. 67–74, 2005.

[43] R. Oono, I. Schmitt, J. I. Sprent, and R. F. Denison, "Multiple evolutionary origins of legume traits leading to extreme rhizobial differentiation," *New Phytologist*, vol. 187, no. 2, pp. 508–520, 2010.

[44] W. Van de Velde, G. Zehirov, A. Szatmari et al., "Plant peptides govern terminal differentiation of bacteria in symbiosis," *Science*, vol. 327, no. 5969, pp. 1122–1126, 2010.

[45] G. Saalbach, P. Erik, and S. Wienkoop, "Characterisation by proteomics of peribacteroid space and peribacteroid membrane preparations from pea (*Pisum sativum*) symbiosomes," *Proteomics*, vol. 2, pp. 325–337, 2002.

[46] N. Hohnjec, F. Lenz, V. Fehlberg et al., "The signal peptide of the *Medicago truncatula* modular nodulin MtNOD25 operates as an address label for the specific targeting of proteins to nitrogen-fixing symbiosomes," *Molecular Plant-Microbe Interactions*, vol. 22, no. 1, pp. 63–72, 2009.

[47] M. H. Meckfessel, E. B. Blancaflor, M. Plunkett, Q. Dong, and R. Dickstein, "Multiple domains in mtenod8 protein including the signal peptide target it to the symbiosome," *Plant Physiology*, vol. 159, no. 1, pp. 299–310, 2012.

[48] P. Mergaert, K. Nikovics, Z. Kelemen et al., "A novel family in *Medicago truncatula* consisting of more than 300 nodule-specific genes coding for small, secreted polypeptides with conserved cysteine motifs," *Plant Physiology*, vol. 132, no. 1, pp. 161–173, 2003.

[49] P. Mergaert, T. Uchiumi, B. Alunni et al., "Eukaryotic control on bacterial cell cycle and differentiation in the *Rhizobium*-legume symbiosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 13, pp. 5230–5235, 2006.

[50] B. Alunni, Z. Kevei, M. Redondo-Nieto, A. Kondorosi, P. Mergaert, and E. Kondorosi, "Genomic organization and evolutionary insights on *GRP* and *NCR* genes, two large nodule-specific gene families in *Medicago truncatula*," *Molecular Plant-Microbe Interactions*, vol. 20, no. 9, pp. 1138–1148, 2007.

[51] A. Kereszt, P. Mergaert, and E. Kondorosi, "Bacteroid development in legume nodules: evolution of mutual benefit or of sacrificial victims?" *Molecular Plant-Microbe Interactions*, vol. 24, no. 11, pp. 1300–1309, 2011.

[52] D. Wang, J. Griffitts, C. Starker et al., "A nodule-specific protein secretory pathway required for nitrogen-fixing symbiosis," *Science*, vol. 327, no. 5969, pp. 1126–1129, 2010.

[53] R. Cabeza, B. Koester, R. Liese et al., "An RNA sequencing transcriptome analysis reveals novel insights into molecular aspects of the nitrate impact on the nodule activity of *Medicago truncatula*," *Plant Physiology*, vol. 164, pp. 400–411, 2014.

[54] A. F. Haag, M. Baloban, M. Sani et al., "Protection of *Sinorhizobium* against host cysteine-rich antimicrobial peptides is critical for symbiosis," *PLoS Biology*, vol. 9, no. 10, Article ID e1001169, 2011.

[55] V. L. Marlow, A. F. Haag, H. Kobayashi et al., "Essential role for the BacA protein in the uptake of a truncated eukaryotic peptide in *Sinorhizobium meliloti*," *Journal of Bacteriology*, vol. 191, no. 5, pp. 1519–1527, 2009.

[56] R. Karunakaran, A. F. Haag, A. K. East et al., "*BacA* is essential for bacteroid development in nodules of galegoid, but not phaseoloid, legumes," *Journal of Bacteriology*, vol. 192, no. 11, pp. 2920–2928, 2010.

[57] M. K. Udvardi and D. A. Day, "Metabolite transport across symbiotic membranes of legume nodules," *Annual Review of Plant Biology*, vol. 48, pp. 493–523, 1997.

[58] V. A. Benedito, H. Li, X. Dai et al., "Genomic inventory and transcriptional analysis of *Medicago truncatula* transporters," *Plant Physiology*, vol. 152, no. 3, pp. 1716–1730, 2010.

[59] P. Masalkar, I. S. Wallace, J. H. Hwang, and D. M. Roberts, "Interaction of cytosolic glutamine synthetase of soybean root nodules with the C-terminal domain of the symbiosome membrane nodulin 26 aquaglyceroporin," *Journal of Biological Chemistry*, vol. 285, no. 31, pp. 23880–23888, 2010.

[60] M. N. Fotelli, D. Tsikou, A. Kolliopoulou et al., "Nodulation enhances dark CO_2 fixation and recycling in the model legume *Lotus japonicus*," *Journal of Experimental Botany*, vol. 62, no. 8, pp. 2959–2971, 2011.

[61] M. Udvardi and P. S. Poole, "Transport and metabolism in legume-rhizobia symbioses," *Annual Review of Plant Biology*, vol. 64, pp. 781–805, 2013.

[62] M. A. Trainer and T. C. Charles, "The role of PHB metabolism in the symbiosis of rhizobia with legumes," *Applied Microbiology and Biotechnology*, vol. 71, no. 4, pp. 377–386, 2006.

[63] M. Libault, A. Farmer, L. Brechenmacher et al., "Complete transcriptome of the soybean root hair cell, a single-cell model, and its alteration in response to *Bradyrhizobium japonicum* infection," *Plant Physiology*, vol. 152, no. 2, pp. 541–552, 2010.

[64] M. Libault, M. Govindarajulu, R. H. Berg et al., "A Dual-targeted soybean protein is involved in *Bradyrhizobium japonicum* infection of soybean root hair and cortical cells," *Molecular Plant-Microbe Interactions*, vol. 24, no. 9, pp. 1051–1060, 2011.

[65] E. H. Newcomb, S. R. Tandon, and R. R. Kowal, "Ultrastructural specialization for ureide production in uninfected cells of soybean root nodules," *Protoplasma*, vol. 125, no. 1-2, pp. 1–12, 1985.

[66] F. J. Bergersen, M. B. Peoples, and G. L. Turner, "A role for poly-β-hydroxybutyrate in bacteroids of soybean root nodules," *Proceedings of the Royal Society B: Biological Sciences*, vol. 245, no. 1312, pp. 59–64, 1991.

[67] M. Cermola, E. Fedorova, R. Taté, A. Riccio, R. Favre, and E. J. Patriarca, "Nodule invasion and symbiosome differentiation during *Rhizobium etli-Phaseolus vulgaris* symbiosis," *Molecular Plant-Microbe Interactions*, vol. 13, no. 7, pp. 733–741, 2000.

[68] C. Wang, M. Saldanha, X. Sheng et al., "Roles of poly-3-hydroxybutyrate (PHB) and glycogen in symbiosis of *Sinorhizobium meliloti* with *Medicago sp*," *Microbiology*, vol. 153, no. 2, pp. 388–398, 2007.

[69] M. Valls, S. Genin, and C. Boucher, "Integrated regulation of the type III secretion system and other virulence determinants in *Ralstonia solanacearum*," *PLoS Pathogens*, vol. 2, no. 8, article e82, 2006.

[70] M. Marchetti, D. Capela, M. Glew et al., "Experimental evolution of a plant pathogen into a legume symbiont," *PLoS Biology*, vol. 8, no. 1, Article ID e1000280, 2010.

[71] IPCC, *Climate Change 2014: Mitigation of Climate Change*, 2014.

[72] S. Díaz, J. P. Grime, J. Harris, and E. McPherson, "Evidence of a feedback mechanism limiting plant response to elevated carbon dioxide," *Nature*, vol. 364, no. 6438, pp. 616–617, 1993.

[73] C. W. Rice, F. O. Garcia, C. O. Hampton, and C. E. Owensby, "Soil microbial response in tallgrass prairie to elevated CO_2," *Plant and Soil*, vol. 165, no. 1, pp. 67–74, 1994.

[74] J. F. Soussana and U. A. Hartwig, "The effects of elevated CO_2 on symbiotic N_2 fixation: a link between the carbon and nitrogen cycles in grassland ecosystems," *Plant and Soil*, vol. 187, no. 2, pp. 321–332, 1996.

[75] S. Zanetti, U. A. Hartwig, A. Lüscher et al., "Stimulation of symbiotic N_2 fixation in *Trifolium repens L.* under elevated atmospheric pCO_2 in a grassland ecosystem," *Plant Physiology*, vol. 112, no. 2, pp. 575–583, 1996.

[76] B. A. Hungate, J. S. Dukes, M. R. Shaw, Y. Luo, and C. B. Field, "Atmospheric science. Nitrogen and climate change," *Science*, vol. 302, no. 5650, pp. 1512–1513, 2003.

[77] A. Rogers, E. A. Ainsworth, and A. D. B. Leakey, "Will elevated carbon dioxide concentration amplify the benefits of nitrogen fixation in legumes?" *Plant Physiology*, vol. 151, no. 3, pp. 1009–1016, 2009.

[78] Y. Luo, B. Su, W. S. Currie et al., "Progressive nitrogen limitation of ecosystem responses to rising atmospheric CO_2 concentration," *BioScience*, vol. 54, no. 8, pp. 731–739, 2004.

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Research Article

Chromium (VI) Uptake and Tolerance Potential in Cotton Cultivars: Effect on Their Root Physiology, Ultramorphology, and Oxidative Metabolism

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Chromium (Cr) is present in our environment as a toxic pollutant, which needs to be removed using phytoremediation technology. In present study, two transgenic cotton cultivars (J208, Z905) and their hybrid line (ZD14) were used to explore their Cr uptake and tolerance potential using multiple biomarkers approach. Four different levels of Cr (CK, 10, 50, and 100 μM) were applied. Cr caused a significant reduction in root/shoot length, number of secondary roots, and root fresh and dry biomasses at 100 μM. Cr accumulated more in roots and was found higher in hybrid line (ZD14) as compared with its parent lines (J208, Z905) at all Cr stress levels (10, 50, and 100 μM). Cr translocation was less than 1 in all cultivars. Ultrastructural studies at 100 μM Cr showed an increase in number of nuclei and vacuoles and presence of Cr dense granules in dead parts of the cell (vacuoles/cell wall). Malondialdehyde (MDA), hydrogen peroxide (H_2O_2), total soluble proteins, superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GR) as a whole were upregulated with elevated levels of Cr. Higher Cr uptake by roots, accelerated metabolism, and Cr sequestration in dead parts of the cell indicate that these cotton cultivars can be useful for Cr accumulation and tolerance.

1. Introduction

Chromium (Cr) like other heavy metals threatens both crop production and human health. To minimize its concentration in environment, various reclamation technologies such as redox processes, ion exchange, and reverse osmosis [1] have been introduced. However, most of them are highly expensive and cumbersome. They need to be replaced by cheaper and more cost-effective plant-based technologies. They are, for example, phytoextraction, phytostabilization, and phytoremediation.

Cr-mediated ground water and soil pollution need to be controlled either by avoiding its spills from various industries such as plating, alloying, tanning of animal hides, inhibition of water corrosion, and textile dyes [2] or by exploiting plant-based phytoextraction technologies. Unlike other toxic pollutants, Cr has several oxidation states. Among these, the trivalent Cr (III) and the hexavalent Cr (VI) are the most abundant. Since both possess different chemical, toxicological, and epidemiological feature, they are differently regulated by Environmental Protection Agency (EPA) [3]. Cr (III) is an essential trace element for human and animal health [4], being necessary for sugar and lipid metabolism [5]. It occurs in the form of oxides, hydroxides, and sulfates [6, 7]. It has relatively less mobility in soil and water due to its binding

tendency towards organic matters. It is rapidly oxidized to Cr (VI) in acidic soils in the presence of Mn, which becomes insoluble in water and alternatively has adverse effects on plant growth and development [8]. Cr (VI) is more toxic than Cr (III) for human beings, plants, aquatic animals, and microorganisms [9]. Due to these properties, Cr (VI) is a priority pollutant [6, 10] among the scientific community.

Cr mostly resides in roots and only a fraction of it is translocated to the shoots [11, 12]. Concentration of Cr in roots is 100-fold higher than the shoots [13] in most cases. Various factors such as pH, oxidative state of Cr and its concentration, salinity, and the presence of dissolved salts play an active role in its uptake from hydroponic media [14]. Upon entry into plants, Cr uses channels of sulfur and iron for upward translocation, which results in silent competition among the three. Resultantly, Cr is mainly confined to roots. This competition seems independent of the form of Cr tested [9].

In plants, Cr induces numerous physiological, biochemical, and ultrastructural alterations. Growth and biomass reduction, chlorosis in young leaves, lowering of pigment contents, disturbance of stomatal conductance alteration of enzymatic function, and damage to root cells and ultramorphological modifications [15, 16] in roots and leaves are some of the reported adverse effects of Cr in plants. Roots are severely damaged due to their direct contact with Cr. For example, shortening and browning of roots as well as presence of less number of root hairs in *Zea mays* L. [17] and adverse effects on lateral roots development and root number in mung bean [18] have been reported.

Very few reports cite strong cytotoxic nature of Cr (VI) in plants. Fasulo et al. [19] observed inhibition of cell proliferation and the presence of multinucleated, highly vacuolated giant cells in *Euglena gracilis*. Mouvet [20] has reported electron-dense bodies in cell walls, cytoplasm, and vacuoles in *Fontinalis antipyretica* L., grown in river water contaminated with Cr and Cu. Condensed and irregular structural features of root tips in bush bean as well as damage to membranes especially the tonoplast have been observed by [21]. Panda [22] found plasmolysed cell, large vacuoles, and presence of dense lysosomes like organelles in Cr (VI) treated roots.

All such types of anomalies result in the production of reactive oxygen species (ROS) [23, 24]. ROS can cause oxidative damage to cell membranes, chlorophyll pigments, lipids, nucleic acids, and proteins [8], which result in altered metabolism and finally cell death. However, plants are capable of mediating the deleterious effects of these ROS [22] by activating an extensive network of antioxidants. It is comprised of various enzymes, such as superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), and low molecular weight antioxidants, such as glutathione [25]. Imbalance between rate of ROS production and antioxidant system activity causes ROS accumulation in plants [8]. Cr-mediated ROS production can be either by direct electron transfer or by inhibition of metabolic reactions [26].

Cotton has been almost unexploited for heavy metal tolerance and accumulation. It can be a potential candidate for heavy metal such as Cr pollution control. Due to rapid cultivation of transgenic cotton cultivars in the industrialized area of the world, it is foremost needed to exploit them for Cr remediation in Cr-polluted areas.

We planned a comprehensive study to compare the Cr uptake potential of two transgenic cotton cultivars (insect and herbicide resistant) and their hybrid line testing various biomarkers as their "quantitative responses" towards Cr stress levels. Biometric parameters such as length, biomass, and tolerance index were measured as indices of metabolic damage. Cr contents levels were determined in both roots and leaves so as to evaluate their accumulation potential. Ultramorphological studies were performed to locate possible Cr detoxification and accumulation sites. Oxidative stress biomarkers such as protein, MDA, and H_2O_2 were studied to know the extent of Cr-induced oxidative damage in roots. And moreover, their antioxidative metabolism was evaluated to explore their possible utilization in remediation of Cr.

2. Materials and Methods

2.1. Plant Materials and Culturing Methods. In our present experiment, we used two transgenic cotton cultivars (J208, Z905) and their hybrid line (ZD14). J208 is an herbicide resistant transgenic cotton cultivar and Z905 is an insect resistant transgenic cotton cultivar. Lint was removed with concentrated hydrogen sulfide (H_2SO_4) followed by constant washing with tap water for about 30 min. Uniform-sized seeds with shining color were first immersed in 70% ethanol followed by washing 3 times with double distilled water (ddH_2O). Then seeds were surface-sterilized with 0.1% $HgCl_2$ solution for 8–10 min, which were washed for 3-4 times first with distilled water (dH_2O) and ddH_2O. Seeds were soaked for 4 hr in dH_2O at temperature 35°C. After that they were sown in presterilized sand in growth chamber for seven days. Temperature of the growth chamber was 28 ± 2°C, relative humidity was kept 60%, and light and dark photoperiod was 16:8 hr. Uniform-sized seven-day-old seedlings were transferred to green house by maintaining 32 ± 2°C day and 30 ± 2°C night temperature, while the light and dark period was 14:10 hr. Seedlings were grown for seven days in adaptation media. The nutrients media composition was 500 μM $(NH_4)_2SO_4$, 500 μM $MgSO_4$, 200 μM K_2SO_4, 1000 μM KNO_3, 600 μM $Ca(NO_3)_2{\cdot}4H_2O$, 200 μM KH_2PO_4, 100 μM Na_2-EDTA, 10 μM $FeSO_4{\cdot}7H_2O$, 0.5 μM $MnSO_4{\cdot}H_2O$, 0.25 μM $ZnSO_4{\cdot}7H_2O$, 0.05 μM $CuSO_4{\cdot}5H_2O$, 100 μM H_3BO_3, and 0.02 μM $(NH_4)_6Mo_7O_{24}$.

2.2. Cr Treatment Process and Measurement of Growth Performance Parameter. 14-day-old seedlings were subjected to exceeding levels of Cr (VI) in the form of potassium dichromate ($K_2Cr_2O_7$). Four levels of Cr (μM) were kept, that is, 0, 10, 50, and 100. Each level was randomly replicated thrice. Each replication consisted of 5 L pot having covered polystyrol plates with seven evenly spaced holes. In each hole, two seedlings were kept. Thus each replication consisted of 14 seedlings and there were 42 plants per treatment. Nutrients solution was constantly aerated with air pump and after every three days solution was changed with the new one. pH of

the culture media was maintained between 5.6 and 5.7 either with 0.1 M HCl or NaOH and was adjusted every other day. Treatment regime was maintained for 7 days. At the end of the treatment, 21-day-old seedlings were subjected to the measurement the growth-related parameters such as roots, shoot lengths, root fresh and dry biomass, and biomass-based root growth inhibition. For length measurement, single randomly selected plant per replication was taken. While for the root biomass determination and root biomass-based inhibition, three randomly selected plants per replication were taken.

2.3. Determination of Cr Contents in Roots and Leaves. Cr contents were analyzed in both roots and leaves using atomic absorption spectrometery (PE-100, PerkinElmer). Number of plants per replication was three. At the end of the experiment, seedlings from both nonstressed and Cr-stressed population were washed with tap and distilled water thrice, respectively. Roots were immersed in 20 mM EDTA-Na_2 for 15–20 min, which were subsequently washed with dH_2O for three-four times. Seedlings were separated into roots and leaves, oven-dried at 80°C for 48 hr, and then ground into powder. 0.2 g of each root and shoot sample were digested with a mixture of 5 mL of HNO_3 + 1 mL of $HClO_4$. The resultant solutions were diluted to 25 mL using 2% HNO_3 and then filtered. The root and leaves filtrates were used for Cr analysis.

2.4. Root Specimen Preparation for Ultramicroscopic Observations. Root ultrathin sections were prepared for ultramorphological studies according to Daud et al. [27]. Briefly, root tips (~2-3 mm) of randomly selected plants of both control and 100 μM Cr-treated were fixed overnight in 2.5% glutaraldehyde (v/v) in 0.1 M PBS (sodium phosphate buffer, pH 7.4). They were vacuum-infiltrated for 15 min and washed three times with the same PBS. The samples were post-fixed in 1% osmium (VIII) oxide (O_sO_4) for 1 hr and were washed three times in 0.1 M PBS (pH 7.4) with a ten-minute interval between each washing. After that, they were dehydrated in a graded ethanol series (50, 60, 70, 80, 90, 95, and 100%) with 15–20 min interval and finally by absolute acetone for 20 min. The samples were then infiltrated and embedded in Spurr's resin overnight. After heating the specimens at 70°C for 9 hr, the ultrathin sections (80 nm) were prepared and mounted on copper grids for viewing in the transmission electron microscope (JEOL TEM-1230EX) at an accelerating voltage of 60.0 kV.

2.5. Measurements of Lipid Peroxidation, Hydrogen Peroxide, and Total Soluble Protein. 0.8 g roots materials of all three cultivars were used for the determination of lipid peroxidation, hydrogen peroxide, and total soluble proteins. Lipid peroxidation was estimated in terms of malondialdehyde (MDA) contents and was determined as 2-thiobarbituric acid (TBA) reactive substances following the method of [28]. For determination of hydrogen peroxide (H_2O_2) content [29], 0.8 g roots were crushed with 8.0 mL of trichloroacetic acid (TCA) (0.1%, w/v) in ice cold conditions and the homogenate was centrifuged at 14,000 g for 20 min. 4 mL reaction mixture contained 1 mL supernatant, 1 mL PBS, and 2 mL potassium iodide (KI) and the absorbance was read at 390 nm. H_2O_2 content was determined using an extinction coefficient of 0.28 μMcm^{-1} and expressed as μmol g^{-1} FW.

In order to determine the total soluble proteins, 0.8 g roots sample was homogenized in 8 mL of 50 mM potassium phosphate buffer (PBS) (pH7.8) under chilled conditions. The crude extract was centrifuged at 14,000 g for 15 min at 4°C and the supernatant was used for the determination of total soluble protein using the method of [30] and bovine serum albumin was used as a standard.

2.6. Samples Preparation for Metabolic Antioxidant Assays. In both Cr nontreated and treated root samples, metabolic antioxidant assays were performed according to established protocols. For each assay, 0.8 g fresh weight of roots was taken and the extraction buffer (PBS 7.8) volume was 8 mL. Superoxide dismutase (SOD) (EC1.15.1.1) activity was determined according to [31] following the inhibition of photochemical reduction due to nitro blue tetrazolium (NBT). Ascorbate peroxidase (APX) (EC 1.11.1.11) activity was measured using the protocol of [32]. Catalase (CAT) (EC1.11.1.6) activity was measured as determined by [33], while for the determination of peroxidase (POD) (EC1.11.1.7) activity, the method of [34] was used. Glutathione reductase (GR) (EC1.6.4.2) activity was assayed according to [35].

2.7. Statistical Analyses. The data obtained were subjected to one-way analysis of variance (ANOVA) using STATIX9. All the results were the means ± SD of three replications. Means were separated by least significant difference (LSD) test at 5% level of significance.

3. Results

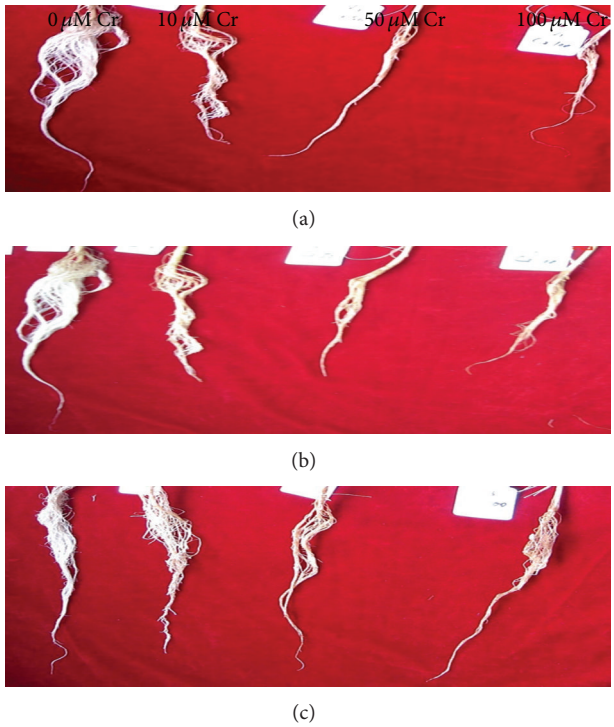
3.1. Cr Stress Differentially Inhibited Root Morphology. Figure 1 shows the root morphology of both transgenic cotton cultivars (J208, Z905) and their hybrid line (ZD14) grown in Cr added nutrients substrate. Root morphology of these cultivars was influenced both in dose-dependent and genotype-dependent manners. In response to 10 μM Cr, reduction in number of root hairs and secondary roots was less. However, both 50 and 100 μM Cr levels demonstrated significant reduction in root lengths and number of secondary roots. J208 was the most sensitive in terms of root morphology, while ZD14 was the least affected cotton hybrid cultivar when exposed to different concentrations of Cr. Moreover, in all cultivars, there were marked reductions in number of root hairs and root vigor as well as induced root pale color at all exceeding levels of Cr compared with control. Degenerative effects regarding root morphology in all these cultivars were in the order of ZD14 < Z905 < J208.

3.2. Cr Stress Variably Decreased the Root Growth Performance. Measurements of growth and biomass performance show significant reduction at 5% probability level in our experimental cultivars (Table 1). Consistent decrease in the root-related mean data reveals that roots were considerably

Table 1: Growth performance of both transgenic cotton cultivars and their hybrid line under chromium stress.

Variety	Cr levels (μM)	Growth performance					
		Length (cm/plant)		Biomass (g/plant)		Growth inhibition	
		Root	Shoot	Fresh	Dry	Fresh biomass	Dry biomass
J208	0	35.83 ± 2.57^{a}	47.20 ± 3.93^{a}	8.32 ± 1.55^{a}	0.67 ± 0.23^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}
	10	31.37 ± 1.70^{b}	44.57 ± 2.29^{a}	5.21 ± 1.35^{b}	0.43 ± 0.22^{ab}	34.61 ± 24.51^{ab}	37.93 ± 20.69^{b}
	50	24.00 ± 2.00^{c}	34.43 ± 2.67^{b}	3.85 ± 1.74^{bc}	0.21 ± 0.00^{b}	49.59 ± 33.52^{a}	66.33 ± 10.48^{a}
	100	17.60 ± 2.75^{d}	30.00 ± 1.74^{b}	2.34 ± 1.06^{c}	0.19 ± 0.00^{b}	69.41 ± 20.37^{a}	69.54 ± 9.48^{a}
Z905	0	22.67 ± 2.52^{a}	30.20 ± 1.71^{a}	7.06 ± 2.16^{a}	0.50 ± 0.13^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
	10	18.83 ± 1.76^{b}	24.57 ± 2.38^{b}	3.82 ± 1.47^{b}	0.35 ± 0.10^{ab}	45.88 ± 11.98^{b}	28.71 ± 18.05^{b}
	50	10.07 ± 0.90^{c}	22.17 ± 3.82^{b}	1.95 ± 0.77^{bc}	0.19 ± 0.10^{bc}	69.26 ± 16.39^{a}	62.29 ± 13.42^{a}
	100	6.67 ± 0.76^{d}	14.60 ± 1.22^{c}	0.78 ± 0.30^{c}	0.10 ± 0.05^{c}	87.76 ± 7.64^{a}	79.57 ± 8.04^{a}
ZD14	0	27.33 ± 2.52^{a}	41.57 ± 3.98^{a}	6.53 ± 2.10^{a}	0.45 ± 0.18^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}
	10	24.87 ± 3.80^{ab}	42.60 ± 2.85^{a}	3.66 ± 0.44^{b}	0.35 ± 0.10^{ab}	38.72 ± 25.55^{b}	16.26 ± 29.45^{ab}
	50	20.33 ± 2.52^{bc}	32.87 ± 1.21^{b}	2.40 ± 0.49^{bc}	0.27 ± 0.10^{ab}	59.05 ± 20.08^{ab}	32.88 ± 37.45^{ab}
	100	17.40 ± 1.51^{c}	27.50 ± 2.30^{c}	1.23 ± 0.50^{c}	0.17 ± 0.05^{b}	80.69 ± 7.92^{a}	58.25 ± 17.23^{a}

Values are the means ± SD of three replications. Variants possessing the same letter are not statistically significant at $P < 0.05$.



(a)

(b)

(c)

Figure 1: Root growth responses of herbicide resistant transgenic cotton cultivar (J208) (a), insect resistant transgenic cotton cultivar (Z905) (b), and their hybrid line (ZD14) (c) under exceeding levels of Cr.

influenced in all these cultivars. As compared to related controls, root length was significantly reduced by 48% (Z905), 32% (J208), and 8% (ZD14) at exceeding levels of Cr in comparison with relevant controls. Statistically significant decline was observed at 100 μM Cr, which was 71% in Z905 followed by 51% in J208 and 15% in ZD14. Compared with related controls, shoot lengths reduced with concomitant increase in Cr levels. At 5% probability level, distinctive reduction in shoot lengths was in order of Z905 (52%) > J208 (36%) > ZD14 (2.3; 34%) relative to their respective controls.

Mean data of Table 1 further reveals reduction in root biomass as well as upregulation in the mean values of biomass-based inhibition. Irrespective of the genotype, root biomasses decreased in Cr-stressed plants in dose-dependent manner. Reduction in fresh biomass reduction (collectively 184%) was more than dry biomass (collectively 165%). In comparison with their controls, marked decline was found in Z905 (89%) at 100 μM Cr, while insignificant decrease was observed in J208 (37%) at 10 μM Cr. Analysis of biomass-based growth inhibition exhibited increase in their mean data at higher Cr stress levels in all experimental cultivars. By comparing the fresh and dry biomass-based growth inhibition, it is evident that fresh biomass was inhibited more (59%) than dry biomass (50%). Intervarietal comparison depicts that fresh biomass-based inhibition was more in Z905 (68%) in comparison with the other two cultivars (ZD14, 59%; J208, 51%), while dry biomass-based inhibition was in the order of J208 (58%) > Z905 (57%) > ZD14 (36%) in comparison with related controls.

3.3. Tolerance of Cotton Cultivars Based on Their Physiological Parameters. Physiology-based tolerance studies show varied genotype-dependent responses (Table 2). Mean tabulated data depict that tolerance of both transgenic cotton cultivars and their hybrid line decrease upon exposure to Cr when compared with controls. In case of root length tolerance index, relative decrease was more in Z905 (70%) than in J208 (51%) and ZD14 (36%) at 100 μM Cr. Plant height tolerance was more in ZD14 followed by J208 and Z905, while root fresh weight tolerance was more in J208 than in ZD14 and Z905, respectively.

Tabulated data further reveal that water contents-based tolerance was more in J208, while the other two cotton cultivars showed a decline, which was 5.93% in Z905 and

Table 2: Tolerance indices of both transgenic cotton cultivars and their hybrid line under Cr stress.

Variety	Cr levels (μM)	Root length	Plant height	Root fresh weight	Root water contents
J208	0	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}
	10	0.88 ± 0.07^{a}	0.91 ± 0.06^{a}	0.66 ± 0.24^{ab}	1.00 ± 0.00^{a}
	50	0.67 ± 0.10^{b}	0.70 ± 0.04^{b}	0.51 ± 0.33^{b}	1.02 ± 0.05^{a}
	100	0.49 ± 0.06^{c}	0.57 ± 0.06^{c}	0.31 ± 0.20^{b}	0.99 ± 0.05^{a}
Z905	0	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}
	10	0.84 ± 0.16^{a}	0.82 ± 0.10^{b}	0.54 ± 0.54^{b}	0.98 ± 0.00^{a}
	50	0.45 ± 0.05^{b}	0.61 ± 0.09^{c}	0.31 ± 0.31^{c}	0.94 ± 0.12^{a}
	100	0.30 ± 0.07^{b}	0.40 ± 0.06^{d}	0.12 ± 0.12^{c}	0.90 ± 0.13^{a}
ZD14	0	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}
	10	0.92 ± 0.22^{ab}	0.98 ± 0.09^{a}	0.61 ± 0.26^{b}	0.98 ± 0.07^{a}
	50	0.75 ± 0.07^{bc}	0.77 ± 0.02^{b}	0.41 ± 0.20^{bc}	0.96 ± 0.11^{a}
	100	0.64 ± 0.05^{c}	0.65 ± 0.02^{c}	0.19 ± 0.08^{c}	0.91 ± 0.03^{a}

Tolerance index of the above parameters was calculated as TI = mean values in treatment/mean values in control. Mean values of TI =/> 1 show the tolerance behavior of the cultivar, while mean values of TI < 1 show the susceptibility of the cultivar towards Cr stress. Values are the means ± SD of three replications. Variants possessing the same letter are not statistically significant at $P < 0.05$.

Table 3: Cr concentration levels in roots and leaves and its translocation from roots to shoot in experimental cotton cultivars.

Variety	Cr levels (μM)	Cr uptake (mg/gDW)		Translocation factor (TF)
		Leaf	Root	
J208	0	0.03 ± 0.01^{d}	0.06 ± 0.03^{d}	0.65 ± 0.40^{a}
	10	0.64 ± 0.08^{c}	23.07 ± 2.92^{c}	0.03 ± 0.00^{b}
	50	0.92 ± 0.03^{b}	38.95 ± 4.45^{b}	0.02 ± 0.00^{b}
	100	1.08 ± 0.13^{a}	59.60 ± 0.00^{a}	0.02 ± 0.00^{b}
Z905	0	0.05 ± 0.02^{d}	1.56 ± 0.61^{d}	0.03 ± 0.01^{a}
	10	0.41 ± 0.07^{c}	646.60 ± 29.29^{c}	0.001 ± 0.00^{b}
	50	1.48 ± 0.32^{b}	1129.2 ± 105.98^{b}	0.001 ± 0.00^{b}
	100	1.97 ± 0.11^{a}	1318.6 ± 150.98^{a}	0.002 ± 0.00^{b}
ZD14	0	0.04 ± 0.02^{d}	0.09 ± 0.03^{d}	0.37 ± 0.20^{a}
	10	0.39 ± 0.05^{c}	39.56 ± 4.06^{c}	0.01 ± 0.00^{b}
	50	0.52 ± 0.06^{b}	55.07 ± 10.98^{b}	0.01 ± 0.00^{b}
	100	0.96 ± 0.00^{a}	111.44 ± 7.72^{a}	0.01 ± 0.00^{b}

Values are the means ± SD of three replications. Variants possessing the same letter are not statistically significant at $P < 0.05$.

4.78% in ZD14. In Z905 and ZD14 the water contents decreased (data not shown), which indirectly reduced their tolerance towards exceeding levels of Cr.

3.4. Cr Accumulation Capacity in Roots and Its Translocation to Shoots. Table 3 shows the Cr levels in roots and shoots of both transgenic cotton cultivars and their hybrid line. According to mean tabulated data, Cr uptake in both roots and leaves was in dose-dependent manner. Statistically significant enhancement was found in all experimental cultivars with the increase in the external application of Cr. The mean data revealed that roots accumulated more Cr as compared with leaves. Greater Cr concentration was found in roots of Z905 in comparison with the other two cotton cultivars. Remarkable relative increase was found in roots of ZD14 followed by Z905 and J208, respectively. Taken together, the Cr contents in both leaves and roots, greater concentrations were found in ZD14 followed by Z905 and J208.

Table 3 furthermore shows the translocation efficiency of both parent lines and their hybrid line. In all cultivars, the translocation factor (TF) values were < 1. In J208 and ZD14, they concomitantly increased with the increase in the external application of Cr as revealed by relative increase in their mean values, while there was a decrease in TF in Z905.

3.5. Cr Stress-Induced Ultramorphological Changes in Roots. Both genotype-dependent and concentration-dependent ultrastructural alterations were observed in root tip cells of the experimental cultivars (Figures 2(a)–2(f)). Comparative ultrastructural studies of our experimental cultivars reveal that the control cells were having typical ultrastructures (Figures 2(a), 2(c), and 2(e)). Intercellular spaces were absent. Plasma membrane was intact with the cell wall. Cell wall size was normal. There was a dense cytoplasm, with numerous mitochondria. Vacuoles were less in number and their size was normal. Nucleus was almost round-shaped having

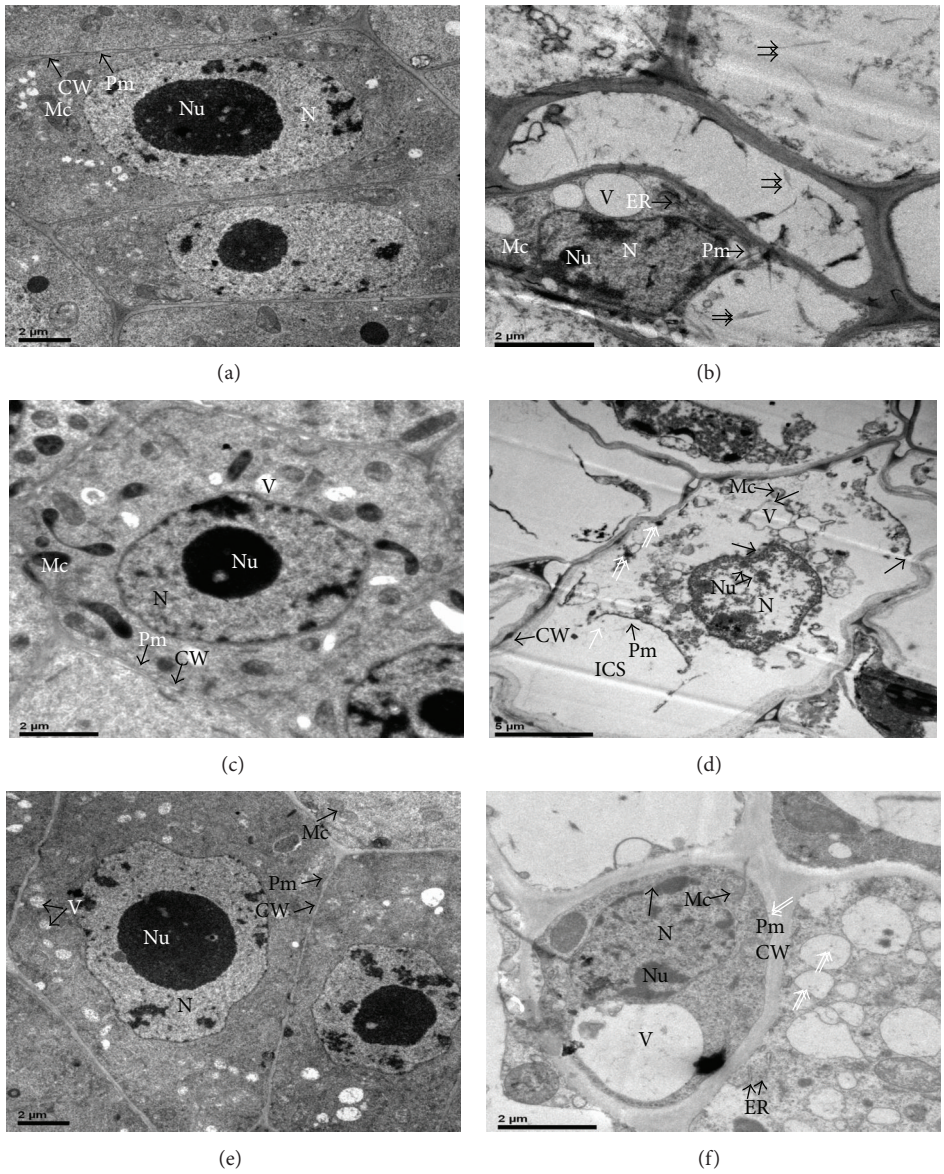


Figure 2: Electron micrographs of herbicide resistant transgenic cotton cultivar (J208) ((a) (CK); (b) (100 μM Cr)), insect resistant transgenic cotton cultivar (Z905) ((c) (CK); (d) (100 μM Cr)), and their hybrid line (ZD14) ((e) (CK); (f) (100 μM Cr)) under exceeding levels of Cr. CW = cell wall; Pm = plasma membrane; Mc = mitochondria, N = nucleus; Nu = nucleoli; V = vacuole; ER = endoplasmic reticulum; ICS = intracellular spaces. Double arrows indicate Cr dense granules while single arrow shows rupturing of membranous structures.

chromatin materials as well as nucleolus. However, genotype-dependent ultrastructural alterations were observed in the Cr-treated samples in all three cultivars (Figures 2(b), 2(d), and 2(f)). Less ultrastructural modifications could be observed in their hybrid line (Figure 2(f)). As a whole, the number of vacuoles increased, shape of nuclei was irregular, and cytoplasm and nucleus were less dense. Moreover, plasmolysis of the plasma membrane was evident and there was an increase in the cell wall size, disruption of plasma, and nuclear membrane as well as the number of nucleoli increased. Also, dense precipitates, most probably Cr, could be noticed in vacuoles and attached to the cell wall. Even in some micrographs, there could be found ruptured mitochondrial membranes. Such alterations were more in J208 followed by Z905 and their hybrid line (ZD14).

3.6. Cr Caused Oxidative Stress as Revealed by Increase in MDA and H_2O_2 Contents and Decrease in Total Soluble Proteins. We also investigated the biomarkers of oxidative stress such as MDA, H_2O_2, and total soluble proteins (Figures 3(a)–3(c)). Mean data regarding MDA contents (Figure 3(a)) and H_2O_2 (Figure 3(b)) revealed that Cr-induced increments were invariably significant. For example, the MDA contents were statistically significant at 10 and 50 μM Cr in comparison with related controls in both transgenic cotton cultivars and their hybrid line. However, at 100 μM Cr, the MDA

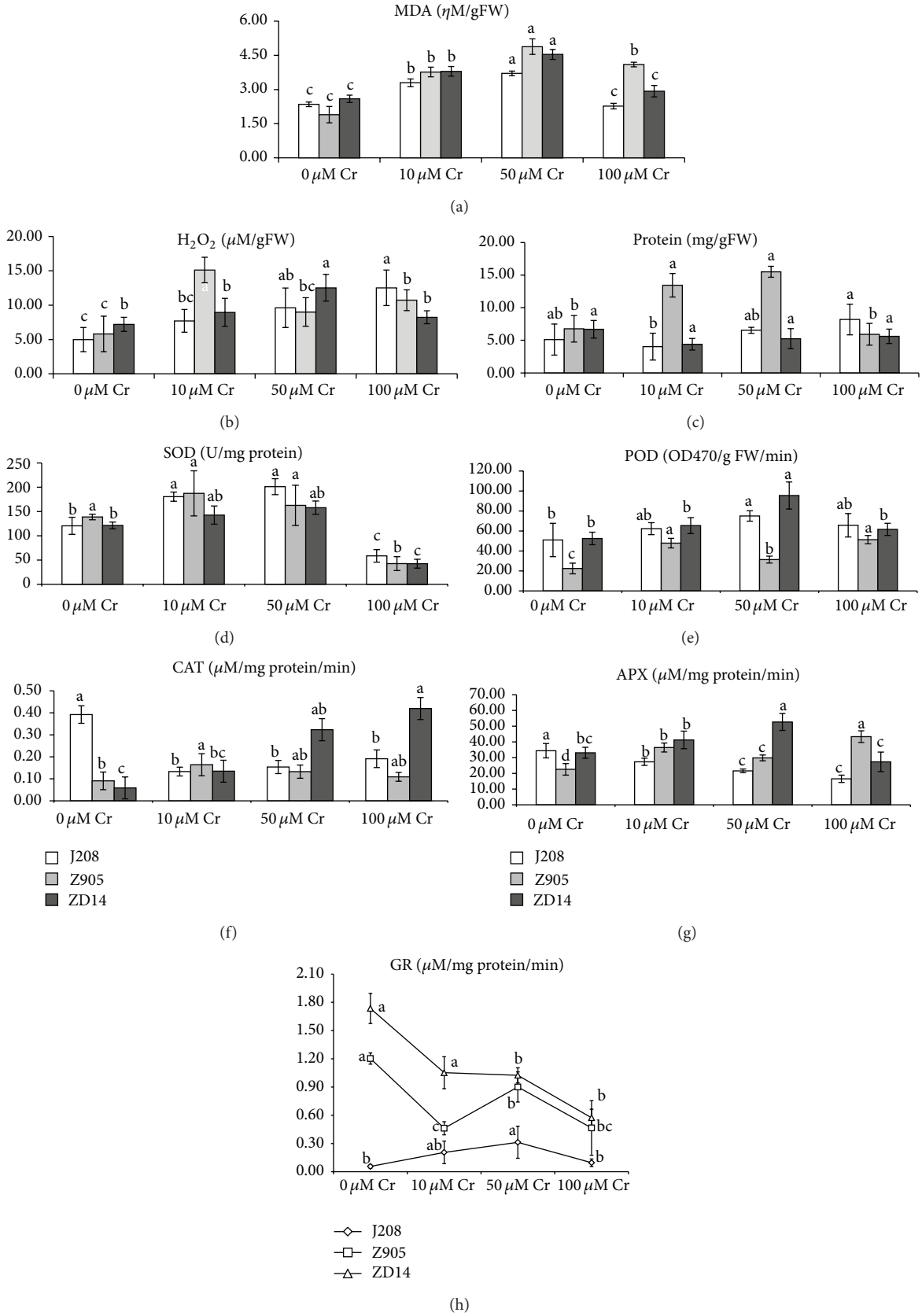


Figure 3: Contents of MDA (a), H_2O_2 (b) and total soluble proteins (c), activities of SOD (d), POD (e), CAT (f), APX (g), and GR (h) in roots of herbicide resistant transgenic cotton cultivar (J208), insect resistant transgenic cotton cultivar (Z905), and their hybrid line (ZD14) under exceeding levels of Cr.

contents of Z905 were significantly higher than its control (i.e., 116% relative to control). Taken together, the MDA contents increased by 200% over the controls and they were found in higher amount (124%) in Z905. However, such trend could not be established in all the experimental cultivars as revealed by the mean data of H_2O_2 contents in roots. In J208, enhancement was regular starting from control up to the highest Cr level. At 100 μM Cr level, mean data showed 151% increase over its relevant control. In Z905, significant and highest increase (160%) could be observed at 10 μM over the control, which was followed by a sharp decline. However, overall an increase was recorded in H_2O_2 contents over its control. Like J208, similar trend was found in ZD14 up to 50 μM Cr. However, at 100 μM Cr level, there was a decline over the other two lower levels of Cr but its contents were higher (14%) than the controls.

Figures 3(a)–3(c) further depict that the soluble protein contents (Figure 3(c)) in roots of Cr challenged cotton cultivars. In both parent lines, a sequential increase in their mean values except at 10 μM Cr in J208 and at 100 μM Cr in Z905 was observed. Moreover, greater relative increase (60.18%) was found in J208 at 100 μM Cr, while in Z905 such increase (129.1%) was found at 50 μM Cr. In their hybrid line, decline was noticed, which was higher at 10 μM Cr (i.e., 34%). However, decline was statistically nonsignificant at 5% probability levels. As a whole, percent relative increase was found higher in Z905 followed by J208 and ZD14. Furthermore, highest enhancement was noted in H_2O_2 (237%), while it was 200% and 70% in MDA and protein, respectively.

3.7. Differential Responses of ROS-Scavenging Enzymatic Antioxidants. Figures 3(d)–3(g) show the metabolic status of various enzymatic antioxidants in cotton cultivars under Cr stress. Regarding SOD activity (Figure 3(d)), the mean values showed concentration-dependent increase in all experimental cultivars up to 50 μM Cr. Up to this level, gradual increase could be observed in both J208 and their hybrid line (ZD14) up to 50 μM Cr, while in Z905 SOD activity first increased (35%) at 10 μM Cr and then decreased but was higher than its control. At 100 μM Cr, its activities significantly decreased. Highest decline was noted in Z905 (69%) as compared to its hybrid line (65%) and J208 (51%). Taken together, regarding relative increase or decrease values, the overall SOD activity was decreased by 6% in Z905 and ZD14 while, in J208, it was enhanced by 22%. However, as whole the mean data reveal 11% relative increase over the controls in SOD activity in these cultivars.

Figures 3(d)–3(g) also show considerable stimulation of the peroxidase activity (Figure 3(e)) in Cr-stressed roots over the related controls. Significant enhancement could only be observed in Z905, while the increase was almost statistically nonsignificant in the other two experimental cotton cultivars. Moreover, in J208, highest relative increase (47%) was found at 50 μM Cr, while, in Z905 and ZD14, it was 128% and 82% at 100 μM Cr, respectively. Overall performance of these cultivars was in the order of Z905 (33%) > ZD14 (41%) > J208 (33%).

Figures 3(d)–3(g) depict differential changes in APX activity (Figure 3(f)) upon application of Cr in the nutrient solutions. In J208, its activity gradually decreased by 37% relative to control, while, in Z905, it is significantly enhanced by 62% over its control. However, in their hybrid line, increase was significant up to 50 μM Cr and at 100 μM Cr there was a sharp decline by 17%. Furthermore, statistical significance was observed at all levels of Cr over the control in Z905, while in J208 a nonstatistical significance was present between 50 and 100 μM Cr. In ZD14 such situation could be found between 0 and 10 as well as between 0 and 100 μM Cr levels.

The spectrometric analysis of root CAT activity (Figure 3(g)) shows almost similar trend-like APX in both transgenic cotton cultivars and their hybrid line except in ZD14, which showed sharp increase at all levels of Cr over its control. Significant inhibition of CAT activity (51% relative to control) in roots of J208 could be found at 100 μM Cr over the control, while significant increase (81, 614%) was found at 10 and 100 μM Cr in Z905 and ZD14, respectively.

By comparing the overall performance of our present experimental cultivars, we can find that ZD14 excels the other two cultivars. Comparison of the overall activities of enzymatic antioxidants shows that there was an increase in their activities, which was in the order of CAT (387% relative to control) > POD (168% relative to control) > APX (48% relative to control) > SOD (11% relative to control).

3.8. Nonenzymatic Antioxidants' Response towards Cr Stress Levels. Responses of both transgenic cotton cultivars and their hybrid line with respect to nonenzymatic antioxidant such as glutathione reduced were quite different from one another (Figure 3(h)). The mean data revealed either increase as in case of J208 or decrease as in case of Z905 or both increase and decrease as in case of ZD14. GR activity in J208 first increased up to 50 μM Cr (by 457%) and then decreased at 100 μM Cr (by 70%). However, the value was still higher at 100 μM Cr as compared with the control. In case of Z905, GR activity showed downward trend, which was highest (78%) at 10 μM Cr. Regarding ZD14, GR activity at 10 μM Cr increased by 11% and then gradually deceased as compared with control. Moreover, at 5% probability level, statistically significant increase could only be noticed at 50 μM Cr in J208 as compared with its control, while such significance was found in Z905 at 10 and 50 μM Cr as compared with control. Varietal comparative performance reveals overall an increase in GR activity by 265% over the control in J208, 65 and 49% decline in Z905 and ZD14, respectively, could be observed.

4. Discussion

Chromium (Cr) is a toxic element to higher vascular plants [36], which causes the oxidative damages to DNA, RNA, proteins, and pigments [8, 37]. Plants provide a unique set up of antioxidant enzymes [38] against such oxidative stress.

In the present experiment, Cr variably influenced the root morphology of cotton cultivars. Dose-dependent reduction in number of root hairs and secondary roots was found. Roots of both parent lines (J208, Z905) were poorly developed with brown coloration. Such observations have also been demonstrated by [17] in *Zea mays* L. [39], in *T. aestivum,* and

[40] in *P. sativum* in Cr-stressful conditions. Pale color and stunted growth of roots might be due to interaction of Cr with various unknown root metabolic processes.

Decrease in root growth in terms of length and biomass has been a well-documented effect of Cr in plants [41]. Its presence in roots may exclude the translocation of essential metals such as Fe, S, and Zn to aerial parts of the plants thus causing indirectly the lowering down of shoot growth. A consistent decrease was found in root growth and biomass. Root fresh biomass reduced significantly as compared with root dry biomass. Similar trend was found in biomass-based growth inhibition, which exhibited an increase in their mean data at higher Cr stress levels in all experimental cultivars. Dose-dependent inhibition was noted in root-related growth parameters, which is in line with the findings of [12] in paddy rice [17], in *Z. mays* [40], in *Pisum sativum* [42], and in *B. oleracea*. Cr-induced decrease in root growth might be either due to an extension in cell cycle, which leads to arrested cell division, cell elongation, and lowering down of mitotic index [12, 43], or incapability of the roots to absorb water and nutrients from the medium [44]. Tolerance indices based on root length and fresh weight are commonly used to quantify plant metal tolerance [45]. Physiology-based tolerance indices overall declined, which was in the order of root fresh weight > root length > plant height > water contents. Mean data depict greater decrease in tolerance in root length of Z905. Further, the water contents-based tolerance was upregulated in J208, while the other two cotton cultivars showed a decline. It conveys a message that Cr caused an insignificant water stress in Z905 and ZD14. Similar results were obtained by [27] during their studies on cotton seedlings under Cd stress. However, Reisinger et al. [46] found increased tolerance in transgenic mustard cultivars.

Heavy metal uptake by roots and its translocation to aerial parts are two important indicators in phytoextraction technology. This signifies that such plant species should be selected by phytoextraction technologists, whose aerial parts are utilized and have no role in our food chain. Cotton, being mostly grown as a fiber crop in most parts of the world, is an ideal species to be used for Cr extraction and uptake. In the present experiment, Cr uptake in both transgenic cotton cultivars and their hybrid was concentration-dependent. More Cr was accumulated in roots than in leaves in all these cultivars. Taken together, the Cr uptake efficiency of both roots and leaves, Cr uptake in roots was more in Z905 as compared with other cultivars. And this could the reason that there was a greater distortion in its plasma membrane integrity as evident from electron micrographs. Similar to our findings, increase in root Cr levels has been found in rice [22], *Typha* [47], and pea [48].

Furthermore, the data about translocation factor reveal that the Cr translocation efficiency was < 1 in all these cultivar. This conveys a message that Cr was not efficiently translocated from root to shoot. That is why Cr mainly accumulated in roots than leaves. Translocation efficiency was higher in ZD14 as compared with its parent lines as revealed by relative increase in its values. Lower Cr translocation from roots to leaves is a good sign that all these cultivars can be exploited for Cr uptake purpose. Low levels of Cr translocation have also been found by [13, 49].

Typical ultrastructures were found in root tip cells of the experimental cultivars in control cells. However, at highest Cr concentration level, noticeable modifications were observed. Less ultrastructural changes were observed in hybrid line as compared to its parent lines. Increase in number of nuclei is an indicator of increased stress tolerance in respective experimental material. Such increase may result in enhanced protein synthesis [27]. Moreover, Cr dense granules were obviously present in vacuoles and attached to the cell wall. Increase in number of nuclei and vacuolar size and its number as well as the presence of Cr dense precipitates in the dead parts of the cell in all these cultivars shows a positive indication that they can be used for phytoremediation purpose in Cr-contaminated areas. Consistent with our results, Panda [22] in rice seedlings, Speranza et al. [50] in the pollen grains of kiwi, and Eleftheriou et al. [51] in *Allium cepa* observed almost similar ultramorphological features in Cr-treated root samples.

In plants, Cr may induce oxidative stress, which results in lipid peroxidation and oxidative damage [52]. Mean data regarding MDA contents revealed that they were significantly increased over the related controls in all three cultivars and were found higher in Z905. However, such trend was not found in H_2O_2 contents of roots. However, overall an increase was recorded in its contents over its control. Increase in MDA contents has also been reported by [53] in germinating pollen of kiwi and *M. sinensis* [54]. In contrast to our findings, there was no change in the MDA content in *S. natans* plants growing in Cr-rich wastewater [55]. An overall increase was also recorded in H_2O_2 contents of roots, which are in line with the findings of [22, 54, 56].

Total soluble protein contents in roots were increased in our experimental materials as a whole. Enhancement in the total soluble proteins is a good indicator that these cultivars are capable to withstand Cr-stressed environment. Another possible reason for such increase might be due to increase in the number of nuclei, which might have led an increase in the mRNA synthesis. Our results are against the findings of Ganesh et al. [57].

In our present experiment, we studied ROS-scavenging antioxidants. The SOD activity as a whole increased up to 50 μM Cr, while, at 100 μM Cr, its activity significantly decreased in all cultivars. Peroxidase (POD) activity in Cr-stressed roots considerably stimulated over the related controls, which was significant in Z905. The APX activity differentially changed. In J208, its activity gradually decreased, while, in Z905, it was significantly enhanced. However, in their hybrid line, increase was found up to 50 μM Cr, while at 100 μM Cr there was a sharp decline. CAT activity was almost similar to APX except in their hybrid line (ZD14). Our experimental cultivars showed differential responses regarding glutathione reductase activity. As a whole, activities of ROS-scavenging antioxidants increased upon exposure to Cr exceeding levels. Such increase has also been found by Liu et al. [58] in *P. sativum* under Cr stress. However, contrasting results were reported by [22] in rice and [55] in Cr hyperaccumulator *S. natans*.

Taken together, an overall increase in the activities of these enzymes reveals that SOD might have been actively involved in H_2O_2 production. This hypothesis can be proved by increase in H_2O_2 contents in roots of all these cultivars. And more importantly to detoxify H_2O_2, ROS-scavenging enzymes were active as a whole. This might be one of the reasons that these cultivars grew well in Cr-stressful conditions and their Cr uptake by roots was appreciable.

5. Conclusions

Based on our present results, it is evident that Cr influenced the physiology of our experimental cotton cultivars. However, their growth and ultramorphology were least affected. Also, there was an increase in number of nuclei and vacuoles as well as presence of Cr-dense granules in root cells. Antioxidative metabolism was also upregulated. Increase in number of nuclei and vacuoles, rise in antioxidant enzymatic activity, lower translocation factor, and higher accumulation of Cr in roots than in leaves show that these cultivars have greater potential of Cr uptake and can be considered potential candidates for reclamation of Cr-polluted areas.

Abbreviations

APX: Ascorbate peroxidase
CAT: Catalase
GR: Glutathione reductase
H_2O_2: Hydrogen peroxide
MDA: Malondialdehyde
POD: Guaiacol peroxidase
ROS: Reactive oxygen species
SOD: Superoxide dismutase.

Conflict of Interests

All authors have no conflict of interests regarding the publication of this paper.

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References

[1] C. Prado, L. Rodríguez-Montelongo, J. A. González, E. A. Pagano, M. Hilal, and F. E. Prado, "Uptake of chromium by *Salvinia minima*: effect on plant growth, leaf respiration and carbohydrate metabolism," *Journal of Hazardous Materials*, vol. 177, no. 1–3, pp. 546–553, 2010.

[2] S. Avudainayagam, M. Megharaj, G. Owens, R. S. Kookana, D. Chittleborough, and R. Naidu, "Chemistry of chromium in soils with emphasis on tannery waste sites," *Reviews of Environmental Contamination and Toxicology*, vol. 178, pp. 53–91, 2003.

[3] D. E. Kimbrough, Y. Cohen, A. M. Winer, L. Creelman, and C. Mabuni, "A critical assessment of chromium in the environment," *Critical Reviews in Environmental Science and Technology*, vol. 29, no. 1, pp. 1–46, 1999.

[4] R. Shrivastava, R. K. Upreti, P. K. Seth, and U. C. Chaturvedi, "Effects of chromium on the immune system," *FEMS Immunology and Medical Microbiology*, vol. 34, no. 1, pp. 1–7, 2002.

[5] J. R. Peralta-Videa, M. L. Lopez, M. Narayan, G. Saupe, and J. Gardea-Torresdey, "The biochemistry of environmental heavy metal uptake by plants: implications for the food chain," *International Journal of Biochemistry and Cell Biology*, vol. 41, no. 8-9, pp. 1665–1677, 2009.

[6] C. Cervantes, J. Campos-García, S. Devars et al., "Interactions of chromium with microorganisms and plants," *FEMS Microbiology Reviews*, vol. 25, no. 3, pp. 335–347, 2001.

[7] T. K. Tokunaga, J. Wan, T. C. Hazen et al., "Distribution of chromium contamination and microbial activity in soil aggregates," *Journal of Environmental Quality*, vol. 32, no. 2, pp. 541–549, 2003.

[8] S. Ali, P. Bai, F. Zeng et al., "The ecotoxicological and interactive effects of chromium and aluminum on growth, oxidative damage and antioxidant enzymes on two barley genotypes differing in Al tolerance," *Environmental and Experimental Botany*, vol. 70, no. 2-3, pp. 185–191, 2011.

[9] H. Oliveira, "Chromium as an environmental pollutant: insights on induced plant toxicity," *Journal of Botany*, vol. 2012, Article ID 375843, 8 pages, 2012.

[10] C. Desai, K. Jain, and D. Madamwar, "Evaluation of In vitro Cr(VI) reduction potential in cytosolic extracts of three indigenous *Bacillus* sp. isolated from Cr(VI) polluted industrial landfill," *Bioresource Technology*, vol. 99, no. 14, pp. 6059–6069, 2008.

[11] L. B. Paiva, J. G. de Oliveira, R. A. Azevedo, D. R. Ribeiro, M. G. da Silva, and A. P. Vitória, "Ecophysiological responses of water hyacinth exposed to Cr^{3+} and Cr^{6+}," *Environmental and Experimental Botany*, vol. 65, no. 2-3, pp. 403–409, 2009.

[12] P. Sundaramoorthy, A. Chidambaram, K. S. Ganesh, P. Unnikannan, and L. Baskaran, "Chromium stress in paddy: (i) nutrient status of paddy under chromium stress; (ii) phytoremediation of chromium by aquatic and terrestrial weeds," *Comptes Rendus: Biologies*, vol. 333, no. 8, pp. 597–607, 2010.

[13] A. Zayed, C. M. Lytle, J.-H. Qian, and N. Terry, "Chromium accumulation, translocation and chemical speciation in vegetable crops," *Planta*, vol. 206, no. 2, pp. 293–299, 1998.

[14] P. Babula, V. Adam, R. Opatrilova, J. Zehnalek, L. Havel, and R. Kizek, "Uncommon heavy metals, metalloids and their plant toxicity: a review," *Environmental Chemistry Letters*, vol. 6, no. 4, pp. 189–213, 2008.

[15] S. K. Panda and S. Choudhury, "Chromium stress in plants," *Brazilian Journal of Plant Physiology*, vol. 17, no. 1, pp. 95–102, 2005.

[16] S. Ali, F. Zeng, S. Cai, B. Qiu, and G. Zhang, "The interaction of salinity and chromium in the influence of barley growth and oxidative stress," *Plant, Soil and Environment*, vol. 57, no. 4, pp. 153–159, 2011.

[17] S. Mallick, G. Sinam, R. K. Mishra, and S. Sinha, "Interactive effects of Cr and Fe treatments on plants growth, nutrition and oxidative status in *Zea mays* L," *Ecotoxicology and Environmental Safety*, vol. 73, no. 5, pp. 987–995, 2010.

[18] S. Samantary, "Biochemical responses of Cr-tolerant and Cr-sensitive mung bean cultivars grown on varying levels of chromium," *Chemosphere*, vol. 47, no. 10, pp. 1065–1072, 2002.

[19] M. P. Fasulo, M. Bassi, and A. Donini, "Cytotoxic effects of hexavalent chromium in *Euglena gracilis*. II. Physiological and ultrastructural studies," *Protoplasma*, vol. 114, no. 2-3, pp. 35–43, 1983.

[20] C. Mouvet, "Accumulation of chromium and copper by the aquatic moss *Fontinalis antipyretica* L. ex Hedw. transplanted in a metal-contaminated river," *Environmental Technology Letters*, vol. 5, no. 12, pp. 541–548, 1984.

[21] M. D. Vázquez, C. H. Poschenrieder, and J. Barceló, "Chromium vi induced structural and ultrastructural changes in bush bean plants (*Phaseolus vulgaris* L.)," *Annals of Botany*, vol. 59, no. 4, pp. 427–438, 1987.

[22] S. K. Panda, "Chromium-mediated oxidative stress and ultrastructural changes in root cells of developing rice seedlings," *Journal of Plant Physiology*, vol. 164, no. 11, pp. 1419–1428, 2007.

[23] J. J. Grant and G. J. Loake, "Role of reactive oxygen intermediates and cognate redox signaling in disease resistance," *Plant Physiology*, vol. 124, no. 1, pp. 21–29, 2000.

[24] A. Polle, "Dissecting the superoxide dismutase-ascorbate-glutathione-pathway in chloroplasts by metabolic modeling. Computer simulations as a step towards flux analysis," *Plant Physiology*, vol. 126, no. 1, pp. 445–462, 2001.

[25] R. Mittler, "Oxidative stress, antioxidants and stress tolerance," *Trends in Plant Science*, vol. 7, no. 9, pp. 405–410, 2002.

[26] S. J. Stohs and D. Bagchi, "Oxidative mechanisms in the toxicity of metal ions," *Free Radical Biology and Medicine*, vol. 18, no. 2, pp. 321–336, 1995.

[27] M. K. Daud, Y. Sun, M. Dawood et al., "Cadmium-induced functional and ultrastructural alterations in roots of two transgenic cotton cultivars," *Journal of Hazardous Materials*, vol. 161, no. 1, pp. 463–473, 2009.

[28] W. Zhou and M. Leul, "Uniconazole-induced alleviation of freezing injury in relation to changes in hormonal balance, enzyme activities and lipid peroxidation in winter rape," *Plant Growth Regulation*, vol. 26, no. 1, pp. 41–47, 1998.

[29] V. Velikova, I. Yordanov, and A. Edreva, "Oxidative stress and some antioxidant systems in acid rain-treated bean plants protective role of exogenous polyamines," *Plant Science*, vol. 151, no. 1, pp. 59–66, 2000.

[30] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[31] W. F. Zhang, F. Zhang, R. Raziuddin et al., "Effects of 5-aminolevulinic acid on oilseed rape seedling growth under herbicide toxicity stress," *Journal of Plant Growth Regulation*, vol. 27, no. 2, pp. 159–169, 2008.

[32] Y. Nakano and K. Asada, "Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts," *Plant and Cell Physiology*, vol. 22, no. 5, pp. 867–880, 1981.

[33] H. Aebi, "Catalase in vitro," *Methods in Enzymology*, vol. 105, pp. 121–126, 1984.

[34] W. Zhou and M. Leul, "Uniconazole-induced tolerance of rape plants to heat stress in relation to changes in hormonal levels, enzyme activities and lipid peroxidation," *Plant Growth Regulation*, vol. 27, no. 2, pp. 99–104, 1999.

[35] M. Jiang and J. Zhang, "Water stress-induced abscisic acid accumulation triggers the increased generation of reactive oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves," *Journal of Experimental Botany*, vol. 53, no. 379, pp. 2401–2410, 2002.

[36] S. K. Yadav, M. Dhote, P. Kumar, J. Sharma, T. Chakrabarti, and A. A. Juwarkar, "Differential antioxidative enzyme responses of *Jatropha curcas* L. to chromium stress," *Journal of Hazardous Materials*, vol. 180, no. 1–3, pp. 609–615, 2010.

[37] I. Sharma, P. K. Pati, and R. Bhardwaj, "Effect of 28-homobrassinolide on antioxidant defence system in *Raphanus sativus* L. under chromium toxicity," *Ecotoxicology*, vol. 20, no. 4, pp. 862–874, 2011.

[38] S. P. Choudhary, M. Kanwar, R. Bhardwaj, J.-Q. Yu, and L.-S. P. Tran, "Chromium stress mitigation by polyamine-brassinosteroid application involves phytohormonal and physiological strategies in *Raphanus sativus* L," *PLoS ONE*, vol. 7, no. 3, Article ID e33210, 2012.

[39] S. Hasnain and A. N. Sabri, "Growth stimulation of *Triticum aestivum* seedlings under Cr-stresses by non-rhizospheric pseudomonad strains," *Environmental Pollution*, vol. 97, no. 3, pp. 265–273, 1997.

[40] E. Rodriguez, R. Azevedo, P. Fernandes, and C. Santos, "Cr(VI) induces DNA damage, cell cycle arrest and polyploidization: a flow cytometric and comet assay study in *Pisum sativum*," *Chemical Research in Toxicology*, vol. 24, no. 7, pp. 1040–1047, 2011.

[41] S. Hayat, K. Gulshan, I. Mohammad et al., "Physiological changes induced by chromium stress in plants: an overview," *Protoplasma*, vol. 249, no. 3, pp. 599–611, 2012.

[42] Y. Ozdener, B. Aydin, S. Fatma Aygün, and F. Yürekli, "Effect of hexavalent chromium on the growth and physiological and biochemical parameters on *Brassica oleracea* L. var. acephala DC," *Acta Biologica Hungarica*, vol. 62, no. 4, pp. 463–476, 2011.

[43] J. H. Zou, M. Wang, W. S. Jiang, and D. H. Liu, "Effects of hexavalent chromium (VI) on root growth and cell division in root tip cells of *Amaranthus viridis* L," *Pakistan Journal of Botany*, vol. 38, no. 3, pp. 673–681, 2006.

[44] J. Barceló, C. Poschenrieder, and J. Gunsé, "Effect of chromium (VI) on mineral element composition of bush beans," *Journal of Plant Nutrition*, vol. 8, pp. 211–217, 1985.

[45] A. Murphy and L. Taiz, "A new vertical mesh transfer technique for metal-tolerance studies in *Arabidopsis*—ecotypic variation and copper-sensitive mutants," *Plant Physiology*, vol. 108, no. 1, pp. 29–38, 1995.

[46] S. Reisinger, M. Schiavon, N. Terry, and E. A. H. Pilon-Smits, "Heavy metal tolerance and accumulation in Indian mustard (*Brassica juncea* L.) expressing bacterial γ-glutamylcysteine synthetase or glutathione synthetase," *International Journal of Phytoremediation*, vol. 10, no. 5, pp. 440–454, 2008.

[47] A. M. Bah, H. Dai, J. Zhao et al., "Effects of cadmium, chromium and lead on growth, metal uptake and antioxidative capacity in *Typha angustifolia*," *Biological Trace Element Research*, vol. 142, no. 1, pp. 77–92, 2011.

[48] K. K. Tiwari, S. Dwivedi, N. K. Singh, U. N. Rai, and R. D. Tripathi, "Chromium (VI) induced phytotoxicity and oxidative stress in pea (*Pisum sativum* L.): biochemical changes and translocation of essential nutrients," *Journal of Environmental Biology*, vol. 30, no. 3, pp. 389–394, 2009.

[49] J. Chatterjee and C. Chatterjee, "Phytotoxicity of cobalt, chromium and copper in cauliflower," *Environmental Pollution*, vol. 109, no. 1, pp. 69–74, 2000.

[50] A. Speranza, A. R. Taddei, G. Gambellini, E. Ovidi, and V. Scoccianti, "The cell wall of kiwifruit pollen tubes is a target for chromium toxicity: alterations to morphology, callose pattern and arabinogalactan protein distribution," *Plant Biology*, vol. 11, no. 2, pp. 179–193, 2009.

[51] E. P. Eleftheriou, I.-D. S. Adamakis, and P. Melissa, "Effects of hexavalent chromium on microtubule organization, ER distribution and callose deposition in root tip cells of *Allium cepa* L," *Protoplasma*, vol. 249, no. 2, pp. 401–416, 2012.

[52] M. O. Montes-Holguin, J. R. Peralta-Videa, G. Meitzner et al., "Biochemical and spectroscopic studies of the response of *Convolvulus arvensis* L. to chromium(III) and chromium(VI) stress," *Environmental Toxicology and Chemistry*, vol. 25, no. 1, pp. 220–226, 2006.

[53] V. Scoccianti, M. Iacobucci, M. F. Paoletti, A. Fraternale, and A. Speranza, "Species-dependent chromium accumulation, lipid peroxidation, and glutathione levels in germinating kiwifruit pollen under Cr(III) and Cr(VI) stress," *Chemosphere*, vol. 73, no. 7, pp. 1042–1048, 2008.

[54] S. A. Sharmin, I. Alam, K. H. Kim et al., "Chromium-induced physiological and proteomic alterations in roots of *Miscanthus sinensis*," *Plant Science*, vol. 187, pp. 113–126, 2012.

[55] B. Dhir, P. Sharmila, P. P. Saradhi, and S. A. Nasim, "Physiological and antioxidant responses of *Salvinia natans* exposed to chromium-rich wastewater," *Ecotoxicology and Environmental Safety*, vol. 72, no. 6, pp. 1790–1797, 2009.

[56] R. K. Rai, M. K. Srivastava, A. K. Khare, R. Kishor, and A. K. Shrivastava, "Oxidative stress response and glutathione linked enzymes in relation to growth of sugarcane plants exposed to hexavalent chromium," *Sugar Tech*, vol. 8, no. 2-3, pp. 116–123, 2006.

[57] K. S. Ganesh, L. Baskaran, S. Rajasekaran, K. Sumathi, A. L. A. Chidambaram, and P. Sundaramoorthy, "Chromium stress induced alterations in biochemical and enzyme metabolism in aquatic and terrestrial plants," *Colloids and Surfaces B: Biointerfaces*, vol. 63, no. 2, pp. 159–163, 2008.

[58] D. Liu, J. Zou, M. Wang, and W. Jiang, "Hexavalent chromium uptake and its effects on mineral uptake, antioxidant defence system and photosynthesis in *Amaranthus viridis* L," *Bioresource Technology*, vol. 99, no. 7, pp. 2628–2636, 2008.