Overexpression of Auxin Binding Protein 57 from Rice (Oryza sativa L.) Increased Drought and Salt Tolerance in Transgenic Arabidopsis thaliana

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Abstract. Auxin plays important roles in many aspects of plant growth and development. It is known that auxin binds to auxin-binding protein (Abp57) and activates the plasma membrane H⁺-ATPases. In order to elucidate the biological function of rice Abp57 in plants, overexpression transgenic arabidopsis lines were generated. We found overexpression transgenic arabidopsis harbouring rice Abp57 showed tolerance to osmotic stress. Phenotypic transgenic arabidopsis showed longer roots than the wildtype when grown on ½ MS media supplemented with various concentrations of NaCl or PEG-6000. Besides, transgenic arabidopsis has relatively lower water loss rate, higher chlorophyll content and higher biomass when growing under osmotic stress compared to the wildtype. These results suggested that Abp57 has important roles in giving tolerance to arabidopsis against drought and salt stresses.

Keywords: Abiotic stress tolerance; auxin; osmotic stress; NaCl

1. Introduction

Abiotic stress adversely affects plant growth and crop yields. Plants as sessile organisms have significantly evolved their efficient defense mechanism to sense and rapidly adapt to abiotic stress conditions. One of the most important components is phytohormone, which has the ability to regulate plant viability under abiotic stress conditions [1,2].

In plants, auxin is an essential phytohormone in regulating various aspects of plant growth and development such as embryogenesis, cell division and elongation, gravitropism as well as apical dominance [3]. Recent studies have shown that auxin also renders plants tolerant against abiotic stress [4]. Plant tolerance to abiotic stress such as drought and salinity was found to highly correlate to the expression of auxin-related genes. Overexpression of the YUCCA6 gene (flavin-containing monooxygenase) involved in auxin biosynthesis in transgenic poplar and potato, for example, enhanced resistance to water deficit and oxidative stress of the plants [5,6] Meanwhile, overexpression of an auxin efflux carrier gene, OsPIN3t also improved drought and salt stress tolerance in plants [7].

Homeostasis of auxin in plants is affected by abiotic stress [8]. The redox state of cells is altered under stress conditions and this affects auxin signaling in plants [9,10]. Previous studies indicate that
double auxin receptor mutants, *tir1 afb2* have displayed higher antioxidant activities and tolerance against stress [11]. In addition to TIR1/AFB (Transport Inhibitor Response 1/Auxin Signaling F-Box Proteins), auxin is also perceived by Auxin-binding Protein 1 (ABP1), which is needed for the activation of ROP GTPases for ROS production [12,13]. ABP1 was said to have negatively regulated activities of TIR1/AFB at the upstream of the auxin-signaling pathway [14]. Expressions of these two auxin receptors have a counter effect on ROS production. ROS as a main factor causing oxidative stress in plants also acts as a secondary signaling messenger, which interacts with auxin in regulating plant growth and development [15]. It was shown that stress-induced ROS could trigger the MAPK cascades that, in turn, repress the auxin-dependent signaling as well as transduce protective oxidative stress signaling in plant cells [16].

Apart from the auxin-binding proteins ABP1 and auxin receptor TIR1/AFBs that is important for abiotic stress response signaling [17,18,19], Kim et al. [20] have isolated a new auxin-binding protein, ABP57 from rice. ABP57 activates plasma membrane (PM) H⁺-ATPase via direct interaction [21]. In plants, the PM H⁺-ATPase couples ATP hydrolysis for proton transport activity. The pH and potential difference created by PM H⁺-ATPase across the plasma membrane are important for secondary transport, stomata aperture and osmoregulation, salinity tolerance, intracellular pH regulation and cellular expansion [22]. Both auxin and PM H⁺-ATPase involvement in plant abiotic stress suggest that *Abp57* are likely to be involved in abiotic stress tolerance in plants. In this paper, the biological function of *Abp57* in response to drought and salt stress was characterized by the generation of transgenic arabidopsis overexpressing Abp57.

2. Materials and methods

2.1. Generation of transgenic arabidopsis overexpressing Abp57

The overexpression construct pH2GW7-Abp57 was obtained from RDA (Suwon, Republic of Korea) and then transformed into *Agrobacterium tumefaciens* (strain GV3101). Transgenic *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) was generated via floral-dip transformation method as described by Clough and Bent [23]. Seeds obtained from transformed arabidopsis were screened on MSO medium supplemented with 20 mg/L hygromycin.

Transgenic seeds from T2 generation were sterilized and germinated on MS media containing hygromycin to distinguish the homozygous lines from segregating lines. The homozygous lines will show 100% survivability while segregating lines will have a survival rate of < 100%. Meanwhile, the ratio of survived seedlings and bleached seedlings were also counted after seven days of germination to determine the copy number of transgene.

2.2. Total RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from one-week-old seedlings of MR219 using TRIzol® reagent (Life Technology, USA) and then subjected to DNase treatment using Ambion® TURBO® DNase. The total RNA was reverse-transcribed into first-strand cDNA with Maxima First Strand cDNA synthesis kit (Thermo Fisher Scientific, USA). Equal amounts of cDNA were used as templates for PCR amplification using DreamTaq Master Mix (Thermo Fisher Scientific, USA). The gene of interest was amplified using gene specific primer pair, *Abp57* 5'-ATGGCAGAGATTGTTAGTTC 3' and 5'-CTAAAATTCAGGCAGCAGTA-3'. Housekeeping gene, 4-HPPD was used as an internal control. Reaction conditions were set up following the kit’s protocol: denaturation at 95°C for 10 min followed by 35 amplification cycles (94°C/30 s, 55°C/30 s, 72°C/2 min) and final extension step at 72°C for 5 min.

2.3. Stress assays of transgenic arabidopsis

Three independent lines of homozygous and single copy transgenic arabidopsis from T3 generations were used to study the effect of water stress and salinity stress on transgenic arabidopsis *Abp57*. Response of transgenic arabidopsis to desiccation and NaCl stress were also tested on soil condition.
Plants were grown for 20 days under normal conditions and watering was stopped for ten days to observe the effect of soil drying on transgenic arabidopsis and Col-0. Dry biomass of the intact plant after the soils drying treatment was determined by drying in oven (70 °C) for 48 hours. Meanwhile, transgenic arabidopsis were also subjected to NaCl solution (200 mM) irrigation for two weeks in another independent experiment for salinity stress.

To analyze the water loss rate between transgenic arabidopsis and control, the detached leaves grown under normal conditions were collected and kept under growth chamber conditions with air flowing. The leaves fresh weight was quantified in one hour intervals for up to six hours and the loss rates were calculated from decreases in the rate of FW at designed time interval.

2.4. Root elongation assay
To further investigate the response of transgenic arabidopsis under moderate water stress and salinity stress, Col-0 or NT and transgenic arabidopsis Abp57 were grown on MS medium supplemented with PEG-6000 ($\psi_w = -0.5$ MPa) and various concentration of NaCl (0, 50, 100, 150 mM) for ten days. The root lengths of seedlings grown under control and stress conditions were measured using Image J (https://imagej.nih.gov/ij/index.html).

2.5. Chlorophyll measurement
For evaluating the effect of desiccation and salinity stress on chlorophyll content of leaves of Col-0 and transgenic arabidopsis, seedlings were cultured on MS medium containing 100 mM NaCl and 150 mM mannitol for two weeks. The total chlorophyll content was estimated according to the method described by Hiscox and Isrealstem [24].

2.6. Statistical analysis
All experiments in the study were repeated at least twice and the data explained are the mean ± SE of these independent experiments. The asterisk and letters above the column of figures indicated significant difference at $p < 0.05$.

3. Results

3.1. Generation of Abp57 overexpressing transgenic arabidopsis
Transgenic arabidopsis were generated to study Abp57 in plants under abiotic stress. The open reading frame (ORF) of Abp57 was over-expressed under the control of CaMV35S promoter in arabidopsis. Progeny segregation analysis (table 1) shows that five independent lines of transgenic arabidopsis have single insertion. Homozygous seeds were obtained from these lines at T3 generation for molecular and physiological analysis. The semi-quantitative PCR analysis (RT-PCR) showed a high transcript level of Abp57 in selected homozygous transgenic lines, whereas no transcript was detected in WT seedlings (figure 1).

3.2. Transgenic arabidopsis Abp57 is more tolerance to abiotic stress
Abp57-overexpression arabidopsis were examined for tolerance to osmotic stress and salinity stress. After a two weeks stress treatment on soil, the transgenic arabidopsis showed mild symptoms and retained more chlorophyll, whereas the wildtype (Col-0) and non-transformant (NT) experienced more severe wilting or necrosis. This result suggested that transgenic arabidopsis Abp57 is comparatively more tolerant to drought and salinity stress than the wildtype (figure 2).

3.3. Phenotype of transgenic arabidopsis under stress condition
The potential effect of Abp57 overexpression on salt tolerance and drought was evaluated by transferring Col-0 and Abp57-overexpression lines seedlings onto the medium supplemented with or without NaCl or mannitol. The chlorophyll content of Col-0 was found to be reduced more than
transgenic lines at 100 mM NaCl (figure 3). Meanwhile, the chlorophyll content of transgenic plants was also higher compared to the Col-0 at 150 mM mannitol treatment.

**Table 1.** Progeny segregation analysis of five independent lines of transgenic arabidopsis. The copy number of transgene was decided based on ratio of survived seedlings and bleached seedlings.

| Line | Hyg<sup>R</sup> | Hyg<sup>S</sup> | Ratio | Transgene copy number |
|------|-----------------|-----------------|-------|-----------------------|
| 1    | 106             | 34              | 3.1 : 1 | 1                     |
| 2    | 84              | 30              | 2.8 : 1 | 1                     |
| 3    | 102             | 32              | 3.2 : 1 | 1                     |
| 4    | 102             | 29              | 3.5 : 1 | 1                     |
| 6    | 48              | 15              | 3.2 : 1 | 1                     |

**Figure 1.** RT-PCR analysis of *Ahp57* overexpression in five independent lines of transgenic arabidopsis and wild type arabidopsis, Col-0. The 4-HPPD was used as an internal control.

**Figure 2.** Comparisons of transgenic arabidopsis lines, Col-0 and non-transformant, NT under well-watering, salt stress (NaCl, 200 mM) and drought conditions (from left to right).
The 2nd International Conference on Biosciences (ICoBio) IOP Publishing
IOP Conf. Series: Earth and Environmental Science 197 (2018) 012038  doi:10.1088/1755-1315/197/1/012038

Figure 3. Chlorophyll content in Abp57-overexpressing transgenic arabidopsis and Col-0 under control condition, salt stress and osmotic stress. Data shown represent means ± S.E of three replicates and experiments were repeated three times. Means with different letters are significantly different at p ≤ 0.05 according to Duncan’s multiple range test.

Stress tolerance of arabidopsis Abp57-overexpression lines were further examined by root length analysis. The transgenic plants had longer roots than Col-0 under osmotic stress conditions. When grown on 1/2 Murashige-Skoog (MS) medium containing PEG6000 ($\psi_w = -0.5$ MPa) and NaCl (50, 100 and 150 mM), the primary roots of Abp57-overexpression lines seedlings were significantly longer than observed in Col-0 after 8 d of growing (figure 4 and figure 5). No significant difference was observed under normal condition.

Abp57 was also investigated for osmoregulation by measuring the fresh weight loss of detached leaves. Both transgenic lines show a significant decrease in the rate of water loss compared with Col-0 (figure 6). This analysis reflects the balance between water supply to the leaf and transpiration rate. Low water loss rate of detached leaves under drought have indicate better capability to maintain water balance in leaves and this is attributable to stress tolerance [25]. Furthermore, the Abp57-overexpression lines also had a higher biomass compared to the Col-0 and NT (figure 7). These results further demonstrated that Abp57-overexpression lines increased tolerance to salt and drought stresses. Taken together, these suggest that Abp57 contributed to the growth of plant under abiotic stress. The transgenic plants enhanced tolerance to osmotic and salinity stress.

4. Discussion

Previous researches have reported that various stress-related genes are involved in plant tolerance to abiotic stress. Expression manipulation of these genes is able to give stress tolerance to crop plants. In this present study, transgenic arabidopsis overexpressing Abp57 was generated for abiotic stress assay.

The transgenic arabidopsis overexpressing Abp57 showed tolerance to drought and salinity stress. This finding is different from previous research of a well-recognized auxin receptor, TIR1/AFB (Transport Inhibitor Response 1/ Auxin signaling F-Box) in auxin signaling. The arabidopsis double mutant, tir1 afb2 showed tolerance to salt stress with enhanced physiological parameters and upregulation of antioxidant enzyme production such as CAT (Catalase) and APX (ASCORBATE PEROXIDASE) [11]. However, downregulation of OsTIR1 and OsAFB2 via OsmiR393 overexpression led to less tolerance to salt and drought in rice. Nevertheless, the IAA synthesis was
not affected by a low expression of OsTIR1 and OsAFB2 in the transgenic rice [26]. This suggested the involvement of other auxin-related genes in auxin signaling. Another class of speculated auxin receptors known as Auxin-binding protein 1, Abp1 was previously found to have an antagonistically regulated TIR1/AFB pathway [14]. However, the function of Abp1 as an auxin receptor is still under debate. The findings of Abp57 with auxin-binding activity suggest the existence of auxin signaling pathways that remain to be elucidated. Overexpression of Abp57 is able to confer drought and salinity stress tolerance in transgenic aridopsis. Auxin-binding activity indicates that Abp57 involved in auxin signaling process and regulate auxin-related mechanism in abiotic stress tolerance.

Figure 4. Effect of osmotic stress on root growth of transgenic aridopsis and Col-0. (a) Comparative growth of seedlings root on MSO and PEG-infused media (ψw = -0.5 MPa). The root length of seedlings were measured from 0 DAG to 10 DAG on (b) MSO and (c) PEG-infused media. Data represent mean ± S.E. of 20 seedlings and experiment was repeated three times.
Figure 5. Effect of salt stress on root growth of transgenic Arabidopsis and Col-0. (a) Comparative growth of seedlings root on MSO and NaCl-containing media. (b) The root length of seedlings was measured at 10 DAG on media containing various concentrations of NaCl. Data represent mean ± S.E of 20 seedlings and experiment was repeated three times. Means with different letters are significantly different at $p \leq 0.05$ according to Duncan’s multiple range test.
It was found that under drought stress conditions, auxin content in the plant increases [27]. This has directly affected the auxin signaling and indirectly impacted homeostasis of ROS, which can significantly affect the physiology and morphology of plants [15]. In this paper, chlorophyll contents of transgenic arabidopsis were maintained at higher levels compared to the control under drought and salt stress conditions. This is consistent with the results of an auxin autotroph tomato plant that experienced pigment deficiency phenotype [28]. Togenetti et al. [10] hypothesized that auxin has a protective effect on photosynthesis against photooxidative inhibition by reorganization of photosynthetic apparatus. Besides, auxin was also needed for maintaining root growth under moderate water stress. Previous studies found that moderate water stress accumulated ABA and increased auxin transport in the root tips [29]. The auxin at the root apex activates the plasma membrane H⁺-ATPase and the proton secretion is essential for maintaining root elongation under water stress. This is also in agreement with the transgenic arabidopsis overexpressing Abp57, which has longer root under water stress and salt stress conditions.

**Figure 6.** Biomass comparison between Abp57-overexpressing lines and control (Col-0 and NT) after two weeks of soil drying. Data represent mean ± S.E (n=3) and experiment was repeated three times. Means with asterisks* denoted significantly differences between lines at p ≤ 0.05 according to Duncan’s multiple range test.

**Figure 7.** Water loss rates of detached leaves from three weeks old irrigated plants of Col-0, NT and two independent transgenic lines (Abp57-Ox1 and Abp57-Ox2). Data representing mean ± S.E (n=15) and experiment was repeated twice.

5. Conclusion
In summary, the overexpression of Abp57 displayed improved water deficit and salt tolerance, as determined via physiological parameters such as chlorophyll content, main root growth, water loss rate and biomass. The relationship between the expression levels of Abp57 and plant tolerance to abiotic stress might be valuable for future crop breeding. Further studies of the molecular basis of the improved stress tolerance of the Abp57 can be carried out to further understanding of the gene-gene interaction in plants.
Acknowledgments
This work was funded by Research University Grant GUP-2017-110, Universiti Kebangsaan Malaysia awarded to Zamri Zainal. Tan L.W. was a recipient of the MyBrain 15, KPT.

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