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SUMMARY OF DOCTORAL THESIS

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Title:

Research on Drought Physiology and Molecular Responses, and Development of Biotechnology Tools for the Drought-Tolerant Wild Watermelon (*Citrullus lanatus* acc. 101117-1)

(乾燥耐性の野生種スイカ（*Citrullus lanatus* acc. 101117-1）における乾燥下の生理および分子応答と、生物工学的技術の開発に関する研究)

Drought has been documented as the major threat to food security worldwide. It is therefore important to study plants that have shown to be drought tolerant. Wild watermelon grows in the harsh desert conditions of the Kalahari Desert in Botswana. Previous studies have shown that various proteins and compounds are induced and accumulated in this plant when exposed to drought stress, which are believed to contribute to drought stress tolerance. It is therefore attractive to study this plant and unravel the drought tolerance mechanisms it possesses. The information acquired in studying this plant can be useful for future plant breeding programs.

Various studies have been performed on wild watermelon to understand the drought tolerance mechanisms, but these studies have always been performed in artificial environment. The extent of similarity or dissimilarity of the experimental results obtained between in artificial environments and in the natural environment has never been addressed. In this part of study, drought physiological responses of wild (*Citrullus lanatus* Acc. 101117-1) and a cultivar (*Citrullus lanatus* L. cv Matsuribayashi-777) watermelons were evaluated in both natural and artificial environments. For the natural environment, the plants were planted in a field in Botswana during the summer of 2017, while for the artificial environment the plants were grown in the growth chamber. The weather data in the natural conditions showed daily variations in factors like temperature, solar radiance, humidity and wild speed, while minimal rainfall was recorded. These variations have been thought to cause additional stress to the plant. Under the natural environment, response to water deficit was very rapid. Down-regulation in the photosynthetic assimilation and stomatal closure was more rapid in the natural conditions under drought stress. Effects on the fluorescence parameters showed that the photochemical quenching reduced rapidly in the early days of the drought stress in the natural environment, as compared to the artificial condition Interestingly, even though the results were different in terms of intensity, the trend of physiological response was similar between the two environments. These observations shows that artificial condition can be used to study effects of environmental stress of plants, but care must be taken when interpreting the results.

Ascorbate peroxidase (APX) plays an important role in detoxifying reactive oxygen species

under environmental stress. Although previous work in wild watermelon has shown an increase in chloroplast APX enzyme activity under drought, molecular entities of APX have remained uncharacterized. In this study, structure and transcriptional regulation of the APX gene family in watermelon were characterized. Five APX genes, designated as *CLAPX1* to *CLAPX5*, were identified from watermelon genome. The mRNA alternative splicing was suggested for *CLAPX5,* which generated two distinct deduced amino acid sequences at their C-terminus, in resemblance to a reported alternative splicing of chloroplast APXs in pumpkin. This observation suggests that two isoenzymes for stromal and thylakoid-bound APXs may be generated from the *CLAPX5* gene. Phylogenetic analysis classified *CLAPX* isoenzymes into three clades, *i.e.*, chloroplast, microbody, and cytosolic. Up-regulation of *CLAPX5-I* and *CLAPX5-II* was observed at the early phase of drought stress, which was temporally correlated with the observed increase in chloroplast APX enzyme activity, suggesting that transcriptional up-regulation of the *CLAPX5* gene may contribute to the fortification of chloroplast APX activity under drought. Our study has provided an insight into the functional significance of the *CLAPX* gene family in the drought tolerance mechanism in this plant.

Ethylene (C_2H_4), a phytohormone that is produced in response to both abiotic and biotic stresses, is an important factor influencing the efficiency of *Agrobacterium*-mediated transformation. In this study, effects of various ethylene inhibitors on the efficiency of *Agrobacterium*-mediated genetic transformation in wild watermelon was comparatively examined. Consequently, in comparison to the application of chemical inhibitors such as $AgNO_3$ and aminoethoxyvinylglycine (AVG), lower ethylene level was observed when the infecting *Agrobacterium* contained a gene for 1-aminocyclopropane-carboxylic acid (ACC) deaminase (acdS), which cleaves ethylene precursor ACC into α-ketobutyrate and ammonia. These observations demonstrated that controlling the ethylene level during co-cultivation and shoot formation, particularly using the acdS-harboring *Agrobacterium*, is advantageous for enhancing the transformation efficiency in this plant.

The study of gene entities suggested to enhance the wild watermelon drought tolerance is important. Loss of function has been widely used as a method of choice for functional studies of genes in plants. Genome editing technologies, such as the CRISPR/Cas9 system, has been an important technology used in functional studies. In this study, therefore, the focus was to develop the CRISPR/Cas9 genome editing system in wild watermelon, as a tool for studying gene functions. The *N*-acetylglutamate kinase (NAGK) gene, a plausible committing step in citrulline biosynthesis in wild watermelon was selected as the target for genome editing. Biochemical analysis showed a reduction of the NAGK enzyme activity in the protein extract from the infected leaves. Analysis of the genome sequence extracted from the agroinfiltrated leaves and the cassette-introduced protoplasts displayed high frequency of various nucleotide substitutions on the targeted locus, but not on the other region of the NAGK gene. Deletion-type mutations, which have been commonly observed in genome editing in other plants, were not observed in the present analysis. Although further research will be needed to achieve maximum efficiency for the gene knockout study, present observations indicated that target locus was specifically edited at a higher frequency in wild watermelon, suggesting that the genome editing technique can be an powerful tool for studying drought-tolerant wild plant resources in the future analyses.