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SUMMARY OF DOCTORAL THESIS

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Title: Functional Analysis of Genes Involved in Ascorbate Biosynthesis and Regulation in *Arabidopsis thaliana*

（シロイヌナズナにおけるアスコルビン酸の生合成と調節に関する遺伝子の機能解析）

Ascorbate (L-ascorbic acid, vitamin C, AsA) is a well-known antioxidant in plants. The concentration of AsA in plant cell is of mM order. The AsA biosynthesis pathway in higher plants has been proposed and designated as D-Mannose (D-Man)/L-Gal pathway. Among the enzymes in this pathway, GDP-L-Gal phosphorylase (GGP) catalyzes GDP-L-Galactose to L-Galactose 1-P. GGP is encoded by a pair of homologues VTC2 and VTC5. VTC2 is reported to be sensitive to light regulation and it contributes more to AsA biosynthesis. However, the function of VTC5 and the relationship between VTC2 and VTC5 are not clear, especially during the development of young seedlings. AsA has multi-functions in plants. It works as antioxidant, enzyme co-factor and directly attends the electron transfer in photosystem. Except those, the change in AsA level can influence cell cycle, cell wall composition, metabolites synthesis, flowering, leaf senescence, oxidative stress-resistance, pathogen-resistance and so on. The reduced total AsA level in AsA deficient mutants induces distinct change in numerous genes expression level including up-regulation and down-regulation. However, to date, there is no report about the exact sensitive AsA-responsive genes. The identification of the AsA-responsive gene should be crucial for understanding the mechanism that AsA influences the above aspects during the growth of plants.

In this research, the total AsA levels of *Arabidopsis* wild type and the AsA deficient mutants at different stages of development were analyzed, different from mature plants, the AsA level in the young seedlings of *vtc2-1* and *vtc5-1 vtc5-2* were same as that in wild type. In the results of gene expression in young seedlings, I found that *VTC2* and *VTC5* complementarily expressed in each other's mutants. This explained that why AsA level was not declined in the mutants in young seedlings. To observe the expression of *VTC2* and *VTC5* in vivo, I cloned their promoter region (up stream of start codon) into luciferase reporter system and transformed into *Arabidopsis* wild type. From the transgenic seedlings, it is clear that *VTC2* promoter controlled luciferase is always more active than that controlled by *VTC5* promoter. Except that, the luciferase activity was analyzed under a diurnal course. The fluorescent signal from *VTC2* promoter::LUC transgenic plants performed same change as the light/dark rhythm. Also high light illumination induced much higher luciferase activity.

The expression analysis of *VTC2* and *VTC5* suggested that, enzyme GGP possesses a special mechanism to protect the development of young seedlings after germination. It conserves two homologous coding genes (*VTC2* and *VTC5*) which can be up-regulated in each other's mutants. However, the exact signal pathway for it is not clear. Except that, the expression level of VTC2 and VTC5 is controlled by illumination condition and light intensity which is in accordance with the AsA pool size. In conclusion, during the biosynthesis of AsA in *Arabidopsis*, the reaction catalyzed

by GGP should be a key step in the D-Man/L-Gal pathway.

Furthermore, to screen the sensitive and fast-response AsA-responsive genes, I used *vtc2-1* which contains the lowest total AsA level in leaves among the AsA deficient mutants. The leaves from *vtc2-1* were fed with L-Galactone-1,4-lactone (L-GalL) the final precursor of AsA biosynthesis, after 16 h under light illumination, the AsA level in it increased dramatically compared to control (fed with H_2O). After analysis of the genes expression of the above feeding samples with microarray, I found a lot of genes are up-regulated or down-regulated in the leaves fed with L-GalL. To find out the most sensitive AsA-responsive genes, several candidates which were up-regulated by more than 4 folds were focused. I further checked the relationship between the expression of the above candidate genes and the AsA level in *Arabidopsis* through real-time PCR (qPCR). After confirming the genes expression in both *vtc2-1* and wild type in feeding experiments and under light/dark rhythm, aspartyl protease (ASP) and C3HC4 type RING zinc finger (AtATL15) were finally selected out as expected AsA-responsive gene. For observing their expression in vivo, the promoter region of ASP and AtATL15 were cloned into luciferase reporter system. The transgenic plant containing AtATL15 promoter::LUC showed same change in luciferase activity as that in AsA level in both feeding samples and light/dark rhythm. However, the ASP promoter::LUC transgenic line was failed to detect the fluorescent signal, revealed that the cis-element of ASP may not localize at the normal 5' region. It is reported that C3HC4 type RING Zinc Finger gene influence the cell cycle. For protease, they are found to be responsible for protein degradation during the process of hormone signal regulation. Therefore, it is hypothesized that ASP and AtATL15 probably work as the AsA-responsive genes in *Arabidopsis*. Also, in the future work, other AsA-responsive genes may possibly be identified.

During the process of screening AsA-responsive genes, I found two homologous genes (At1g80240, *DGR1* and At5g25460, *DGR2*) up-regulated by the feeding with L-GalL. The deduced amino acid sequence showed high identity (63%). *DGR* genes belong to the DUF (Domains of Unknown Function) 642 family. In the DUF642 family, there are some other genes including At5g11420, At4g32460, At2g41800 and At3g08030. Through the bioinformatic analysis, DGR are proposed to conserve the galactose binding domain. Galactose is one of the intermediate in the D-Man/L-Gal pathway, and it is the main composition in cell wall polysaccharides, therefore I am interested in studying the function of DGR. After expression analysis of DUF642 family genes in the leaves fed with L-Gal, I confirmed that among the DUF642 family, only *DGR1* and *DGR2* were responsive to the feeding of L-GalL. Then the qPCR and GUS stain results showed that *DGR* genes both expressed in leaf, root, primordia, petal and silique. From the expression level, *DGR2* is dominant. In root and petal, *DGR* genes showed complementary expression. *DGR1* localized at root cap and the up part of petal, oppositely, *DGR2* localized at whole root except root cap and the middle and down part of petal. This suggest they may not be functional redundant.

The T-DNA insertion mutant of *DGR2* performed slow development in leaf and root compared to same growing period wild type. However, there is no difference in the *DGR1* T-DNA insertion mutant although the expression of *DGR1* is suppressed dramatically. The phenotype revealed again that *DGR2* should be the dominant one that influences the development of *Arabidopsis* obviously. It has been reported that DGR proteins localized at cell wall, and because they conserve the galactose binding domain also, therefore, I hypothesize that DGR may attend the cell wall biosynthesis or cell wall assembly and further influence the cell expansion and the size of tissues. This is needed to be confirmed in the future.