CHAPTER V

Discussion

Plant aromatic PTases are divided into three groups based on their aromatic substrates: (I) the enzymes involved in shikonin and ubiquinone biosynthesisin which their substrate is p-hydroxybenzoate (PHB) (Ohara, et al., 2009; Ohara, et al., 2006; Yazaki, et al., 2002) (II) the enzymes in the plastoquinone, tocopherol and tocotrienol biosynthesis with the substrate homogentisate (HGA) (Matsuzuka, et al., 2013; Sadre, et al., 2010; Schledz, et al., 2001), and (III) the enzymes in the prenylated flavonoid biosyhthesis with flavonoids as the substrates (Akashi, et al., 2009b). Mostly, plant aromatic PTases are membrane bound proteins consisting of 6 − 9 transmembrane α-helixes. The homogentisate phytyltransferase (HPT or VTE2) is thought to be the rate-limiting step enzyme that catalyzes the prenylation reaction in the initial step of tocopherol biosynthetic pathway. HPT catalyzes the reaction by condensation of the aromatic head group precursor, homogetisate (HGA) and the phytyl tail precursor, phytyl diphosphate (PDP) to produce the first intermediate, 2-methyl-6-phythylbenzoquinol (MPBQ) for the production of tocopherol. According to the phylogenetic tree analysis, the flavonoid PTases showed similarity with HPT indicating that the flavonoid PTases are evolved from HPT in tocopherol biosynthesis.

In this study, we isolated *ctl* and *alrc2* cDNA from *C. ternatea* L. and *A. lakoocha* Rox. Construction of the phylogenetic tree showed that *ctl* and *alrc2* are the member of HPT family, in which HGA is their aromatic substrate (prenyl acceptor) and phytyl diphosphate (PDP) is their prenyl substrate (prenyl donor). both appeared to be most closely related to HPT of *Glycine max* and *Morus natabilis*. Their amino acid sequences display two aspartate (Asp) rich conserved motifs which are particularly found in UbiA prenyltransferase family. The motif I: NQXXDXXXD is

corresponding to prenyl diphosphate binding and catalytic reaction by chelation of Mg^{2+} and the motif II: KDXXDXD helps stabilizing the substrate binding (Huang, et al., 2014; Melzer and Heide, 1994). The activity of these enzyme required Mg^{2+} or related ion. The Asp rich motif is important for substrate binding affinity. It has been reported that point mutation at Asp rich motif resulted in the declination of substrate binding affinity (Ohara, et al., 2009). From our results, the motif I of CTL and ALRC2 showed high similarity with a group of the enzymes in tocopherol and tocotrienol biosynthesis that can bind to PDP or GGPP, while their motif II showed high similarity between flavonoid PTase and HPT or HGGT group. Like the identified aromatic PTases, CTL and ALRC2 are located in the same clan with HPT group in phylogenetic analysis and they have an N-terminal signal peptide which destines these translated proteins to localize in chloroplast membrane, as were the results predicted by WoLF PSORT and Protcomp. Both proteins were predicted to be transmembrane proteins with nine transmembrane α-helices. Altogether, the results suggested that CTL and ALRC2 are likely to be the group of HPT enzymes.

To characterize the *ctl* and *alrc2* functions, overexpression of these genes in tomato leaves were conducted by agroinfiltration. In recent years, transient gene expression mediated by agroinfiltration has been used as a tool to demonstrate transcription and protein expression analysis. It is rapid, simple, and effective procedure to generate the recombinant protein within a few days by injection of *A. tumefaciens* into leaves or fruits of plants (Leckie and Neal Stewart, 2011; Zottini, et al., 2008). In this study, agroinfiltration technique was used to follow the expression of the recombinant genes in tomato leaves. The *ctl* and *alrc2* genes were constructed into binary vector pGWB6 carrying GFP at the N-terminus and under control of 35S CaMV promoter. *A. tumefaciens* stain GV3101 containing pJL3:p19 that expressing RNA silencing suppressor protein (p19) was used to improve agroinfection

efficiency (Kanagarajan, et al., 2012; Wydro, et al., 2006). The mRNA expression of these genes showed the highest level on the first day postinfiltration (dpa) and decreased afterward. The recombinant protein expression were detected by western blot analysis and it has been found that the expression of CTL showed the highest level at 3 dpa and slightly decrease until 9 dpa while ALRC2 showed little expression at 3 and 6 dpa. It was common that the genes must be expressed first in order to encode their proteins later. The results clearly demonstrated that the genes and their corresponding proteins were expressed oppositely during the days after infiltration. Similarly the level of protein expression via agrobacterium infiltration followed by GFP protein or GUS activity showed high expression after 3 dpa and decrease after that (Kim, et al 2009, Orzaez, et al. 2006, Yasmin and Debener, 2010). In addition, these results suggested that the CTL had higher expression than ALRC2 in this system and the protein expression can increase to the maximum level at 3 dpa as well as slightly CTL expression showed high activity to induced α-tocopherol accumulation at first day after agroinfiltration. It may be influenced by the presence of 5′-UTRs and 3′-UTRs of mRNA that form complexes with protein in post-transcription process for control transcription mRNA stability and translation in chloroplast (Choquet. and Wollman, 2002, Cohen and Mayfield, 1997, Del Campo, 2009, Gao, et al. 2012, Monde, 2000 and Robida, 2002.). Furthermore, overexpression of these genes clearly led to the increase of α-tocopherol accumulation in tomato leaves. The successful detection of increased α-tocopherol content from agroinfiltration tomato leaves in this study was analyzed by TLC. The *ctl* and *alrc2* genes isolated from *C. ternatea* and *A. lakoocha*. could enhance the α-tocopherol content up to 2.4 fold and 1.4 fold, respectively within 3 dpa when compared with the control. The increase of the α-tocopherol content was parallel with the increase of the protein expression level. Similar results have been reported in overexpression of HPT (also called VTE2) genes isolated from other species. The overexpression of *A.*

thaliana VTE2 (*At-VTE2*) in *Arabidopsis* have been reported to increase the total tocopherol level up to 4.4 fold in leaves and 40% in seeds (Collakova and DellaPenna, 2003a). Moreover, *At-VTE2* has also been found to enhance α-tocopherol level up to 5.5 and 2 fold in tobacco and lettuce transgenic leaves, respectively (Harish, et al., 2013a; Harish, et al., 2013b; Koeun, et al., 2007) and also 106 % increasing of α-tocopherol level in potato tuber (Crowell, et al., 2008). The α-tocopherol levels from transgenic expression of lettuce HPT (*LsHPT*) and apple HPT (*MdHPT*) have been reported to raise to 18 and 3.6 fold in lettuce and tomato leaves, respectively (Ren, et al., 2011; Seo, et al., 2011). It was obvious that the overexpression of HPT led to the high accumulation of α-tocopherol; however, the levels of increment appear to be varied and seem to be depending on the species whose HPT genes are isolated from.

Our results also indicated that the accumulation of α-tocopherol was affected by the activity of the *ctl* and *alrc2* genes. However, the limitation of transient expression method should be taken into account when monitoring the phenotype after infiltration. In this case, the genes and their proteins could be detected up until 6 dpa while it was observed that at 9 dpa the accumulation of α-tocopherol of control was higher than both recombinant gene infiltration samples because the infiltrated area may be damaged due to the stress of agroinfiltration. Therefore, this result suggested that the phenotype of these genes transiently expressed in tomato leaves should be monitored within a week of post-infiltration.

According to the biosynthetic pathways of tocopherol and chlorophyll, tocopherol production is somewhat related to chlorophyll degradation. It has been known that HGA and PDP are the substrates of HPT and HGA is derived from shikimate pathway but PDP can be derived from de novo synthesis of MVA or MEP pathway and chlorophyll degradation. During the stress conditions, chlorophyll is

degraded by chlorophyllase to produce chlorophyllide and free phytol. Phytol is then stepwise phosphorylated by phytyl kinase (VTE5) and unknown phytylphosphate kinase to produce PDP as a precursor for chlorophyll, tocopherol, phylloquinone and fatty acid alcohol ester biosynthesis (Ischebeck, et al., 2006; Valentin, et al., 2006). The correlation between chlorophyll degradation and tocopherol accumulation is still not clear although it is possible that phytol involved in tocopherol biosynthesis may derive from chlorophyll degradation. It has been reported that phytol is incorporated to tocopherol in seedling *Arabidopsis* by feeding experiment and affected increasing of tocopherol in overexpressed HPT and TC tobacco cell suspensions (Harish, et al., 2013b; Rise, et al., 1989). In addition, it has been found that chlorophyll content was declined about 20% while the tocopherol content was increased in overexpressed HPT plant (Lee, et al., 2007). To demonstrate the effect of the recombinant protein (CTL and ALRC2) on tocopherol accumulation and chlorophyll degradation in plant, the transformed plant extracts were analyzed by spectrophotometry method. The result showed that CTL and ALRC2 transiently expressed leaves were related with the chlorophyll decline. Total chlorophylls slightly continuous decreased after agroinfiltration when compare with control at 1 – 6 dpa. At 9 dpa, the α-tocopherol accumulation droped while the total chlorophyll degradation slightly increased that could be effect of senescence during stress. GC-MS after silylation. At 3 and 6 dpa, the CTL and ALRC2 transiently expressed leaves contained pools of phytol, fatty acid e.g. palmitic acid (16:0), stearic acid (18:0) and linolenic acid (18:3), and together with α-tocopherol accumulation. These results suggested that overexpression of the recombinant CTL and ALRC2 proteins might induce chlorophyll hydrolysis to release free phytol as a precursor of tocopherol. However, the expression of genes related in chlorophyll degradation should be further investigated to reveal the correlation between α-tocopherol and chlorophyll accumulation. In addition, the pools of those fatty acids highly

accumulated in the transgenic leaves were likely the result of free phytol increment since it serves as a precursor of fatty acid biosynthesis (Ischebeck, et al., 2006). Although fatty acid can be derived from the degradation of the chloroplast envelop membrane where they are its major components (Poincelot, 1976; Whitaker, 1986) or from chloroplast lipid in response to pathogen attack (Upchurch, R.G. 2008, Walley, J.W., *et al.* 2013), these causes were unlikely to affect the increase of fatty acid accumulation in transgenic CTL and ALRC2 tomato leaves because if these processes occurred, they would lead to plant cell death but the sign of cell death was not detected at that moment.

Overexpression of CTL and ALRC2 increased the amounts of MPBQ and DMPBQ in transgenic tomato leaves. MPBQ, DMPBQ and γ-tocopherol are stepwise intermediates in α-tocopherol biosynthesis pathway. These compounds are synthesized by HPT, tocopherol cyclase (TC), and γ-tocopherol methyltransferase (γTMT), respectively. In this study, GC-MS could detect MPBQ and DMPBQ but could not detect γ-tocopherol. It is possible that γTMT activity may rapidly convert γ-tocopherol to α-tocopherol under stress condition (Collakova and DellaPenna, 2003b). To identify MPBQ and DMPBQ, their fragmentation patterns were compared with the patterns from previous reports (Kobayashi and DellaPenna, 2008; Porfirova, et al., 2002; Sussmann, et al., 2011) instead of standard compounds because MPBQ and DMPBQ are not commercially available. The mass spectrum of derivatized α-tocopherol showed molecular ion signal at m/z 502 and heterocyclic chromanol ring at m/z 237 (Liebler, et al., 1996; Mottier, et al., 2002). The suspected peaks of MPBQ and DMPBQ were at retention time of 7.923 and 8.203 minute and their expected m/z were 546 and 560, respectively. However, the expected molecular ions of derivative compounds was only detected for DMPBQ but not detected for MPBQ. This would be due to its low amount or instability of MPBQ. Considering

fragmentation patterns of these compounds, MPBQ showed major fragment of quinol head group at m/z 281 and 321 and DMPBQ showed the major fragment of quinol head group at m/z 281 and 335. Moreover, the quinol head group of these compounds showed fragment ion at m/z 207 which was calculated from m/z 281 deleted by m/z 73 of TMS (trimethylsilane) derivative. The phytyl tail fragment of MPBQ and DMPBQ displayed at m/z 265 and m/z 265 and 155, respectively. These patterns of MPBQ and DMPBQ were in agreement with previous reports; therefore, GC-MS is a suitable detection method for MPBQ and DMPBQ and these results confirmed the presence of MPBQ and DMPBQ in the transgenic tomato leaves expressing CTL and ALRC2. Overexpression of CTL increased the accumulation of MPBQ and DMPBQ since 1 dpa while overexpression of ALRC2 increased little accumulation of intermediates after 3 dpa. This result indicated that CT has higher potential to catalyze reaction than ALRC2.