博 士 論 文 の 要 約

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Molecular Genetic Study on Tocopherol Synthesis in Soybean Seeds

（ダイズ種子におけるトコフェロール生合成に関する分子遺伝学的研究）

Chapter 1 Introduction

Tocopherols (vitamin E) are lipophilic antioxidant, which prevent oxidation of unsaturated fatty acids. There are four isoforms of tocopherols (α-, β-, γ-, and δ-Toc). Among them, α-tocopherol (α-Toc) has the highest vitamin E activity in human owing to its highest affinity with the hepatic tocopherol transfer protein. In addition to vitamin E activity, α-Toc is also reported to be able to prevent aging-related diseases, such as cardiovascular diseases and cancer.

The soybean oil has a relatively high total tocopherol content compared with other oilseed crops. However, the content of α-Toc is mostly less than 10 % of the total Toc content in soybean. Increasing the seed α-Toc content may therefore open up opportunities for new food and industrial uses of soybean.

For this reason, increasing α-Toc contents is an important breeding target of soybean seed quality. However, the molecular mechanism of tocopherol biosynthesis is not fully identified. The purposes of the present study were first to determine the molecular genetic basis for an elevated α-Toc ratio detected in a wild soybean (*Glycine soja*) accession, and second to characterize the thermal responses of tocopherol biosynthesis and determine the association with DNA polymorphisms in the three genes encoding γ-tocopherol methyltransferase (*γ-TMT1* to *γ-TMT3*).

Chapter 2 Identification of quantitative trait loci for increased α-tocopherol biosynthesis in wild soybean using a high-density genetic map

The wild soybean is a huge reservoir of potentially useful variants for the improvement of

soybean cultivars. To date, it has been used to improve yield, stress tolerances, disease resistances, and nutritional components of seeds in soybean breeding.

Dwiyanti et al. (2016) discovered 11 wild soybean accessions with high α-Toc ratios. Sequencing analyses of the promoter and 5′-untranslated region of *γ-TMT3* classified these 11 accessions into four haplotypes, of which one was identical to the *γ-TMT3* sequence of a high α-Toc cultivar Keszthelyi Aproszemu Sarga (KAS). The molecular genetic analysis for the high α-Toc traits of wild accessions with novel *γ-TMT3* promoter haplotypes is useful in broadening the genetic diversity of α-Toc biosynthesis in soybean.

In this chapter, I identified quantitative trait loci (QTLs) for a high α-Toc trait detected in a wild accession B04009, and discussed the candidates for the QTLs, based on the results obtained from sequencing analyses.

A recombinant inbred line (RIL) population was developed from a cross between the low α-Toc breeding line TK780 and the high α-Toc wild accession B04009. The seed tocopherol contents and compositions were evaluated in the RILs cultivated in the greenhouse of ambient temperatures of 25 °C to 30 °C in short day condition. The α-Toc content correlated closely with the ratio of α-Toc to γ-Toc contents (α/γ ratio). QTL analysis using a high-density map constructed with 7,710 single nucleotide polymorphisms (SNPs) generated by restriction site-associated DNA sequencing detected six QTLs involved in Toc biosynthesis. Of these, three located in chromosomes (Chr) 9, 11, and 12 produced consistent large effects on α-Toc contents and ratios in a 2-year trial. The B04009 allele at QTLs in Chr9 and Chr12 and the TK780 allele at the QTL in Chr11 each promoted the conversion of γ-Toc to α-Toc, which elevated the seed α-Toc contents and ratios. The opposite effects by the three QTLs resulted in the transgressive segregation in the seed α-Toc contents and α/γ ratios in the RIL population. The QTL with the largest effect was located close to the QTL in Chr9 as previously detected in the cross between a low α-Toc cultivar Ichihime and KAS (Dwiyanti et al., 2011), suggesting that the high α-Toc trait in B04009 may be controlled by the same QTL as detected in the Ichihime × KAS cross. The other two QTLs in Chr11 and Chr12 were the novel QTLs which have not been detected so far. A survey on the Williams 82 genome database revealed that three genes (*γ-TMT1*, *γ-TMT2* and *γ-TMT3*) encoding γ-tocopherol methyltransferase, which convert γ-Toc to α-Toc in the last step of tocopherol biosynthesis, were co-located in the QTLs in Chr9 (*γ-TMT3*) and Chr12 (*γ-TMT1* and *γ-TMT2*), whereas the QTL in Chr11 had no known genes involved in Toc biosynthesis; there were two zinc finger transcription factors

(Glyma.11G220400 and Glyma.11G226400) and S-adenosyl-L-methionine-dependent methyltransferases superfamily protein (Glyma.11G222800).

The sequence analyses for three *γ-TMT* genes and the three annotated genes in Chr11 revealed non-synonymous substitutions in *γ-TMT2* and Glyma.11G222800 between TK780 and B04009; the coding sequences were identical in *γ-TMT1*, *γ-TMT3,* and two zinc finger transcription factors on Chr11. In addition, a number of DNA polymorphisms were detected in the promoter and introns region for all of the genes sequenced; some were located in known cis elements in the promoter region.

Chapter 3 Molecular mechanism on thermal response of α-tocopherol biosynthesis

Temperature during seed development is one of the environmental factors that influence the α-Toc contents and ratios in seeds. It is well known that the α-Toc contents and ratios increase as temperatures rise during seed maturation. In this chapter, I evaluated how the *γ-TMT* genes function in the Toc biosynthesis in response to temperatures.

The α-Toc ratios in seeds were analyzed in 15 cultivars and 10 wild accessions in 20 °C and 21 cultivars and 11 wild accessions in 30 °C. In both thermal conditions, α-Toc ratios and α/γ ratios were highly correlated to each other. The increment of α-Toc ratios was mainly associated with the conversion efficiencies from γ-Toc to α-Toc. Expression analyses of immature cotyledons developed in different thermal conditions were performed in TK780, KAS and B04009. Of the three *γ-TMT* genes, *γ-TMT2* was upregulated by high temperature, and this upregulation was marked particularly in KAS and B04009. The expression levels of *γ-TMT3* were markedly high in KAS, compared with TK780 and B04009; the expression levels were slightly but significantly higher in B04009 than TK780 at both temperatures. These findings suggest that different expression levels of *γ-TMT3* and *γ-TMT2* may be the molecular bases underlying the two QTLs for α-Toc contents and ratios in Chr9 and Chr12.

Of the three candidate genes for the QTL in Chr11, Glyma.11G222800 encoding S-adenosyl-L-methionine-dependent methyltransferase superfamily protein exhibited higher expressions in TK780 than B04009 in both 20 °C and 30 °C conditions. In addition, two non-synonymous substitutions were detected in Glyma.11G222800 between TK780 and B04009; TK780 had the amino acid residues conserved in the homeolog and homologs in

other plant species. Therefore, B04009 possibly had a missense variant of the protein. A further study is needed to determine whether this methyltransferase protein has any function to convert γ-Toc to α-Toc.

The methylation of the promoter regions of *γ-TMT2* and *γ-TMT3* was also analyzed as a causal factor for different expression profiles between thermal conditions. A part of the *γ-TMT3* promoter of TK780 was methylated in the 20 °C condition, but not in the 30 °C condition, whereas no methylation was detected in B04009. However, the presence of methylation was not directly related to the expression levels of *γ-TMT3* in TK780 grown in different temperatures. Therefore, the observed methylation in the *γ-TMT3* promoter was not a main factor, if any, to control the Toc biosynthesis in response to different thermal conditions in TK780.

The association analysis was performed between the SNPs in the *γ-TMT1*, *γ-TMT2* and *γ-TMT3* sequences and α-Toc ratios for 16 soybean cultivars and 9 wild soybean accessions. The SNPs were obtained from whole genome resequencing data. The α-Toc ratios were significantly associated with one SNP in the intron of *γ-TMT1*, two in the promoter of *γ-TMT2*, and seven in the promoter and genic regions of *γ-TMT3*. Particularly, three SNPs in the promoter region of *γ-TMT3* showed the highest association with α-Toc ratios, suggesting that the SNPs in *cis*-elements of *γ-TMT3* were involved in the different controls of α-Toc biosynthesis among soybean cultivars and wild accessions.

Chapter 4 General Discussion

In this study, I identified two major QTLs and one minor QTL conferring higher α-Toc contents in a cross between a low α-Toc soybean breeding line TK780 and a high α-Toc wild accession B04009. Of these, the QTL in Chr9 was most likely identical to the QTL detected in the Ichihime × KAS cross, and harbored *γ-TMT3*. The results suggest that the elevated α-Toc ratios detected in both cultivated and wild soybeans are partly attributed to the same QTL. The other two QTLs were novel QTLs that had not been reported so far. The genomic region flanking QTL in Chr12 contained tightly linked *γ-TMT1* and *γ-TMT*2, whereas there were no genes involved in tocopherol biosynthesis in the QTL in Chr11 region. The sequence analysis suggested that a missense variant of Glyma.11G222800 in B04009 is a possible factor for the negative effect of wild allele on α-Toc contents in the RIL population. A further study is

needed to confirm whether the variant is a responsible factor for the QTL. The wild allele at the QTL in Chr12 had a positive effect on the increment of α-Toc contents, suggesting that it would be a useful gene source in breeding high α-Toc cultivars.

The α-Toc ratios in seeds were influenced by the temperatures during maturation, and increased as the temperatures rose from 20°C to 30°C. In both thermal conditions, the α-Toc ratios in cultivars/accessions were highly correlated with the α/γ ratios, indicating that the different accumulations of α-Toc are mainly attributed to the variation in the conversion efficiency from γ-Toc to α-Toc. In particular, *γ-TMT*2 was up-regulated in high temperatures in all of the accessions tested, but the extents were marked in the high α-Toc accessions, KAS and B04009. *γ-TMT*2 may therefore have an important role in the accumulation of α-Toc as promoted by high temperature. The expression levels of *γ-TMT3* were significantly higher in B04009 than TK780 at both temperatures. Accordingly, the different expression levels of *γ-TMT3* and *γ-TMT2* may be the molecular bases underlying the two QTLs for α-Toc contents and ratios in Chr9 and Chr12 detected in the TK780 × B04009 cross. Association analysis further suggested that three SNPs in the promoter region are critical in different regulations of *γ-TMT3* expression.

The different protein structures, different expression profiles, and different thermal responses of expressions may therefore indicate that the three *γ-TMT* genes have been subfunctionalized to each other, and possess different functions in the adaptation to oxidative stresses that occur during various phases of development. A further study should be directed to their roles in the adaptation to the oxidative stresses. The findings obtained in this study would be helpful in understanding of the molecular mechanisms underlying α-Toc biosynthesis in soybean seeds and developing the cultivars with high α-Toc contents in seeds.