EFFECTS OF OVEREXPRESSION OR SUPPRESSION OF HETEROLOGOUS OR HOMOLOGOUS GENES ON PHENOTYPIC ALTERATION IN TRANSGENIC PLANTS

外来および内生遺伝子の過剰発現および発現抑制が形質転換体における表現型の変化に及ぼす影響

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Abstract

Understanding how genes work together and interact is the focus of functional genomics, a branch of genomics that attempts to find the correlation between genome and phenotype. Changes in gene expression have a significant impact on phenotypic responses. In the present study, two distinct gene expression patterns were demonstrated in two different model plants in order to investigate the effect of gene expression on phenotypic responses.

A floral homeotic gene from dicotyledonous *Arabidopsis thaliana* (*AtLFY*) was transferred to a monocot liliaceous ornamental plant *Tricyrtis* sp. by *Agrobacterium*-mediated transformation. Nine independent transgenic plants with overexpressing *AtLFY* have been obtained, and all of them exhibited dwarf phenotypes compared with the vector control. These transgenic plants could be classified into three types according to the degree of dwarfism: one showed an extremely dwarf phenotype with smaller leaves (Type I); two showed moderately dwarf phenotypes (Type II); and six showed slightly dwarf phenotypes (Type III). All of Type I, Type II and Type III transgenic plants produced flower buds 1-3 weeks earlier than the vector control. Vector control and Type III transgenic plants produced 1-4 apical flower buds, whereas Type I and Type II transgenic plants produced only a single apical flower bud. Type I and Type II transgenic plants rarely produced fully-opened flowers. Quantitative real-time reverse transcription-polymerase chain reaction analysis showed that the *At*LFY expression level generally correlated with the degree of dwarfism. These results indicate that morphological alterations observed in the transgenic plants were induced by overexpression of *AtLFY*. Lower levels of ectopic expression of *LFY* may be valuable for producing dwarf and early flowering ornamental plants.

Homologous expression of metabolic genes was demonstrated in a dicotyledonous medicinal plant, *Artemisia annua* L, to examine the role of key enzyme genes on biochemical production. A multifunctional sesquiterpene oxidase gene, *CYP71AV1*, which is responsible for the conversion of artemisinin precursors, was successfully cloned into an RNA silencing plasmid using the gateway pANDA vector. Three independent RNAi suppression of CYP71AV1 gene (siCYP) transgenic plants

were obtained and used for biochemical analysis, in comparison with transgenic *A. annua* with sense overexpression of CYP71AV1 gene (oxCYP) and wild type. Results revealed variations in terpenoid compounds among transgenic lines. Reduced numbers of terpenoid compounds were detected in siCYP transgenic lines. Some useful sesquiterpenes such as copaene, trans-β-farnesene and aromadendrene were also absent. On the other hand, the levels of some terpenoids significantly increased in oxCYP transgenic lines. The contents of artemisinin derivatives were observed at relatively high levels in oxCYP transgenic lines, while only slightly decreased in siCYP transgenic lines. Overall results suggest the diverse effect of different *CYP71AV1* constructs on alteration of metabolites in *A. annua*.

The present study demonstrated the phenotypic response of model plants to different patterns of gene expression. Overexpression of *AtLFY* successfully induced dwarfism and early flowering in transgenic *Tricyrtis* sp., which are attractive traits, especially for ornamental plants. Transgenic *A. annua* plants with over- and suppressed expressions of *CYP71AV1* exhibited variations in metabolic compositions, which may be valuable materials for functional genomic studies and pharmaceutical applications.

Abstract (Japanese)

遺伝子がどのように連携して相互作用するかを理解することは，ゲノムと表現型の関係を解明する機能ゲノミクスにおいて重要である．遺伝子発現の変化は表現型に大きな影響を及ぼす．本研究では，表現型応答に対する遺伝子発現の影響を明らかにするために，2 つの異なるモデル植物における 2 つの異なる遺伝子発現パターンを調査した．

まず，双子葉植物シロイヌナズナの花のホメオティック遺伝子 (*AtLFY*) を，アグロバクテリウム法により単子葉鑑賞植物のホトトギス (*Tricyrtis* sp.) に導入した．*AtLFY* を過剰発現する 9 系統の形質転換体が得られ，それらの全てがベクターコントロールと比較して矮性の表現型を示した．形質転換系統は，矮化の程度により 3 つのタイプに分類することができた：1 系統は著しく矮化しており小さな葉をつけていた (タイプ I)；2 系統は中程度の矮化を示した (タイプ II)，残りの 6 系統はわずかな矮化を示した (タイプ III)．タイプ I〜III いずれの形質転換体も，ベクターコントロールより 1〜3 週間早期に花蕾を形成した．ベクターコントロールおよびタイプ III 形質転換体は 1〜4 の頂生花蕾を形成したが，タイプ I および II 形質転換体は単一の頂生花蕾のみを形成した．タイプ I および II 形質転換体の花は，ほとんど展開しなかった．リアルタイム定量 RT-PCR を行ったところ，*AtLFY* の発現レベルと矮化の程度の間には相関がみられた．これらの結果は，形質転換体における形態変化が *AtLFY* の過剰発現によって誘導されたことを示している．また，外来 *LFY* の低発現レベルが矮性および早咲きの観賞植物の作出に有効であることも示している．

次に，薬効成分の生合成における重要な酵素遺伝子の役割を調査するために，双子葉薬用植物のクソニンジン (*A. annua*) を用いた実験を行なった．アルテミシニン前駆体の変換に関与する多機能セスキテルペンオキシダーゼ遺伝子 (*CYP71AV1*) を過剰発現する形質転換体 (oxCYP 系統)，RNAi により *CYP71AV1* の発現が抑制された形質転換体 (siCYP 系統) および野生型の個体について，テルペノイド化合物の分析を行った．siCYP 系統では，テルペノイド化合物の数の減少がみられ，copaene や trans-β-farnesene，aromadendrene のような有用なセスキテルペンも存在していなかった．一方，oxCYP 系統においては，一部のテルペノイドのレベルが有意に増加した．アルテミシニン誘導体の含有量は，oxCYP 系統では比較的高レベルで検出されたが，siCYP 系統ではわずかに減少していた．これらの結果は，*CYP71AV1* の発現レベルの変化により代謝産物の変化が誘導されたことを示している．

本研究は，2 種類のモデル植物において異なる遺伝子発現レベルによる表現型の変化を示すものである．*AtLFY* の過剰発現は，ホトトギスにおいて矮化および早期開花を誘導した．これにより，鑑賞植物の育種における *LFY* を

用いた形質転換の有用性が示された．一方，*CYP71AV1* の過剰発現および発現抑制は，クソニンジンにおける代謝産物の変動を誘導した．*CYP71AV1* の発現が変化した形質転換体は，機能ゲノムの研究および製薬に関して有用な材料となる可能性がある．

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List of abbreviations

ADS	Amorpha-4,11-diene synthase
AG	*AGAMOUS*
ALDH1	Aldehyde dehydrogenase
AP1	*APETELA1*
AP3	*APETELA3*
AtLFY	*LEAFY* from *Arabidopsis thaliana*
CAL	*CAULIFLOWER*
CaMV	Cauliflower mosaic virus
CPR	Cytochrome P450 oxidoreductase
CPS	β-caryophyllene synthase
CYP71AV1	Cytochrome P450-dependent hydroxylase
DBR2	Artemisinic aldehyde reductase
DMAPP	Dimethylallyl diphosphate
DXR	1-deoxyxylulose 5-phosphate reductoisomerase
DXS	1-deoxyxylulose 5-phosphate synthase
FLO	*FLORICAULA*
FPS	Farnesyl diphosphate synthase
FT	*FLOWERING LOCUS* T
GST	Glandular trichomes
GUS	β-glucuronidase
HMGR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
HPT	Hygromycin phosphotransferase
IPP	isopentenyl diphosphate

LFY	*LEAFY*
MEP	methylerythritol phosphate pathway
MS	Murashige and Skoog (1962)
MVA	mevalonic acid pathway
NOS	Nopaline synthase
NPTII	Neomycin phosphotransferase II
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription-polymerase chain reaction
SQS	squalene synthase

Chapter 1

General introduction

Functional genomics is established to find correlation between genome of organism and its phenotype. It provides insights into how genes function and biological processes react under different circumstances. As sessile organisms, plants are exposed to a wide variety of environmental conditions, resulting in a significant reduction in survival. To cope with changes in environment, plants have evolved advanced mechanisms of gene regulation through signaling cascades. As a result, plants accumulate transcription factors that activate related gene expression and allow plants to adapt to environmental challenges (Mirouze and Paszkowski 2011). Major differences in plant phenotypes under certain conditions are strongly influenced by changes in regulatory mechanisms associated with altered gene expression, as well as their genetic background. To investigate effect of gene expression on phenotypic responses, two different expression patterns were demonstrated in different model plants. The ectopic or heterologous expression, which refers to introduction of gene(s) from different species or cell types, was demonstrated in a liliaceous ornamental plant. On the other hand, homologous expressions which transfer the target genes into the same host were conducted in a medicinal plant.

Among several phenotypic parameters, growth and floral morphologies are obvious distinguishing features that are regulated by several interactions of gene regulatory networks, including floral homoeotic genes. Among several floral meristem identity genes in *Arabidopsis thaliana*, *LEAFY* (*LFY*) encodes a plant-specific transcription factor that controls the transition of plant development from vegetative to reproductive (Schultz and Haughn 1991; Weigel et al. 1992) was focused. This gene appears to play a key role in regulating floral identity (Weigel and Nilsson 1995) and its homology has been extensively investigated in many other species. *LFY* is highly expressed in young meristems from early stages of development (Figure 1.1). Upregulation of *LFY* is important for flower identity as it activates expression of other

floral meristem identity genes such as *APETALA1* (*AP1*) and *CAULIFLOWER* (*CAL*) (Parcy et al. 1998). The roles of *LFY* or its homologs were investigated in many dicotyledon species. *FLORICAULA* (*FLO*), an *LFY* homolog from *Antirrhinum majus*, promoted floral meristem and subtending bract and its mutants accelerated flower-to-inflorescence conversions (Coen et al. 1990). Similarly, the *LFY* homolog from *Impatiens balsamina* (*IbLFY*) is highly expressed in leaf primordia, axillary meristem, and floral meristems. Ectopic expression or heterologous expression of *IbLFY* in *Arabidopsis* induced phenotypic alterations such as altered leaf shape, early flowering, shoot-to-flower conversions, and a loss of inflorescence indeterminacy (Ordidge et al. 2005). Since *LFY* is found as a single gene in most plants including monocotyledonous species, gene duplications can occur when the gene is expressed in a heterologous host (Moyroud et al. 2010). Hence, it is interesting to investigate the roles of *LFY* from *A. thaliana* (*AtLFY*) in monocotyledonous plant, which remains elusive. In this present study, *Tricyrtis* sp. was used as a model plant due to a well-established protocol for producing transgenic *Tricyrtis* sp. (Nakano and Otani 2020). In addition, transgenic *Tricyrtis* sp. has shown various phenotypic alterations, such as shortened nodes, changes in flower form, and alteration of flower color (Nakano et al. 2006; Kamiishi et al. 2012). Therefore, heterologous expression of *AtLFY* is clearly observed in this monocotyledonous host (**Chapter 2**).

In many plant species, phenotypic variations can be noticed in terms of metabolomic patterns. *Artemisia annua* L. is a dicotyledon plant that produces a variety of useful compounds such as terpenoids, flavonoids, coumarins, sterols, phenols, lipids, and other hydrocarbons (Bhakuni et al. 2001; Czechowski et al. 2018), was used as a model plant to investigate homologous expressions of terpenoid biosynthetic genes. Among several compounds produced in this plant, artemisinin is a main bioactive compound that plays a central role in combating the malaria-causing parasite *Plasmodium falciparum* (Klayman 1985; Dondorp et al. 2009). Artemisinin is synthesized by artemisinin biosynthetic pathway with the activity of several functional enzymes (Figure 1.2); however, production occurs in certain parts of the plant such as leaves and flower buds and the contents vary from 0.01 to 1.1 % DW in wild type *A. annua* L. depending on cultivar and environmental factors (Abdin et al. 2003; Wallaart

et al. 2000). One of the most promising strategies for improving artemisinin production is genetic engineering of key gene(s) in biosynthetic pathway using *Agrobacterium tumefaciens*-mediated transformation (Banyai et al. 2010; Alam and Abdin, 2011; Chen et al. 2013). Several reports revealed the role of target gene in manipulating the production of *A. annua* metabolites. Overexpression of *FPS* successfully improved artemisinin content in transgenic *A. annua* plant (Chen et al. 2000; Han et al. 2006; Banyai et al. 2010). Transgenic lines with overexpressing *HMGR* gene enhanced artemisinin content to 0.6 mg/g DW (Nafis et al. 2010), whereas 1.73 mg/g DW artemisinin was obtained from co-overexpression of *HMGR* and *ADS* genes (Alam and Abdin 2011). In addition, suppressing the expression of the gene in competing pathways also affected terpenoid production. Zhang et al (2009) demonstrated that suppression of squalene synthase (*SQS*) gene resulted in an approximately 3.14-fold increase in artemisinin content compared to controls, while the levels of four major sterols dropped in the suppressed lines. Introducing an RNAi fragment of the β-caryophyllene synthase (*CPS*) into *A. annua* plants downregulated the CPS transcript and showed an obvious reduction in β-caryophyllene content. In contrast, artemisinin content increased to 54.9% compared to controls (Chen et al. 2011). The cited reports suggested that manipulation of the key gene(s) induces diverse biochemical responses in *A. annua*. Therefore, overexpression and suppression of gene(s) in cyclization steps of artemisinin biosynthesis were conducted in *A. annua* to observe the role of the tested gene in biochemical production (**Chapter 3**).

Results of phenotypic alterations obtained from heterologous expression of *AtLFY* in *Tricyrtis* sp. and biochemical patterns of transgenic *A. annua* with overexpression and suppression of target gene were discussed and concluded (**Chapter 4**).

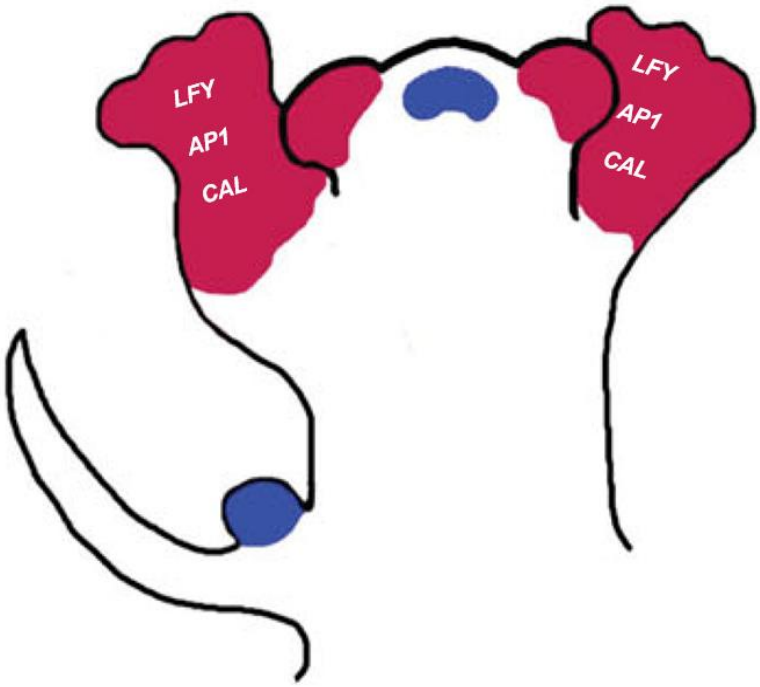

Figure 1.1 Expression of floral identity genes (*LFY*/*AP1*/*CAL*) in the *Arabidopsis* inflorescence shoot apex (Benlloch et al. 2007).

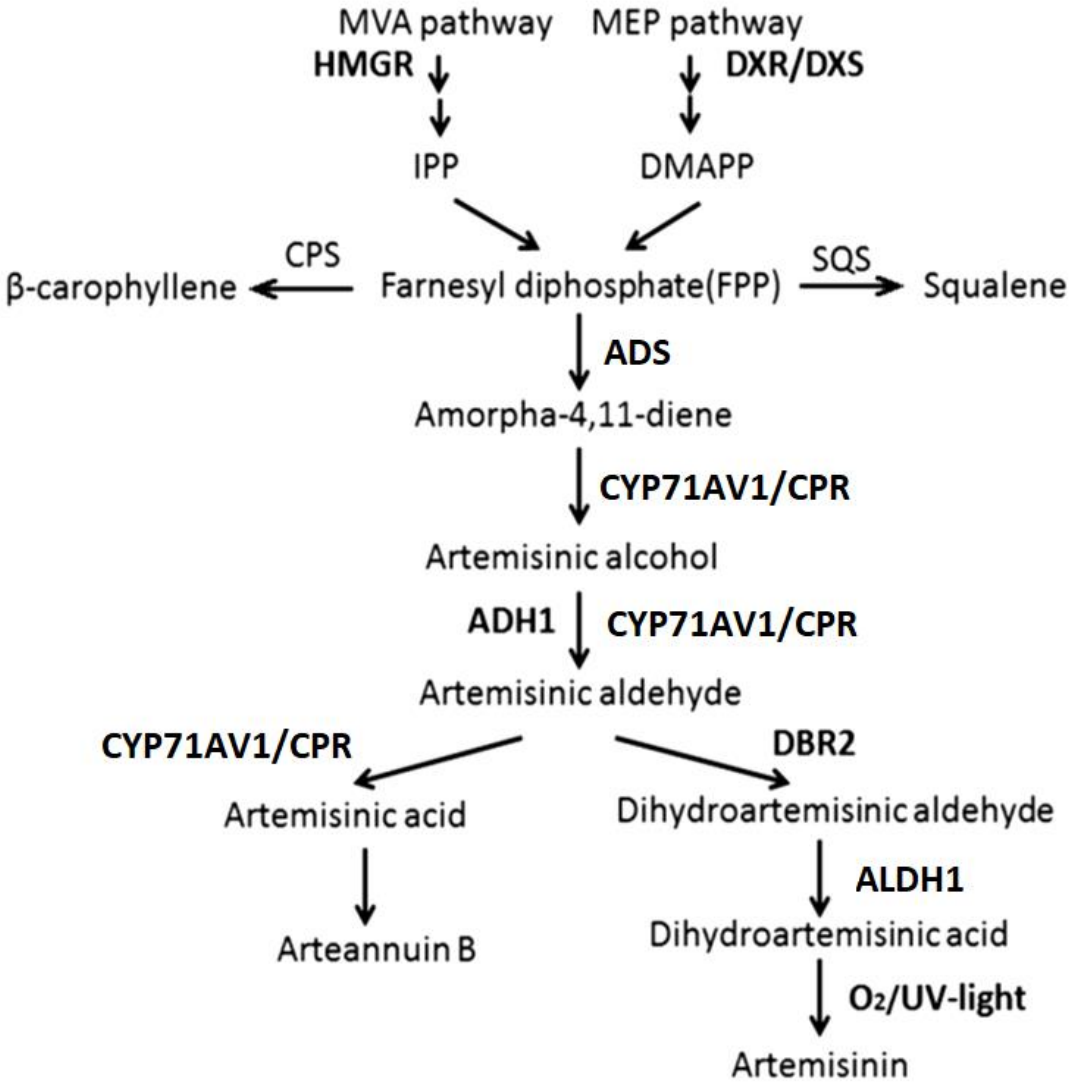

Figure 1.2 Artemisinin biosynthetic pathway in *Artemisia annua* L. *HMGR*: 3-hydroxy-3-methylglutaryl-coenzyme A reductase, *DXS*: 1-deoxyxylulose 5-phosphate synthase, *DXR* 1-deoxyxylulose 5-phosphate reductoisomerase, *FPS*: farnesyl diphosphate synthase, *ADS*: amorpha-4,11-diene synthase, *CYP71AV1*: cytochrome P450-dependent hydroxylase, *CPR*: cytochrome P450 oxidoreductase, *DBR2*: artemisinic aldehyde reductase, *ALDH1*: aldehyde dehydrogenase, *DMAPP*: dimethylallyl diphosphate, *IPP*: isopentenyl diphosphate, *CPS*: β-caryophyllene synthase, *SQS*: squalene synthase, MEP: methylerythritol phosphate pathway, MVA: mevalonic acid pathway (modified from Shi et al. 2017).

Chapter 2

Heterologous expression of *LEAFY* from *Arabidopsis thaliana* in monocotyledon *Tricyrtis* sp.

(This part was performed at Niigata University)

2.1 Introduction

2.1.1 Function of *LEAFY* (*LFY*), a floral homeotic gene in ornamental plants

LEAFY (*LFY*) gene encodes a plant-specific transcription factor that plays an important role in transition from vegetative to reproductive development (Schultz and Haughn 1991; Weigel et al. 1992; Parcy et al. 1998). LFY can be found in many species of terrestrial plants including mosses and angiosperms. Although the sequences are highly conserved throughout the kingdom Plantae, no apparent homologs are identified (Maizel et al. 2005). Unlike most other transcription factors, LFY does not belong to a multigene family.

LFY activates downstream genes by recognizing pseudo-palindromic sequences in promoters of the target genes (Parcy et al. 1998; Busch et al. 1999; Lohmann et al. 2001; Lamb et al. 2002; Hong et al. 2003). It has two domains: a partially conserved N-terminal domain that is thought to contribute to transcriptional activation and a highly conserved C-terminal domain that is responsible for DNA binding (Coen et al. 1990; Maizel et al. 2005). *LFY* is strongly expressed throughout the young floral meristems (Weigel et al. 1992) but the expression is not restricted to floral tissues. During vegetative growth, expression of *LFY* is low in leaf primordia and gradually increases until floral transition (Blázquez et al. 1997). When the expression reached a certain level, LFY induces the expression of other floral meristem identity genes such as *AP1* and *CAL* genes. The levels of LFY are considered to be an important parameter

that determines when flower transitions occur (Blázquez et al. 1997). LFY also interacts with co-regulator such as UNUSUAL FLORAL ORGANS (UFO), an F-box component of an SCF ubiquitin ligase, to regulate floral homeotic gene expression (Lee et al. 1997; Chae et al. 2008). After floral meristem is developed, LFY controls spatial patterning by stimulating the expression of floral organ identity *ABC* genes such as *AP1*, *AP3*, or *AGAMOUS* (*AG*) (Blázquez and Weigel, 2000; Lamb et al. 2002; Benlloch et al. 2007; Liu et al. 2009). The expression of LFY also increases in newly formed leaves.

2.1.2 The effect of *LFY* on the induction of phenotypic changes

LFY appears to play an important role in controlling flowering time and initiation of floral meristem (Blázquez and Weigel, 2000; Parcy, 2005). In arabidopsis, *lfy* mutants exhibited an increased number of secondary inflorescence shoots and abnormal flowers (Weigel et al. 1992). Although inflorescence branches of *lfy* mutants form normally early in development, they later become flowerlike with shorter internodes and fewer axillary buds (Huala and Sussex 1992). The *lfy* mutants could produce flowers; however, the numbers of flowers varied depending on the strength of the allele. The extremely phenotypic change was observed when only 6.3% of *lfy* mutant could produce flowers with an average of 2.3 flowers/plant. Nevertheless, daylength had an effect on their phenotypes, as they produced more inflorescence branches with fewer flowers in short days. In addition, the *lfy* mutants are slightly delayed in their transition from vegetative to inflorescence (Blázquez et al. 1997). Constitutive expression of *LFY* induces early flowering and the transformation of all shoots into flowers, suggesting the crucial role of *LFY* in regulating floral identity (Weigel and Nilsson 1995).

On the other hand, ectopic expression of *LFY*-homologous genes in other plant species has been demonstrated. Overexpression of *EjLFY-1* from *Eriobotrya japonica* Lindl in strawberry (*Fragaria ananassa*) shortened flowering induction and development of flower and fruit set (Liu et al. 2017). Though transgenic plants with *EjLFY-1* expression showed no significant changes in vegetative growth, flowers, or fruit development, asexual progeny showed a stronger early-flowering phenotype, including reduced vegetative growth and aberrant floral organs in individual plantlets. The role of LFY or its homologs in inducing early-flowering phenotype has been

observed not only in herbaceous plants (Weigel and Nilsson 1995; He et al. 2000) but also in woody plants like poplar (Rottmann et al. 2000).

Interestingly, overexpression of *LFY*-homologous genes induced dwarfism and early flowering in *A. thaliana* and *Nicotiana tabacum*, as well as the conversion of inflorescence meristems to floral meristems in *A. thaliana* (Weigel and Nilsson 1995; Ahearn et al. 2001). Dwarfness and early flowering are attractive traits, especially for ornamental plants. To create dwarf and/or early flowering plants, several molecular techniques have been used as tools in including mutation induction (Schum and Preil 1998) and *Agrobacterium*-mediated transformation. To date, dwarf plants of *Nierembergia scoparia* (Godo et al. 1997), *Antirrhinum majus* (Hoshino and Mii 1998), *Petunia hybrida* (Winefield et al. 1999), *Angelonia salicariifolia* (Koike et al. 2003), *Gentiana* sp. (Mishiba et al. 2006) and *Kalanchoe blossfeldiana* (Christensen et al. 2008) have been produced by transformation with wild type strains of *Agrobacterium rhizogenes* or *rol* genes from *A. rhizogenes*. Dwarf plants have also been produced in *Torenia fournieri* (Niki et al. 2006) and *Tricyrtis* sp. (Otani et al. 2013) by introducing gibberellin 2-oxidase genes, which catalyzes the conversion of bioactive gibberellins or their immediate precursors to inactive forms. However, almost all of these transgenic plants exhibited undesirable morphologies such as decreased apical dominance, wrinkled leaves, and/or only small or no flowers in addition to dwarfism. On the other hand, only few reports have demonstrated the production of early-flowering transgenic ornamental plants. For example, early-flowering transgenic *Lilium longiflorum* plants were created through overexpression of *FLOWERING LOCUS T* (*FT)*, a key regulator that activates floral homeotic gene (Leeggangers et al. 2018).

Although *LFY* may be an alternative gene for creating dwarf and/or early flowering transgenic plants, there have been few reports of LFY being used to genetically change ornamental plants. Therefore, the effect of *LFY* from *A. thaliana* (*AtLFY*) on morphological alteration of the ornamental plant was examined in this present study. *Tricyrtis* spp., liliaceous geophytes native to Japan, was used as a model plant, according to the following reasons: high transformation efficiencies, relatively small plant sizes, ease of cultivation, and taking only one year from *in vitro* regeneration to flowering (Nakano and Otani 2020). Transgenic *Tricyrtis* sp. plants with altered

flower forms (Nakano et al. 2006) and altered flower colors (Kamiishi et al. 2012) have already been produced. In addition, direct evidence supporting the modified ABCE model for the molecular mechanism of two-layered petaloid tepal development has successfully been obtained by using *Tricyrtis* sp. as a model plant (Otani et al. 2016). The results obtained from this present study showed a possibility of genetic transformation with *LFY* for producing dwarf and early flowering in monocotyledonous ornamental plants.

2.2 Materials and methods

2.2.1 Plant material preparation and embryogenic callus cultures

Potted plants of a tetraploid somaclonal variant of *Tricyrtis* sp. 'Shinonome' (Nakano et al. 2006) were used as plant materials for embryogenic callus induction. The plants were cultivated in the greenhouse without heating. Tepal-derived embryogenic callus cultures were induced and maintained as previously described by Nakano and Otani (2020).

2.2.2 Preparation of *Agrobacterium tumefaciens* solution

Agrobacterium tumefaciens strain EHA101/pIG-*AtLFY* was used for the transformation of *AtLFY* into *Tricyrtis* sp, while *A. tumefaciens* strain EHA101/pIG121-Hm (accession number AB489142, GenBank/EMBL/DDBJ databases) was also used as vector control. The T-DNA region of the binary vector pIG-*AtLFY* contains *AtLFY* (accession number NM125579, GenBank/EMBL/DDBJ databases) under the control of the CaMV35S promoter, *NPTII* under the control of the *NOS* promoter, and *HPT* under the control of the CaMV35S promoter. A single colony was picked up and pre-cultured in liquid YEP medium containing 50 mg l^{-1} kanamycin and 50 mg l^{-1} hygromycin for more than 24 h at 28°C in a reciprocating shaker. *Agrobacterium* cells were collected using centrifugation and re-suspended to a final OD_{600} of 0.2 in liquid *Tricyrtis* inoculation medium containing 50 mg l^{-1} of acetosyringone before co-cultivation.

2.2.3 *Agrobacterium tumefaciens*-mediated transformation and transgenic confirmation

Embryogenic calli of *Tricyrtis* sp. were co-cultivated with *Agrobacterium* solution, as previously described (Nakano and Otani 2020). Thereafter, co-cultivated calli were transferred to *Tricyrtis* embryo selection medium containing 40 mg l^{-1} hygromycin and 300 mg l^{-1} cefotaxime and incubated at 25°C under continuous illumination with white fluorescent light. Hygromycin-resistant somatic embryos were transferred to *Tricyrtis* plantlet selection medium and incubated at 25°C under continuous illumination. Transgenic plants and the vector control plants were transplanted to pots and cultivated in a growth chamber at 25°C under a 16-h photoperiod. The leaves were harvested to confirm the presence of the transgene *HPT* by PCR analysis using the primer set hpt290-F (5'-GTG CTT TCA GCT TCG ATG TAG G-3') and hpt290-R (5'-GCT CGT CTG GCT AAG ATC GG-3').

2.2.4 Morphological characterization of transgenic plants

After two years of cultivation, morphological characteristics of transgenic plants were observed. Shoot length, number of nodes per shoot, internode length, stem diameter, leaf length, leaf width, flowering date of the first flower, number of flowers per shoot, flower length, and flower diameter were recorded.

2.2.5 Expression analysis of *AtLFY* using quantitative real-time RT-PCR analysis

Transgene transcripts in young leaves of transgenic plants were quantified by real-time RT-PCR analysis using the DNA Engine Opticon System (MJ Research, Waltham, MA, USA) as previously described (Kamiishi et al. 2012). The primer sets used were an *AtLFY*-specific primer set, AtLFY RT-F1 (5'-CGC CGT CAT TTG CTA CTC TCC-3'), and AtLFY RT-R2 (5'-TGC GTC CCA GTA ACC ACT TCC-3'); and a *Thact2* (actin gene of *Tricyrtis* sp.; accession number AB196261 in the Gen- Bank/EMBL/DDBJ databases)-specific primer set, Thact1-F (5'-CCG ACT CCC TCA TGA AAA TCC-3') and Thact2-R (5'-CTC GAG CTC CTG TTC GTA GTC A-3'). The relative amount of

AtLFY transcripts was calculated using the comparative cycle threshold method, and the results were normalized to *Thact2*.

2.3 Results and discussion

2.3.1 Ectopic expression of *AtLFY* induced dwarfism in *Tricyrtis* sp.

Transgenic plants carrying the *LFY* gene from *A. thaliana* (*AtLFY*) were successfully demonstrated in liliaceous monocotyledon *Tricyrtis* sp. A total of nine independent transgenic lines were obtained by *Agrobacterium*-mediated transformation and the presence of *HPT* was confirmed by gene-specific PCR analysis. After cultivation for two years in a growth chamber, they were subjected to morphological characterization during flowering season. Morphological characteristics of transgenic plants were obviously different from the vector control plants, and all of the transgenic plants showed diverse phenotypes from the vector control plants.

Transgenic plants were classified into three types according to the degree of dwarfism (Table 2.1; Figure 2.1): an extremely dwarf Type I line (L1); moderately dwarf Type II lines (L2 and L3); and slightly dwarf Type III lines (L4, L5, L6, L7, L8 and L9). All morphological characteristics of vegetative organs of Type I plants differed significantly from the vector control plants. Type I plants had the shortest shoot length of 5.7 cm, which was 26% of the vector control plants. Moreover, Type I plants had the smallest number of nodes per shoot, stem diameter and leaf size. Type II plants had a shoot length of 11.2 cm, which was approximately half of that of the vector control plants. Reductions in stem diameter and leaf size were also observed in Type II lines, but the degree of alterations was lower than in Type I plants. Type III plants exhibited less dwarfism compared with Type I and Type II plants.

In this present study, the ectopic expressions of *LFY* from *A. thaliana* in an ornamental monocotyledonous plant, *Tricyrtis* sp. have been investigated. It was manifest that the morphological characteristics of the *AtLFY*-overexpressed plants were obviously different from those of vector control and non-transgenic plants. The

transgenic *Tricyrtis* plants with *LFY* from *A. thaliana* appeared to have dwarfed vegetative growth such as shortened shoot lengths, shortened internodes and reduction of leave size. Previous studies have reported that overexpression of *LFY* showed a protuberant influence in alterations of plant morphology in a broad spectrum of plant species, mostly in dicotyledonous species. Transgenic apples with overexpressing *AtLFY* exhibited a columnar phenotype with shorter internodes and more leaves (Flachowsky et al. 2009). Overexpression of *JcLFY* from *Jatropha curcas* decreased branch number and heights in transgenic arabidopsis but has no effect on flowering time and floral organs in Jatropha (Tang et al. 2016).

2.3.2 Ectopic expression of *AtLFY* induced early flowering in transgenic *Tricyrtis* sp.

In addition to dwarfism, overexpression of *LFY* altered floral characters. It has been reported on the different roles of dicotyledon overexpressing *LFY* that had an effect on flowering patterns and flowering time in different monocotyledonous species such as rice (Chujo et al. 2004) and *Lolium* grass (Gocal et al. 2001). Alterations in flower development were also observed in this present study. Vector control and Type III transgenic plants produced 1–4 apical flowers, whereas Type I and Type II transgenic plants produced only a single apical flower (Table 2.1). The *AtLFY*-overexpressing characteristics of Type I and Type II transgenic lines exhibited mostly at vegetative growth but had no significant effect on reproductive organs (flower numbers and flower size) when compared with vector control plants. There had no significant difference on reproductive parameters according to statistical analysis. Among the transgenic plants, Type I and Type II transgenic plants rarely had fully-opened flowers, which is an undesirable effect found together with the degree of alteration (Figure 2.1). By contrast, Type III transgenic plants could produce high flower buds per shoot without abnormality in the numbers and size of flowers. They also showed the fully-opened function of the flowers as similar to non-transgenic and vector control plants. In addition, the Type III transgenic plants significantly exhibited moderate dwarfism with a more compact plant shape and leave size when compared to non-transgenic and vector control plants. Therefore, it was suggested that the Type III transgenic plants may have more attractive

opportunities for ornamental implementation than the Type I and Type II transgenic plants.

Interestingly, all Type I, Type II and Type III plants produced flowers 1–3 weeks earlier than the vector control plants (Table 2.1), indicating the role of *LFY* in inducing early flowering in transgenic *Tricyrtis* sp. plants. Production of early-flowering transgenic ornamental plants was reported in transgenic *Oncidium* sp. by overexpressing the *Oncidium*-derived MADS-box gene (Thiruvengadam et al. 2012), suppression of miR159 in transgenic *Sinningia speciosa* (Li et al. 2013), and overexpressing *FT* in transgenic *Lilium longiflorum* (Leeggangers et al. 2018). Similarly, inductions of early flowering plants by overexpressing *LFY* have already been reported in a variety of plants. In *Brassica juncea*, the flowering time of transgenic plants was reduced by 19% when compared with the control (Roy et al. 2009). In transgenic citrus, the regenerated shoot produced flowers within five weeks after regeneration of *in vitro* plantlet (Peña et al. 2001). In contrast to previous reports, which found that dwarf transgenic plants produced small flowers (Godo et al. 1997; Hoshino and Mii 1998; Winefield et al. 1999; Koike et al. 2003; Mishiba et al. 2006; Niki et al. 2006; Christensen et al. 2008; Otani et al. 2013), no differences in the length and diameter of fully opened flowers were observed between all three types of transgenic plants and the vector control plants in the present study.

2.3.3 Correlation between the degree of morphological alteration and *At*LFY expression level

Relative expression level of *AtLFY* in transgenic plants was determined by quantitative real-time RT-PCR analysis (Figure 2.2). *AtLFY* transcripts were detected in all Type I, Type II and Type III plants, but not in the vector control plants. Among nine transgenic lines, the highest expression level of *AtLFY* was observed in Type I line, followed by Type II and Type III lines, respectively. The degree of dwarfism generally correlated with the *AtLFY* expression level as shown by quantitative real-time RT-PCR analysis. These results indicate that the morphological alterations observed in transgenic *Tricyrtis* sp. plants were obtained from ectopic expression of *AtLFY*. Previously, overexpression of the *LFY* gene from *A. thaliana* has been reported to induce columnar

phenotype and shortened internodes (Flachowsky et al. 2009). Although the length of the regrowing shoot had no correlation with the amount of LFY mRNA transcript, increased shoot diameter and LFY transcript level were found to have a positive correlation.

Although dwarf transgenic *Tricyrtis* sp. plants with early flowering were successfully obtained, the adverse effect on morphological alterations was also observed in flowers of the *AtLFY* highly expressed plants. Only a few unopened flowers were produced in these transgenic plants. These might be suggested as results of vector construction and promotor activity. Although strong constitutive promoters are often used to drive the transgene expression in molecular breeding, they often produce undesirable morphologies. Our previous study reported that driving the transgene under a strong constitutive CaMV 35S promoter affected the production of flowers in transgenic plants. Overexpressing the gibberellin 2-oxidases (*TfGA2ox*) driven by CaMV 35S promoter in *Tricyrtis* sp. exhibited severely dwarf transgenic plants, but the plants produced abnormal or no flowers (Otani et al. 2013). By contrast, overexpression of *OsGA2ox1* driven by OsGA3ox2 promoter in rice resulted in induction of dwarf phenotypes with no alteration in flower morphology (Sakamoto et al. 2003). These reports suggested that overexpressing the target genes under weaker or tissue-specific promoters could reduce the expression level of transgene but still maintain the role in flower development.

2.4 Conclusion

In the present study, the role of *LFY* in regulating plant morphology and flowering time was demonstrated in ornamental monocotyledonous plants. Dwarf transgenic *Tricyrtis* sp. plants with early flowering phenotypes were successfully obtained by ectopic expression of the *LFY* gene from the cruciferous plant *A*. *thaliana*. Despite the successful introduction of dwarf and early flowering traits, Type I and Type II transgenic plants with higher levels of *AtLFY* expression produced non-fully-opened flowers, which greatly reduce the ornamental value. On the other hand, no such unfavorable characteristics were observed in Type III transgenic plants with lower levels of *AtLFY* expression. Thus, moderate levels of *AtLFY* expression in Type III transgenic plants may be necessary for producing dwarf and early flowering transgenic plants without alterations of floral characteristics. Higher expression levels of *AtLFY* in the present study might be caused by a strong activity of the CaMV35S promoter. Therefore, efficient production of dwarf transgenic *Tricyrtis* sp. plants with a normal number of fully opened flowers may be achieved by using weaker or tissue-specific promoters. The result provides a possibility of genetic transformation with *LFY* for producing dwarf and early flowering of the rhizomatous monocotyledonous plant, which can be promoted to use as a potted plant or for landscaping purposes.

Figure 2.1 Type I (L1), Type II (L3) and Type III (L9) transgenic plants containing *At*LFY and the vector control plant (VC) of *Tricyrtis* sp. at the flowering stage.

Table 2.1 Morphological characteristics of transgenic plants containing *AtLFY* and the vector control plants of *Tricyrtis* sp. at the flowering stage [a].

Plant lines	Shoot length (cm) [b]	No. of nodes per shoot [b]	Internode length (cm) [c]	Stem diameter (cm) [d]	Leaf length (cm) [e]	Leaf width (cm) [e]	Flowering date of the first flower [f]	No. of flowers per shoot [b]	Flower length (cm) [g]	Flower diameter (cm) [g]
Vector control	21.7 ± 0.7 a	10.0 ± 0.6 a	2.2 ± 0.1 a	3.5 ± 0.3 ab	7.4 ± 0.2 a	2.9 ± 0.1 a	Sep. 25	1.7 ± 0.3 a	2.5 ± 0.1 a	3.6 ± 0.1 a
Type I transgenic line										
L1	5.7 ± 0.8 c	6.0 ± 0 b	0.9 ± 0.1 c	1.5 ± 0 c	3.4 ± 0 d	1.8 ± 0 f	Sep. 3	1.0 ± 0 a	2.4 ± 0 a	3.4 ± 0 a
Type II transgenic lines										
L2	11.2 ± 0.4 bc	7.3 ± 0.3 ab	1.5 ± 0.1 bc	2.0 ± 0 c	4.5 ± 0.4 bcd	2.0 ± 0.1 ef	Sep. 5	1.0 ± 0 a	2.5 ± 0 a	3.6 ± 0 a
L3	11.2 ± 0.4 bc	7.0 ± 0 ab	1.6 ± 0.1 bc	2.3 ± 0.3 bc	4.2 ± 0.5 bcd	2.3 ± 0 cde	Aug. 30	1.0 ± 0 a	2.4 ± 0 a	3.5 ± 0 a
Type III transgenic lines										
L4	15.1 ± 0.4 b	8.7 ± 0.7 ab	1.4 ± 0.1 bc	2.8 ± 0.2 abc	6.7 ± 0.2 abc	2.5 ± 0.1 bcd	Aug. 29	2.3 ± 0.3 a	2.5 ± 0.1 a	3.6 ± 0.2 a
L5	15.1 ± 2.3 b	9.7 ± 1.2 a	1.4 ± 0.1 bc	2.2 ± 0.2 bc	5.7 ± 0.4 abc	2.3 ± 0.1 cde	Sep. 11	2.0 ± 0.6 a	2.5 ± 0.1 a	3.5 ± 0.2 a
L6	15.3 ± 2.2 b	8.7 ± 0.9 ab	1.8 ± 0.1 ab	2.7 ± 0.4 abc	7.0 ± 0.4 ab	2.5 ± 0 bc	Sep. 7	2.7 ± 0.7 a	2.4 ± 0.2 a	3.6 ± 0.1 a
L7	16.1 ± 1.4 ab	9.3 ± 0.3 a	1.7 ± 0.1 bc	2.8 ± 0.4 abc	7.0 ± 0.1 ab	2.5 ± 0 bc	Sep. 5	2.7 ± 0.7 a	2.5 ± 0.2 a	3.6 ± 0.2 a
L8	16.2 ± 0.4 ab	9.3 ± 0.7 a	1.8 ± 0.1 ab	2.3 ± 0.3 bc	7.1 ± 0.4 ab	2.9 ± 0.1 ab	Sep, 12	2.0 ± 0 a	2.6 ± 0.2 a	3.7 ± 0.3 a
L9	16.4 ± 0.7 ab	9.0 ± 0.6 ab	1.8 ± 0 ab	4.0 ± 0 a	5.4 ± 0.3 bc	2.5 ± 0.1 abc	Sep. 16	1.7 ± 0.3 a	2.5 ± 0.2 a	3.6 ± 0.2 a

[a] Values represent the mean ± standard error of clonally propagated three plants for each line. Values within the same column followed by different letters are significantly different at the 0.01 level with the Tukey–Kramer's test. [b] The longest shoot was investigated for each plant. [c] The most basal internode of the longest shoots was measured for each plant. [d] Middle position of the longest shoots was measured for each plant.[e] Randomly selected three expanded leaves of the longest shoots were investigated for each plant. [f] The first flower among three plants for each line. [g] All of fully opened flowers were investigated for each plant. Type I and Type II transgenic plants often produced non-fully opened flowers.

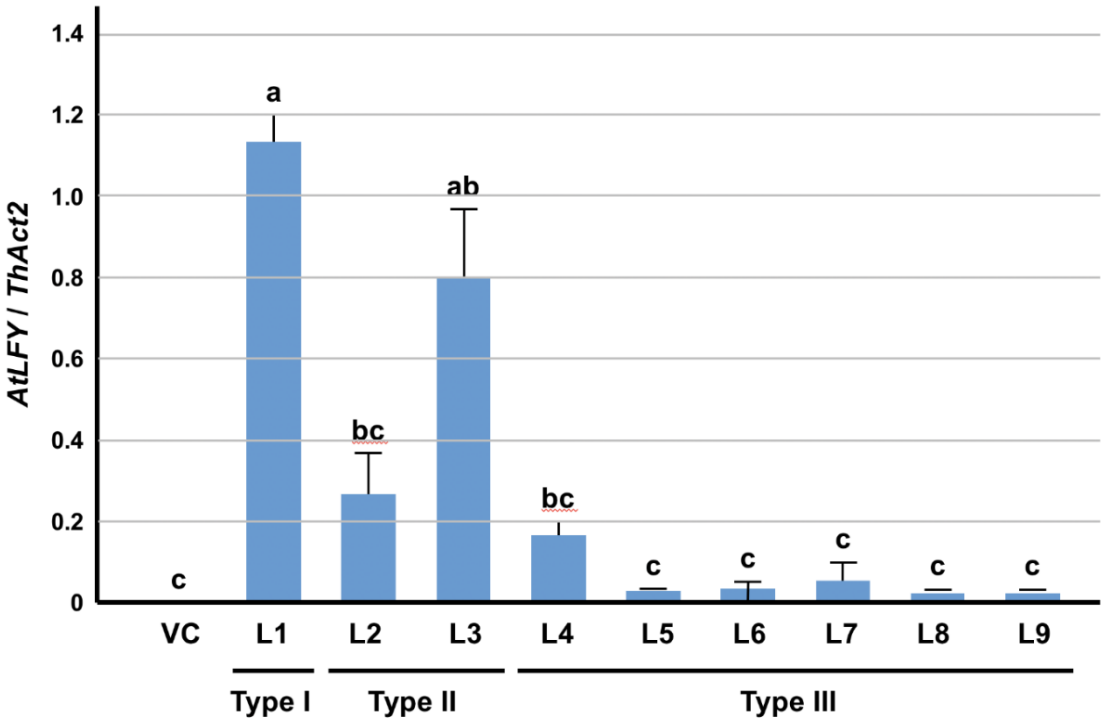

Figure 2.2 Quantitative real-time RT-PCR analysis of *AtLFY* transcripts in young leaves of transgenic plants containing *AtLFY* (L1–L9) and the vector control plant (VC) of *Tricyrtis* sp. Relative amount of *AtLFY* transcripts was normalized to *Thact2*. Values represent the mean ± standard error of triplicate. Values with different letters are significantly different at the 0.01 level with the Tukey–Kramer's test

Chapter 3

Homologous sense overexpressed and RNAi suppressed expressions of a dicot gene in *Artemisia annua*

This part was performed at Mahidol University

3.1 Introduction

3.1.1 Homologous overexpression and suppression of a gene in dicotyledonous species

A demonstrated in Chapter 2, overexpression of target gene in heterologous host exhibited diverse phenotypic responses depending on expression level, insertion sit and copy number of transgenes. Therefore, to investigate phenotypic response of gene expression, overexpression or suppression of target gene was performed in a homologous host. Homologous expressions of a gene in the same host have been effectively used to investigate gene function in metabolic pathways. Based on base-pairing between nucleic acids, a double-stranded DNA molecule is made up of two strands that are reverse complements of each other. Positive-sense DNA is generally defined as nucleotide sequences that correspond directly to the sequence of RNA transcripts. Overexpression of sense fragment has been applied to regulate plant phenotypes as well as plant metabolite production in several plant species including dicotyledonous plants. Transgenic citrus with overexpression of a gibberellin-related gene under control of 35S promoter significantly promoted taller phenotypes, which were correlated with higher levels of GA1 in growing shoots (Fagoaga et al. 2007). Moreover, overexpression of endogenous phytoene synthase increased levels of β-carotene, lutein, violaxanthin, chlorophyll and abscisic acid in transgenic *Arabidopsis thaliana* (Lindgren et al. 2003). Another powerful tool for investigating gene function is RNA interference (RNAi) technology, which is a process of sequence-specific gene silencing and an epigenetic phenomenon. When double-stranded RNA (dsRNA) is introduced into a cell, it causes specific mRNA molecules to break down. This

technology has been widely used in regulating the pathway by inhibiting competitive pathways in order to investigate how they affect the biosynthesis of a desirable molecule. In *A. annua*, DNA-directed RNAi-technology was used to suppress the activity of squalene synthase gene, resulting in increased accumulation of artemisinin (Ali et al. 2017). Kumar et al. (2016) suppressed the activity of cinnamate-4-hydroxylase gene in phenylpropanoid/lignin biosynthesis pathway using RNAi, which decreased p-coumaric acid, total phenolics, and anthocyanin but increased artemisinin. These suggested roles of RNAi technology in rerouting metabolic flux to production of the target compound.

3.1.2 *Artemisia annua* as a source of useful biochemical constituents

The model plant for phenotypic investigation in homologous system was selected based on the following criteria;

1) It has a well-established protocol for genetic transformation.
2) It demonstrates phenotypic responses at the OMICs level.
3) It is effective for functional genomic studies of plant responses to external and internal signals.

According to these criteria, *Artemisia annua* L. or qinghao was chosen. *A. annua* L. is an annual herb native to China but it can grow as a horticultural or medicinal plant in a variety of cool temperate and subtropical areas in northern, middle and eastern parts of Asia (Ferreira et al. 2005). It has been used in China for over 2,000 years to treat several infectious diseases (Chang, 2016; Graziose et al. 2010). In 1972, Chinese researchers isolated qinghaosu, or artemisinin, a C15 terpenoid compound from the leaves of the *A. annua* plant (Klayman, 1985). The discovery of artemisinin was honored with a Noble Prize in Physiology or Medicine in 2015. Artemisinin is a sesquiterpene trioxane lactone with a peroxide structure that is required for combating against *Plasmodium falciparum*, the malaria-causing parasite that is becoming increasingly drug-resistant. Artemisinin is recommended by WHO to use for the malarial patient, as it has less toxic and side effects than other substances. Artemisinin has also been used to treat hepatitis B (Romero et al. 2005), schistosomiasis (Borrmann et al. 2001), and a

variety of cancer cell lines (Efferth et al. 2001; Singh and Lai, 2001). Artemisinin is the only commercial and economical source of artemisinin, but production and accumulation of this compound occur at relatively low levels (0.01-0.8%) and vary depending on genotype and environmental limitations (Abdin et al. 2003; Ferreira et al. 2005; Wallaart et al. 2000). Nevertheless, levels of artemisinin vary from organ to organ, as artemisinin is mainly found in leaves and inflorescences, with a smaller amount in stem and none in pollen and roots.

In addition to artemisinin, *A. annua* has been reported to produce a variety of active constituents including terpenoids, flavonoids, coumarins, sterols, phenols, lipids, and other hydrocarbons (Bhakuni et al. 2001; Czechowski et al. 2018). High content of flavonoids in *A. annua* leaf extract exhibited a potent antioxidant activity (Bilia et al. 2006), suggesting that *A. annua* might be used as a promising source of antioxidants (Zheng and Wang, 2001; Cai et al. 2004). Recently, leaf extracts were reported to have anti-coronavirus activities (Farmanpour-Kalalagh et al. 2022: Fuzimoto et al. 2021: Nair et al. 2022; Nie et al. 2021). Furthermore, artemisinin biosynthesis involves with production of other terpenoid compounds including mono-, sesqui-, di- and triterpene. Our previous study revealed a specific relationship between biosynthetic proteins and the production of terpenoid compounds in *A. annua* under a specific light spectrum after 7 days of artificial light exposure (Sankhuan et al. 2022). These suggested that *A. annua* L. is a highly attractive model plant for investigating metabolomic responses to a variety of environmental changes.

3.1.3 The impacts of transgenic *Artemisia annua* plants on production of artemisinin (C15) and other bioactive terpenoids

Biosynthesis of artemisinin involves several enzymes in terpenoid biosynthesis. Thus, many approaches have been devoted to enhance artemisinin production using genetic engineering. Some artemisinin biosynthetic genes were overexpressed, resulting in higher quantities of artemisinin. For example, overexpression of *HMGR* showed its impact on promoting artemisinin content to 0.06% DW (Nafis et al. 2011). Higher accumulation of artemisinin was also achieved by increasing the expression of *FPS* transcripts from *G. arboretum* (Chen et al. 2000) and *A. annua* (Han et al. 2006; Banyai et al. 2010). Nevertheless, it was suggested that overexpression of multiple genes into

A. annua may be more efficient to manipulate artemisinin biosynthetic pathway. Transgenic *A. annua* with co-expression of *HMGR* and *ADS* contained artemisinin contents up to 0.17% DW, which is three times more than those overexpressing with *HMGR* alone (Alam and Abdin, 2011). Although artemisinin is successfully improved by regulation of a key gene in transgenic plants, influence of transgene expressions on terpenoid change is valuable to investigate in order to understand how gene expression interferes with terpenoid biosynthesis metabolic flux. Manipulation of GA involving gene altered characteristic of trichome and phytochemical profile of transgenic *A. annua* (Inthima et al. 2017). The levels of total sesquiterpene (C15) increased in transgenic *A. annua* with overexpression of *ADS*, whereas those of *FPS-ADS* co-expression increased in total triterpene (C30) contents, indicating that the metabolic flux of artemisinin production might be interfered by inserted genes (Sankhuan et al. 2018). Furthermore, variations in terpenoid content in *A. annua* result in a variety of anti-malarial effects in leaf extracts (Karaket et al. 2014). It was found that transgenic *A. annua* with higher content of squalene exhibited greater antimalarial activity when compared to transgenic plant with high artemisinin, suggesting the synergistic role of terpenoid compounds in enhancing the bioactivity of plant extracts. Accordingly, alteration of gene expression affects metabolite production in both targeted and non-targeted compounds and subsequently interferes with properties of the plants.

3.1.4 The enzymatic functions and the key-enzyme genes at intermediate and cyclization steps of artemisinin (C15) biosynthesis

Artemisinin is a product of isoprenoid biosynthesis pathway that involves several biosynthetic enzymes. The biosynthesis of artemisinin starts with the formation of a common precursor, isoprenyl diphosphate (IPP), which can be synthesized by two different pathways: cytosolic mevalonic acid (MVA) pathway and plastidic 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. Subsequently, several steps of enzymatic and non-enzymatic reactions convert IPP into artemisinin end-product (Figure 3.1). In this present study, genes encoding enzymes that function at intermediate formation and cyclization of artemisinin backbones such as farnesyl pyrophosphate synthase (*FPS*), amorpha-4, 11-diene synthase (*ADS*) and cytochrome P450 monooxygenase (*CYP71AV1*) were emphasized.

Farnesyl pyrophosphate synthase (*FPS*) is one of the key genes involved in the regulation of isoprenoid biosynthesis. The *FPS* encodes prenyltransferase that catalyzes two sequential condensations of dimethylallyl pyrophosphate (DMAPP) with two molecules of isopentenyl pyrophosphate (IPP) to form farnesyl pyrophosphate (FPP) (Lange et al. 2000), which is a key intermediate in the biosynthesis of sterols and a large variety of sesquiterpenes including artemisinin (Newman and Chappell 1999). The *FPS* is commonly found in a wide range of plants, including *Arabidopsis thaliana* (Delourme et al. 1994), *Ginkgo biloba* (Wang et al. 2004), *A. annua* (Matsushita et al. 1996*)* and other medicinal plants. Microdissection and RT-PCR revealed that transcripts of FPS expressed in apical and sub-apical cells of *A. annua* glandular trichomes (GSTs) (Nguyen et al. 2011). Since *FPS* produces substrate FPP for isoprenoid biosynthesis, over-expression of this gene in *A. annua* is a promising idea to increase artemisinin and related terpenoid accumulation. The *FPS* from *G. arboreum* was previously introduced into *A. annua* using *Agrobacterium*-mediated transformation, leading to increased artemisinin in both hairy roots and transgenic shoots (Chen et al. 2000). In 2006, overexpression of *A. annua FPS* resulted in a 34.4 % increase in artemisinin levels compared to non-transformed plants (Han et al. 2006). More recently, artemisinin was over-produced by the integration of the *FPS* into *A. annua* genome (Banyai et al. 2010). Interestingly, growth retardation was observed in some transgenic lines, indicating that the transgene insertion affected growth and biomass of transgenic plants.

The rate-limiting enzyme of artemisinin biosynthesis is amorpha-4,11-diene synthase (ADS). ADS converts FPP intermediate into sesquiterpene skeleton, amorpha-4,11-diene. Therefore, this enzyme reroutes metabolic flux of FPP pool to artemisinin biosynthesis (Bouwmeester and Wallaart, 1999). The transcriptional analysis revealed that transcripts of ADS were exclusively found in *A. annua* (Komori et al. 2013). The lack of ADS transcripts in other *Artemisia* species suggests that *ADS* is unique to the artemisinin biosynthetic pathway. Expression of this gene is predominantly found in GSTs and young leaf tissue (Olofsson et al. 2011). Overexpressing *ADS* has been demonstrated to drive the pool of FPP from squalene and sterol biosynthesis into artemisinin production. It was found that artemisinin, artemisinic acid and arteannuin B were significantly increased in the leaves of transgenic *ADS* plants (Ma et al. 2015).

Moreover, the effect of *ADS* overexpression not only interfered with levels of artemisinin-related compounds but also non-amorphadiene sesquiterpene and other volatile profiles.

ADS is hydroxylated to produce artemisinic alcohol, which is catalyzed by a cytochrome P450 monooxygenase (*CYP71AV1*) (Teoh et al. 2006). This enzyme belongs to the cytochrome P450 family and plays a key role in the biosynthesis of sesquiterpene lactone artemisinin. CYP71AV1 is a multifunctional sesquiterpene oxidase that catalyzes the conversion of ADS to artemisinic acid which is the final enzymatic intermediate and a precursor of arteannuin B biosynthesis (Maes et al. 2011). CYP71AV1 acts in cooperation with cytochrome P450 oxidoreductase (CPR) to increase the ability to generate more oxygenated products (Ro et al. 2006). Co-expression of *CYP71AV1* and its redox partner *CPR* in *A. annua* has previously been demonstrated (Shen et al. 2012). When compared to non-transgenic plants, transgenic plants had a 38 % higher artemisinin content. Four artemisinin biosynthetic genes (*ADS*, *CYP71AV1*, *CPR*, and *ALDH1*) were recently overexpressed (Shi et al. 2017). Artemisinin content was found to be 3.4-fold higher in transgenic plants.

Overexpression and suppression technologies are becoming more attractive in agricultural approach. Despite their diverse applications, phenotypic variations resulting from overexpression and suppression of target genes have been observed. To examine the correlation between functional gene and metabolite production in *A. annua*, manipulations of genes at intermediate and cyclization steps of artemisinin biosynthesis were demonstrated in this present study. These will enable us to better understand the role of gene expression in the metabolic pathway and allow us to further implement this knowledge to induce novel compounds or high-producing lines for pharmaceutical application.

3.2 Materials and methods

3.2.1 Homologous overexpression and suppression of key enzyme genes involved in artemisinin biosynthesis

3.2.1.1 Oligonucleotide primer design for vector construction and amplification of target sequences

RNA interfering sequences (triggers) were designed from the full sequence of *FPS* (GenBank Acc. No. GQ40346), *ADS* (GenBank Acc. No. KJ609176) and *CYP71AV1* (GenBank Acc. No. KJ609177). The triggers were amplified from p33FPS, p33CYP and p33ADS, using specific primers in Table 3.1. The PCR program was 94ºC for 5 min pre-denaturation followed by 35 cycles of 94ºC for 30 s denaturation, 58ºC for 30 s annealing, 72ºC for 2 min extension. PCR condition was ended with final extension step of 72ºC for 5 min. PCR product was electrophoresed on 1% agarose gel. The gel was then stained with ethidium bromide and amplified bands were observed in UV transilluminator.

3.2.1.2 Sub-cloning of target sequences to a cloning vector

The triggers were purified and were inserted into a shuttle vector pENTR™/D-TOPO® following the protocol provided in pENTR™/DTOPO® cloning kit (Invitrogen™, USA). The ligates were then transformed into *E. coli* DH5α using heat shock method (Sambrook 2001). The *E. coli* transformants were grown and selected in 50 mg L^{-1} kanamycin-containing medium. The positive clones were picked up to check the presence of insert by colony PCR. A positive colony with a positive PCR result was then cultured in Luria-Bertani (LB) liquid medium supplemented with the same selective antibiotic for overnight at 37°C in an orbital shaker. The *E. coli* cultures were used for plasmid extraction by alkaline lysis method (Birnboim and Doly, 1979). The plasmid was re-dissolved in 20 µl of 10% TE and kept at -20°C before sequencing analysis was performed.

3.2.1.3 Sub-cloning of trigger sequences to pANDA vector

LR clonase reaction between the entry clone (pENTR vector) and recombinant pANDA vector was performed to produce the final RNAi vector. The resultant vectors were transformed into *E. coli* and selected on LB medium containing 50 mg L^{-1} of both kanamycin and hygromycin. The presence of sense sequences was confirmed by PCR, while the antisense sequences were detected by strand-specific RT-PCR using specific primers. To confirm the presence of triggers, the RNAi vectors were sequenced.

3.2.1.4 Introduction of RNAi vector into *A. tumefaciens* EHA105

The pANDA vector containing the target sequence was introduced into *A. tumefaciens* EHA105 by freeze-thaw method following the procedure performed by Xu and Qingshun (2008). The transformants were then selected on LB medium containing 50 mg L^{-1} kanamycin and 50 mg L^{-1} hygromycin. The colony PCR was performed in the same manner as previously described.

3.2.2 Production of siRNA transgenic *A. annua* plants

3.2.2.1 Plant material preparation

Artemisia annua L. plants (wild type code no. 007) and all transgenic lines were produced and multiplied at Laboratory of Plant Physiology and Agri-Biotechnology, Department of Biotechnology, Faculty of Science, Mahidol University. *In vitro* plantlets were maintained on 0.2% Gelrite®-solidified MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and sub-cultured at three-week intervals. They were incubated at 25±2°C, 65±5% relative humidity, 16 hours photoperiod with a photosynthetic photon flux density of 60±5 µmol $m^{-2}s^{-1}$.

3.2.2.2 Preparation of *Agrobacterium* solution

A single colony was picked up from the plate and transferred to liquid LB medium containing 50 mg L^{-1} of kanamycin and hygromycin. Erlenmeyer flask containing bacterial solution was incubated in gyratory shaker at 28°C. After 16-18 hours of

incubation, bacterial sediment was collected at 1,500 g for 10 min. The pellet was then re-suspended in 30 ml LB medium containing 100 µM acetosyringone and used for *Agrobacterium* infection.

3.2.2.3 *Agrobacterium*-mediated transformation using Ti-plasmid vectors

Fully expanded leaf discs of 3-week-old plantlets were excised and soaked in a bacterial flask containing 30 ml of LB medium supplementing 100 µM acetosyringone. Infected flasks with leaflets were then placed in desiccators and evaporated for 20 min (Banyai et al, 2010a). Infected leaves were transferred to solidified MS medium containing 100 µM acetosyringone and incubated in dark at 25±2°C for 5 days.

3.2.2.4 Plant regeneration and selection of putative transformants

After 5 days of co-cultivation, infected leaflets were transferred to 0.7% agar solidified MS medium containing 0.1 mg L^{-1} NAA, 1 mg L^{-1} BA and 30 mg L^{-1} meropenem for shoot regeneration. The regenerants were transferred to hygromycin-containing media in order to eliminate the escape of non-transformed cultures.

3.2.2.5 DNA isolation and transgene confirmation by PCR

Fresh leaves of non-transformed *A. annua* L plants and putative transgenic plants were excised from *in vitro* plantlets. Genomic DNA was performed using the modified CTAB (cetyltrimethylammonium bromide) method. PCR was demonstrated using thermal cycler (Biometra, Germany). Specific forward and reverse were used for amplification of hygromycin (*HPT*) resistance gene (forward 5'-GTGCTTTCAGCTTCGATGTAGG-3' and reverse primer 5'- GCTCGTCTGGCTAA GATCGG-3'). The PCR reaction was performed following these conditions: 35 cycles of 4 min at 94ºC, 1 min at 55ºC, and 1 min at 72ºC. PCR products were separated in 1% agarose gel using electrophoresis, then the gel was stained with GelRed® (Biotium, USA) prior to analysis by Image Gel Doc XR+ Imaging System with Image Lab Software version 5.0 build 18 (BIO-RAD, USA).

3.2.3 Phenotypic examination of transgenic *A. annua* plants

3.2.3.1 *Ex vitro* transplantation

Wide type, transgenic *A. annua* with oxCYP expression (oxC2, oxC4 and oxC28), which were obtained from transformation of oxCYP (Figure 3.6a) into A. annua and siCYP transgenic lines (siC2, siC9, siC40) were maintained on solidified MS basal medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and incubated at 25±2°C, 65±5% relative humidity, 16 hours photoperiod with a photosynthetic photon flux density of 60±5 μmol m^{-2} s^{-1}. The *in vitro* plantlets with well-performed root growth were transferred to semi-closed vessels, each with a 0.45 μm membrane filter to allow air ventilation. Vermiculite was used as the supporting material, supplemented with sugar-free MS liquid medium. The plantlets were acclimated for 15 days under the same conditions before transfer to *ex vitro* conditions in pots containing vermiculite and then incubated under 25±2 °C, 65±2 %RH, 16 h photoperiod with 60±5 μmol m^{-2} s^{-1} white LED lamps.

3.2.3.2 Analysis of terpenoid profiles by GCMS

Fresh leaves of non-transformed *A. annua* plants and transgenic plants were excised from *ex vitro* transplant. The 50 mg of leaf samples were ground to a fine powder using mortar and pestle in liquid nitrogen. The samples were extracted in 500 μl dichloromethane. After filtration, 240 μl of filtrate was mixed with 10 μl of 1 mg mL^{-1} internal standard (C17; heptadecanoic acid, methyl ester). Dichloromethane extract was then subjected into GCMS (HP-6890) with a HP5-ms capillary fused silica column (30 m, 0.25 mm I.D.; 0.25 m film thickness). The temperature program and other operating conditions were set according to Karaket et al. 2014. The compound identities were searched in Wiley 10 No.14 Library. Only the compounds with 80% match quality were reported and used for further study.

3.3 Results and discussion

3.3.1 Sequencing analysis and cloning efficiency of inverted repeat elements of target genes

The widely used RNA silencing transgenes in transgenic plants have been achieved by introduction of antisense and sense transcripts of the mRNA target, inverted repeat (IR) elements or intron-embedded IR (Frizzi and Huang, 2010). Among several transgene design, IR element, although not highly transcribed, triggered gene silencing with more efficiency than the single-copy antisense or sense transgene (Chuang and Meyerowitz, 2000) and became widely applied in plant biotechnology. Thus, RNAi construct used in this study was designed to contain an IR sequence separated by a spacer sequence.

The IR transgene contained a fusion of a sense and an antisense fragment of target gene that later produced a hairpin RNA when transcribed. To generate RNAi constructs for gene suppression, approximately 300 bp (Figure 3.2) of target genes (*FPS*, *ADS* and *CYP71AV1*) were amplified as sense fragments by PCR. The resulting PCR products were detected in 1% agarose gel as shown in Figure 3.3. After PCR products were purified and cloned into Gateway pENTR/D-TOPO cloning vector, the positive clones were selected in kanamycin-containing medium. Among 30 tested colonies, colony PCR screening could amplify the trigger sequences in 5 clones of pENTR-Fi, 5 clones of pENTR-Ai and 6 clones of pENTR-Ci. Unfortunately, no positive sequencing result was obtained from pENTR-Fi and pENTR-Ai when these plasmids were sent for sequencing analysis (Table 3.2). Only one clone (16.67%) of the tested pENTR-Ci exhibited the presence of sense *CYP71AV1* fragment and showed 100% identity to the reported sequences of *CYP71AV1* (GenBank Acc. No. KJ609177), as present in Figure 3.4. The low cloning efficiency might be caused by the false-positive PCR from colony PCR screening, which can cause by cross-contamination from another DNA fragment or a non-specific PCR condition. When the annealing temperature is set too low, the designed primers may amplify some genomic sequences. Primer design and optimization of the PCR condition are critical steps. It was suggested to perform colony PCR using a combination of a vector-specific primer and an insert-specific primer, with

increased annealing temperature, reduced annealing and reduced extension times to prevent non-specific binding between primers and target sequence.

Subsequently, sense fragment of *CYP71AV1* in entry clone (pENTR-Ci) was transferred into a destination vector, the pANDA vector by an LR clonase reaction. The pANDA vector has a corn ubiquitin1 promoter, GUS linker and kanamycin- and hygromycin-resistance genes as selectable markers (Miki and Shimamoto, 2004). The positive colonies were extracted and used for PCR amplification of sense and antisense *CYP71AV1*. Sense sequences were amplified by CYP-Fi and CYP-Ri primers, while the antisense sequences were amplified by strand-specific PCR using AsCYP-FW and AsCYP-RW primers (Table 3.1). Sense and RNAi *CYP71AV1* fragments were detected in pANDA-Ci7. RNAi vector containing sense and antisense *CYP71AV1* fragments was confirmed by sequencing analysis. The sequence of sense *CYP71AV1* was searched against nucleotide database of NCBI. Sequence similarity was observed with 100% similarity to the registered sequences of *CYP71AV1* (GenBank Acc. No. KJ609177). On the other hand, pairwise alignment performed to confirm the sequence similarity. The result perfectly showed the alignment between sequencing data and RNAi sequences in Figure 3.5. Finally, the T-DNA region of binary pANDA-Ci7 or siCYP vector contained sense and antisense fragments of *CYP71AV1* under control of ubiquitin promoter was demonstrated in Figure 3.6b.

3.3.2 Transgenic siCYP production and evaluation

Although *Agrobacterium*-mediated transformations have been widely applied in *Artemisia annua*, the transformation frequency remained low (Han et al. 2006; Shen et al. 2012). Vacuum force was applied during transformation and successfully improved transformation efficiency in *A. annua* (Banyai et al. 2010) as vacuum force increased the surface attachment between *Agrobacterium* solution and plant tissue. Therefore, vacuum treatment was applied in this study. *Agrobacterium* transformation was carried out using one-month-old leaflets detached from *in vitro* plantlets. The infected leaf discs were incubated on MS medium supplemented with 100 µM acetosyringone for 5 days. Due to the high rate of shoot induction, solidified MS medium containing 0.1 mg L^{-1} NAA and 1 mg L^{-1} BA was used as the regeneration medium (Banyai et al. 2005). A

sign of shoot primordium formation was noticed after 2 weeks of cultivation. After one month, shoot direct organogenesis was observed at the leaf blade and leaf stalk positions (Figure 3.7a), similar to the previous study by Banyai et al (2010). Forty regenerated shoots were obtained from regeneration process, approximately 57.14% of explants. All shoots derived from plant regeneration were transferred to selection medium containing 10 mg L^{-1} hygromycin and 30 mg L^{-1} meropenem to obtain hygromycin-resistant plants. After 30 days of selection, only 3 plantlets; siC2, siC9 and siC40 (7.5% of regenerated shoots) survived in selection medium and these plants were able to produce shoot and root vigorously. Subsequently, all hygromycin-resistant plantlets were tested for the presence of marker gene in transgenic *A. annua* using *HPT*-specific primers. Although few numbers of regenerants were obtained from transformation, all surviving plantlets exhibited PCR-positive results with the size of 290 bp (Figure 3.7b, Table 3.3).

Productions of artemisinin and other related terpenoid compounds in transgenic *A. annua* plants are regulated by expressions of several key enzymatic genes involved in artemisinin biosynthesis. Expression of gene in transgenic plants can be influenced by multiple copies of transgene and insertion site (Gelvin 1998; Napoli et al. 1990). In addition to gene manipulation, external factors such as developmental stage and cultivation conditions also affect the expression level. Thus, transcriptional analyses of artemisinin biosynthetic genes in transgenic *A. annua* plants should be further performed.

Unlike transgenic *Tricyrtis* sp. expressing *AtLFY*, transgenic *A. annua* with manipulation of biosynthetic gene did not show apparent morphological alterations. Since artemisinin is mainly produced in leaves of *A. annua*, leaf factors including leaf numbers, leaf FW, number and size of glandular secretory trichome and total leaf biomass are the most important factors affecting artemisinin yield. However, alteration of metabolic genes influences hormonal balances (eg. gibberellins and cytokinin) that might interfere with plant growth and reproductive success (Bauer-Panskus et al. 2020). Genetic inheritance and the environment that parents experience significantly impact the morphological traits of progenies (Herman and Sultan, 2011). Therefore, progenies of transgenic *A. annua* plants should be subjected to morphological studies.

In addition to overexpression and suppression using RNAi technology, genome editing and induction of targeted suppression using CRISPR/Cas9 is the most advanced system that has been successfully applied in *A. annua*. Inhibition of a competitive gene (Koerniati and Simanjuntak, 2020) and suppression of MYB transcription factor, *AaTAR2* (Zhou et al. 2020) improved artemisinin production. Although the efficiency of genome editing in Asteraceae plants like *A. annua* is much lower than that in Arabidopsis due to larger genome (Zhou et al. 2020; Shen et al. 2018), recent studies have shown the possibility to apply this technology for creating artemisinin-rich plant and functional genomic studies in *A. annua*.

3.3.3 Biochemical analysis of transgenic *Artemisia annua* with oxCYP or siCYP expressions

In order to investigate the effect of genetic manipulation on metabolomic responses, biochemical analysis was performed in transgenic *A. annua* with oxCYP or siCYP expressions and wide type. The oxCYP transgenic plants were obtained from *Agrobacterium*-mediated transformation with oxCYP vector in our previous study. The *in vitro* plantlets were maintained and multiplied in the Laboratory of Plant Physiology and Agri-Biotechnology, Department of Biotechnology, Faculty of Science, Mahidol University. *In vitro* plantlets with well-performed shoot and root growths were used for this present study. The wild type and transgenic *A. annua* with oxCYP or siCYP expressions were acclimatized and transplanted to pots using vermiculite as supporting materials. The plants were cultivated in a plant factory with white light-emitting diode (LED) lamps at 25±2°C, 50±5% relative humidity and 60±5 μmol m^{-2} s^{-1} light intensity for 16 h photoperiod. All plant lines were multiplied by cutting propagation (Figure 3.8). After 2 weeks, leaf samples were collected from the cutting plants for GC-MS analysis.

Variations in phytochemical contents were evaluated among transgenic lines in comparison with wild type. Results revealed 21 volatile compounds with more than 80% match quality to the mass spectral library (Wiley 10 no. 14). These compounds were categorized into two major groups; terpenes and other compounds. Terpenoids were the most abundant group in this study, consisting of 6 monoterpenes (C10), 12 sesquiterpenes (C15), and a diterpene (C20). The rest were categorized as other non-

terpenoid compounds (Table 3.5). Our study was conducted in young cutting plants, thus, no structural complex compounds such as triterpene (C30), phytosterol and fatty acids were observed. Out of 21 detected biochemical compounds, the highest number of biochemical compounds (approximately 90.48%) were found in transgenic oxC28 plant, followed by transgenic oxC2, siC2 and wild type (Table 3.4). Sesquiterpenes were the most abundant compounds with 11 compounds reported. Six were monoterpenes, while the rest comprised one each of diterpene and non-terpene. When compared with wild type, transgenic *A. annua* with oxCYP expression tends to produce a greater number of volatile compounds, except in transgenic oxC4 plant that produced a smaller number of monoterpenes. Similar to wild type, the transgenic siC2 produced a total of 16 volatile compounds. On the other hand, numbers of biochemical compounds slightly decreased in other transgenics of siCYP (siC9 and siC40). Sesquiterpenes were significantly affected, as about half of the compounds in wild type were reduced in these transgenic plants. Overall results suggested that transgenic *A. annua* with oxCYP expression produced higher volatile sesquiterpenes than transgenic plants with siCYP expression.

Diagrams revealed variations in terpenoid composition, especially in transgenic oxC4 plant (Figure 3.9). The wild type contained 49.54% sesquiterpenes, 45.33% monoterpenes, 3.02% diterpene and 2.11% other compounds. Transgenic oxC4 plant showed a distinct composition compared with other lines of oxCYP expressing plants, as a higher content of total sesquiterpenes (76.32%) was reported. On the other hand, no obvious differences were found among transgenic oxC2, oxC28 and wild type. In siCYP transgenic plants, the patterns of terpenoid composition were similar, as 50% of the detected terpenes were identified as monoterpenes. Variations in both types and contents of metabolites were observed among different plant lines (Table 3.5). However, not all oxCYP transgenic plants produced high levels of compounds. Similar pattern was also observed in transgenic siCYP expressing plants, as some contained higher contents than wild type while some produced less.

Among terpenoid compounds detected in this present study, six compounds were remarkably observed (Figure 3.10). Sesquiterpenes copaene (Figure 3.10a) and aromadendrene (Figure 3.10b) were particularly found in oxCYP transgenic plants, but

they were not present in wild type or siCYP-expressing plants. Both copaene and aromadendrene have been reported to exhibit several pharmaceutical activities. Copaene had significant anticarcinogenic and antioxidant properties (Türkez et al. 2014), while aromadendrene synergistically interacts with the other compounds to promote antibacterial activities (Mulyaningsih et al. 2010). Artemisinin G, an artemisinin derivative, was detected at relatively high levels in both transgenic oxCYP and siCYP plants, but it was absent in wild type (Figure 3.10c). Although the bioactivity against malarial parasites has not been clearly explained, this compound can be cost-effectively converted to artemisinin via bioconversion or chemical synthesis (Nair and Basile, 1993). Variations in trans-beta-farnesene were also observed among oxCYP transgenic lines. The transgenic oxC4 line produced slightly higher content than the wild type, while 50% of the contents were significantly reduced in two other lines (Figure 3.10d). Trans-beta-farnesene has been shown to have anticancer, antibacterial, and antifungal properties (Türkez et al. 2014). However, this compound was not found in all siCYP expressing lines. Bicyclogermacrene, a major component in essential oils that has been shown to exert strong antibacterial, anti-inflammatory, and antioxidant properties (Costa et al. 2012), was also reported in this study. About 5% relative content was found in wild type, all oxCYP transgenic lines and one line of siCYP expressing lines (Figure 3.10e). A monoterpene, beta-pinene slightly increased in transgenic oxC4 line, while all siCYP transgenic lines contained less level when compared with wild type and oxCYP transgenic lines (Figure 3.10f). This compound possessed a wide range of pharmacological activities, including antitumor, antimicrobial, antimalarial, antioxidant and anti-inflammatory properties (Salehi et al. 2019).

Results demonstrated that different *CYP71AV1* constructions (oxCYP or siCYP) affected production of *A. annua* metabolites. Differences in terpenoid types and content among transgenic lines might be explained by different insertion sites of the T-DNA containing the target gene in *A. annua* genomes, copy number of transgene and interference from epigenetic processes. Our previous study revealed that gene silencing was detected in transgenic *A. annua* plants containing two copies of the transgene, resulting in reduced artemisinin content and yield (Banyai et al. 2010). The T-DNA insertion at different positions also interfered the terpenoid compositions in transgenic

A. annua plants and affected bioactive properties of the plant extracts (Karaket et al. 2014).

3.4 Conclusion

In the present study, homologous expressions of a gene in artemisinin biosynthesis pathway were demonstrated in both aspects of sense overexpression and suppression. According to plasmid construction, RNA silencing plasmid of *CYP71AV1* was successfully developed using gateway pANDA vector. The presence of target fragments in pANDA-derived vector was also confirmed by sequencing. The siCYP plasmid was transformed into *Agrobacterium tumefaciens* strain EHA105 in order to transform into *A. annua* plant. After transformation process, shoot direct organogenesis formation was observed at 45 days in regeneration medium. However, only three independent-siCYP transgenic lines were obtained. PCR positive lines were further used for phytochemical compound evaluation, in comparison with oxCYP transgenic lines and wild type. Phytochemical fingerprint revealed variations in terpenoid compounds among the tested plants. Some useful compounds were promoted in oxCYP transgenic lines. On the other hand, copaene, trans-β-farnesene and aromadendrene, which exhibited a variety of bioactivities, were absent in siCYP transgenic lines, suggesting the diverse effect of different *CYP71AV1* constructs on metabolite production in *A. annua*. The progenies of transgenic *A. annua* will be further subjected to expression and morphological analyses.

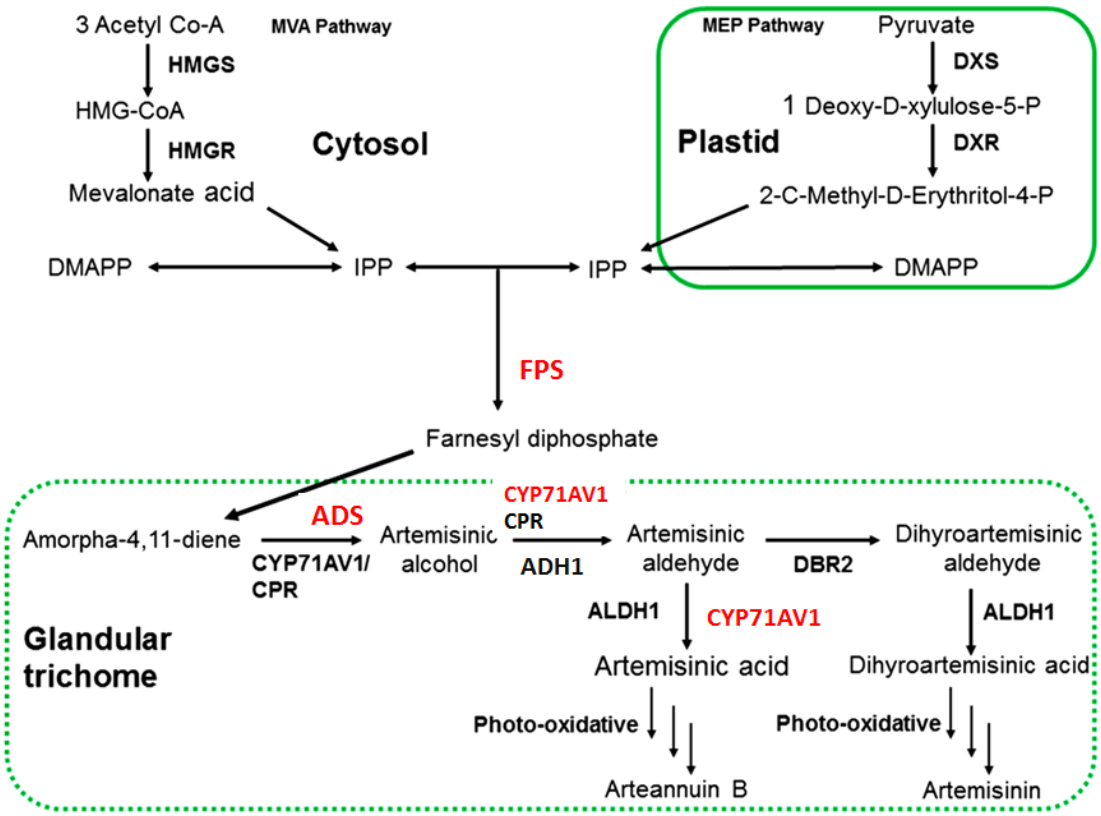

Figure 3.1 Proposed isoprenoid and artemisinin biosynthesis pathway of *Artemisia annua* L. (He et al. 2017)

Table 3.1 Gene-specific sequence for construction of siRNA vectors

Primer name	**Sequence (5'-3')**
ADS-Fi	GGATCCCGGCAACTACTAAAAGAAGCTTTG
ADS-Ri	GAATTCAGCTAACGATTGCTTGAACGCTCC
CYP-Fi	GGATCCCCATGGCGACTTCCCATTA
CYP-Ri	GAATTCACGTAACTGCCTCCAGTATTCACC
FPS-Fi	GGATCCATTCGACGATGATTCCCGTCAATG
FPS-Ri	GAATTCCGTTCGCAGCAATCATACCAACC
AsCYP-FW	GCGACGTAACTGCCTCCAGT
AsCYP-RW	GGCGACTTCCCATTATTGGTC

***FPS* sequence (acc. no. GQ40346)**

ATGAGTAGCATCGATCTGAAATCCAAGTTTTTAAAAGTGTATGATACACTTAAATCAGAGCTTATTAACGATCCCGCCTTCGAATTCGACGA
TGATTCCCGTCAATGGATTGAAAAGATGCTTGACTACAACATACCTGGAGGAAAGCTGAACCGGGGATTATCTGTTGTCGACAGTTATCAG
CTTCTTAAAGGAGGAGAACTGTCTGATGACGAGATTTTTCTTTCATCTGCCCTTGGTTGGTGTATTGAATGGCTTCAAGCATACTTTCTTGT
GCTTGATGATATAATGGACGAGTCTCATACACGCAGAGGGCAACCCTGTTGGTTTAGATTACTCAAGGTTGGTATGATTGCTGCGAACGAT
GGAATTCTTCTTCGCAACCATGTCCCAAGAATTCTTAAGAAACATTTCCGTGGAAAGCCTTACTATGAGGATCTTGTGGACCTGTTCAACG
AGGTTGAATTCCAAACAGCCTCTGGTCAGATGATTGATTTGATCACTACACTTGTTGGAGAGAAAGATCTCTCGAAGTATTCATTGTCTATT
CACCGCCGAATTGTTCAATACAAAACAGCTTACTACTCATTTTACCTTCCAGTTGCCTGTGCACTCCTTATGTTTGGAGAGGATCTTGACAA
GCACGTTGAAGTGAAGAACATGCTCGTTGAAATGGGTACCTATTTTCAAGTTCAGGACGATTATCTAGACTGTTTTGGTGCTCCCGAGGTG
ATTGGAAAGATTGGAACTGATATTGAAGACTTTAAGTGCTCCTGGTTAGTTGTCAAGGCATTGGAACTCGCCAATGAGGAACAAAAGAAA
GTCCTACATGAGAACTATGGGAAAAAGGACCCCGCGTCTGTTGCTAAAGTGAAGGAAGTATACCACACTCTCAATCTTCAGGCTGTATTC
GAAGATTACGAGGCCACAAGTTACAAGAAGCTTATCACATCGATTGAAAATCGCCCAAGCAAAGCAGTCCAAGCGGTGCTGAAATCTTTC
TTGGGTAAAATCTACAAGAGGCAAAAGTAG

***ADS* sequence** (acc. no. KJ609176)

ATGTCACTTACAGAAGAAAAACCTATTCGCCCCATTGCCAACTTTCCTCCAAGCATTTGGGGAGATCAGTTTCTCATCTATGAAAAGCAAG
TAGAGCAAGGGGTGGAACAGATAGTGAATGATTTAAAAAAAGAAGTGCGGCAACTACTAAAAGAAGCTTTGGATATTCCTATGAAACATG
CCAATTTGTTGAAGCTGATTGATGAAATCCAACGCCTTGGAATACCGTATCACTTTGAACGGGAGATTGATCATGCATTGCAATGTATTTAT
GAAACATATGGTGATAACTGGAATGGTGACCGCTCTTCCTTATGGTTCCGTCTTATGCGAAAGCAAGGATATTATGTTACATGTGATGTTTTC
AATAACTATAAAGACAAAAATGGAGCGTTCAAGCAATCGTTAGCTAATGATGTTGAAGGTTTGCTTGAGTTGTACGAAGCAACTTCTATGA
GGGTACCTGGGGAGATTATATTAGAAGATGCTCTTGGTTTTACACGATCTCGTCTTAGCATTATGACAAAAGATGCTTTTTCTACAAACCCC
GCTCTTTTTACCGAAATACAACGGGCACTAAAGCAACCCCTTTGGAAAAGGTTGCCAAGAATAGAGGCGGCGCAGTACATTCCTTTCTAT
CAACAACAAGATTCTCATAACAAGACTTTACTTAAACTTGCTAAGTTAGAGTTCAATTTGCTTCAGTCATTGCACAAGGAAGAGCTCAGCC
ATGTGTGCAAATGGTGGAAAGCTTTCGATATCAAGAAGAACGCACCTTGTTTAAGAGATAGAATTGTTGAATGCTACTTTTGGGGACTAGG
TTCAGGCTATGAGCCACAGTATTCCCGGGCTAGAGTTTTCTTCACAAAAGCTGTTGCTGTTATAACTCTTATAGATGACACTTATGATGCGT
ATGGTACTTATGAAGAACTTAAGATCTTTACTGAAGCTGTTGAAAGGTGGTCAATTACATGCTTAGACACACTTCCAGAATACATGAAACC
GATATACAAATTATTCATGGATACATACACAGAAATGGAAGAATTTCTTGCAAAGGAGGGAAGAACAGATCTATTTAACTGCGGCAAAGAA
TTTGTGAAAGAGTTTGTTAGAAACCTGATGTTTGAAGCAAAATGGGCAAATGAGGGACACATACCAACCACTGAAGAGCATGATCCAGTT
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CTCTGCACCTCCTCTTTTTAGATACTCAGGTATACTTGGTCGACGCCTAAATGATCTCATGACCCACAAGGCCGAGCAAGAAAGAAAACAT
AGTTCATCGAGCCTTGAAAGTTATATGAAGGAATATAATGTCAATGAGGAGTATGCCCAAACCTTGATTTACAAGGAAGTAGAAGATGTGT
GGAAAGATATAAACCGAGAGTACCTCACAACTAAAAACATTCCAAGGCCGTTATTGATGGCTGTGATCTATTTGTGCCAGTTTCTTGAAGT
TCAATATGCAGGAAAGGATAACTTCACACGTATGGGAGACGAATACAAACATCTCATAAAGTCTCTACTCGTTTATCCTATGAGTATATGA

***CYP71AV1* sequence (acc. no. KJ609177)**

ATGGCACTCTCACTGACCACTTCCATTGCTCTTGCAACGATCCTTTTGTTCGTTTACAAGTTCGCTACTCGTTCCAAATCCACCAAAAAAA
GCCTTCCTGAGCCATGGCGACTTCCCATTATTGGTCACATGCATCACTTGATTGGTACAACGCCACATCGTGGGGTTAGGGATTTAGCCAG
AAAGTATGGATCTTTGATGCATTTACAGCTTGGTGAAGTTCCAACAATCGTGGTGTCATCTCCGAAATGGGCTAAAGAGATTTTGACAACG
TACGACATTACCTCTGCTAACAGGCCCGAGACTTTAACTGGTGAGATTGTTTTATATCACAATACGGATGTTGTTCTTGCACCTTATGGTGA
ATACTGGAGGCAGTTACGTAAAATTTGCACATTGGAGCTTTTGAGTGTTAAGAAAGTAAAGTCATTTCAGTCGCTTCGTGAAGAGGAGTG
TTGGAATTTGGTTCAAGAGATTAAAGCTTCAGGTTCAGGGAGACCGGTTAACCTTTCAGAGAATGTTTTCAAGTTGATTGCAACGATACTT
AGTAGAGCCGCATTTGGGAAAGGGATCAAGGACCAGAAAGAGTTAACGGAGATTGTGAAAGAGATACTGAGGCAAACTGGTGGTTTTG
ATGTGGCAGATATCTTTCCTTCAAAGAAATTTCTTCATCATCTTTCGGGCAAGAGAGCTCGGTTAACTAGCCTTCGCAAAAAGATCGATAAT
TTAATCGATAACCTTGTAGCTGAGCATACTGTTAACACCTCCAGTAAAACTAACGAGACACTCCTCGATGTTCTTTTAAGGCTCAAAGACA
GTGCTGAATTCCCATTAACATCTGATAACATTAAAGCCATCATTTTGGATATGTTTGGAGCAGGCACAGACACTTCCTCATCCACAATCGAA
TGGGCTATTTCGGAACTCATAAAGTGTCCGAAAGCAATGGAGAAAGTACAAGCGGAATTGAGGAAAGCATTGAACGGAAAAGAAAAGA
TCCATGAGGAAGACATTCAAGAACTAAGCTACTTGAACATGGTAATCAAAGAAACATTGAGGTTGCACCCTCCACTACCCTTGGTTCTGC
CAAGAGAGTGCCGCCAACCAGTCAATTTGGCTGGATACAACATACCCAATAAGACCAAACTTATTGTCAACGTCTTTGCGATAAATAGGG
ACCCTGAATATTGGAAAGACGCTGAAGCTTTCATCCCTGAACGATTTGAAAATAGTTCTGCAACTGTCATGGGTGCAGAATACGAGTATCT
TCCGTTTGGAGCTGGGAGAAGGATGTGTCCTGGAGCCGCACTTGGTTTAGCTAACGTGCAGCTCCCGCTCGCTAATATACTATATCATTTCA
ACTGGAAACTCCCCAATGGTGTGAGCTATGACCAGATCGACATGACCGAGAGCTCTGGAGCCACGATGCAAAGAAAGACTGAGTTGTTA
CTCGTTCCAAGTTTCTAG

Figure 3.2 Full nucleotide sequences of *FPS*, *ADS* and *CYP71AV1*. The yellow highlights indicate the selected sequences for RNAi constructs

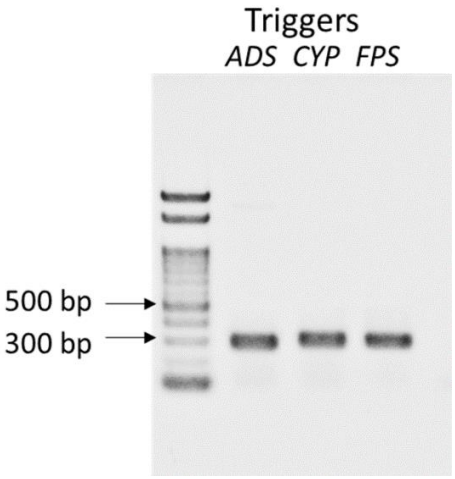


Figure 3.3 PCR amplification of RNAi trigger sequences

Figure 3.4 Sequencing analysis of pENTR-Ci vector containing sense *CYP71AV1* fragment. DNA sequencing chromatogram analysis by SnapGene® software from Insightful Science; available at snapgene.com (**a**). The sequences identification by nucleotide BLAST (NCBI) (**b**).

Table 3.2 Construction efficiency of siRNA vectors

Vector	Number of tested sample [a]	Number of positive sample [b]	Construction efficiency (%) [c]
pENTR-Fi	5	0	0
pENTR-Ai	5	0	0
pENTR-Ci	6	1	16.67

[a] Number of sample subjected to nucleotide sequencing process [b] Number of the plasmid that showed readable sequencing signal with 100% similarity to the target gene [c] Construction efficiency = (number of positive sample/number of tested sample) x 100

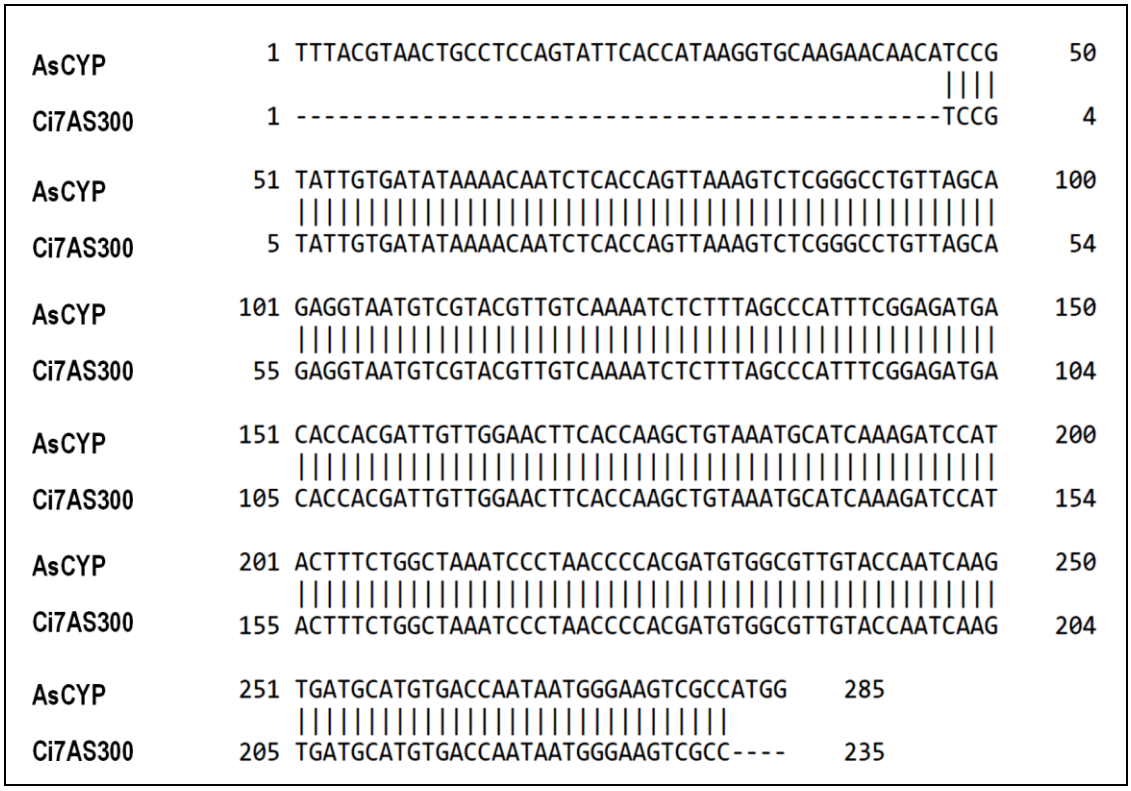


Figure 3.5 Pairwise alignment of sequencing data (Ci7AS300) and antisense sequence of *CYP71AV1* (AsCYP)

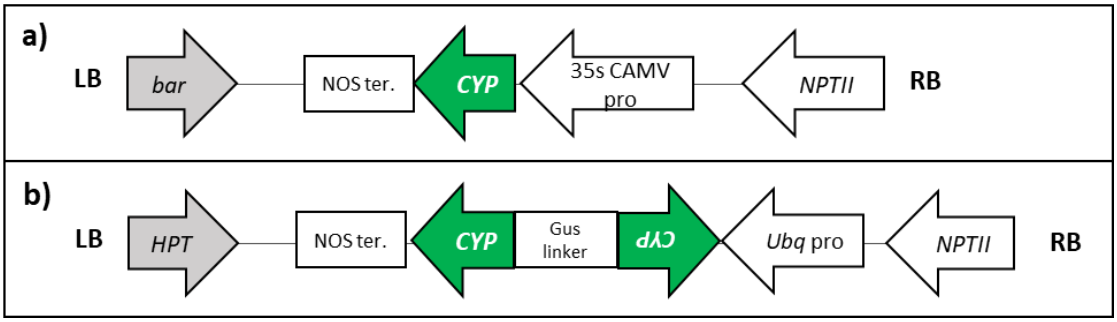


Figure 3.6 T-DNA structure of (**a**) vector with sense *CYP71AV1* (oxCYP) and (**b**) silencing vector (siCYP)

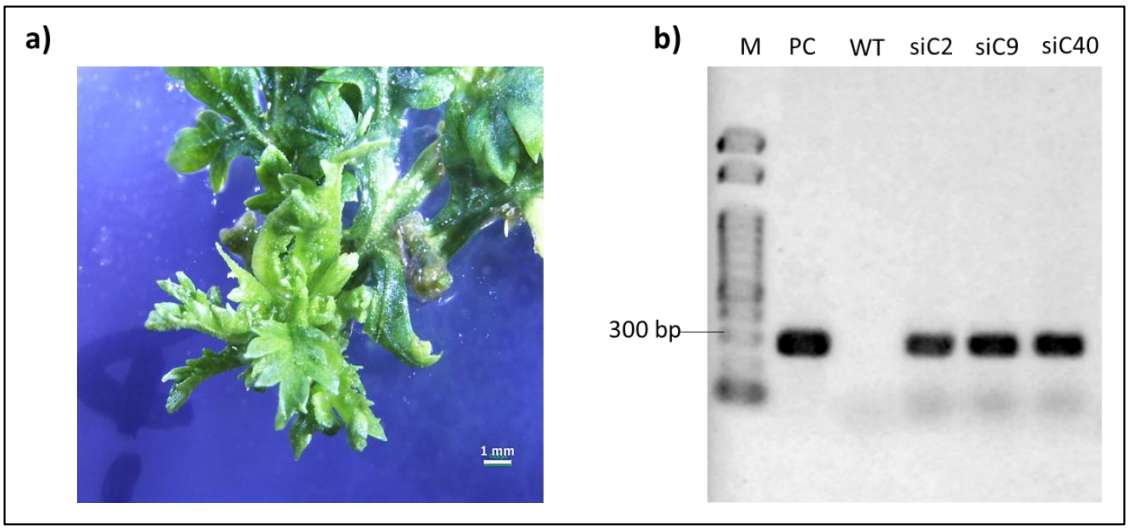


Figure 3.7 Production and confirmation of siCYP transgenic plantlets. Leaf-derived shoot organogenesis of transformed plantlets from leaf discs of *Artemisia annua* were obtained after culturing in regeneration medium for 1 month (**a**). Detection of HPT genes in transgenic plantlets by PCR analysis (**b**). M, 100 bp DNA-ladder as molecular markers; PC, binary plasmid pIG121Hm as a positive control; siC2, siC9 and siC40, transgenic plantlets obtained from *A. tumefaciens* strain pANDA/Ci.

Table 3.3 Transgenic selection and confirmation by *HPT*-specific primers

Transgenic plantlets	Survival shoots in medium with 10 mg L^{-1} hygromycin[1]	Plantlets with PCR-positive of *hptII* gene
siC2	✓	✓
siC9	✓	✓
siC40	✓	✓

[1] About 7.5% of hygromycin resistant shoots were obtained from 40 biological replications (1 shoot segment/replication). The hygromycin resistant shoots were selected after 30 days of continuous supplementation with 10 mg L^{-1} hygromycin-containing medium. The survival shoots with vigorous shoot and root formations were further tested for PCR.

Figure 3.8 Plant material preparation for phytochemical analysis. The single-node cuttings of *A. annua* were cultured in pots containing vermiculite. After 2 weeks, the leaves were collected for GCMS analysis.

Table 3.4 Numbers of biochemical compounds detected in leaves of different *A. annua* lines

Compound type	WT	Transgenic *A. annua* with oxCYP expression			Transgenic *A. annua* with siCYP expression		
		oxC2	oxC4	oxC28	siC2	siC9	siC40
Monoterpenes	6	6	2	6	6	5	4
Sesquiterpenes	8	11	11	11	8	6	6
Diterpenes	1	1	1	1	1	1	-
Other	1	-	-	1	1	-	-
Total	16	18	14	19	16	12	10

Figure 3.9 Phytochemical variations of wild type and transgenic *A. annua* with oxCYP or siCYP expression

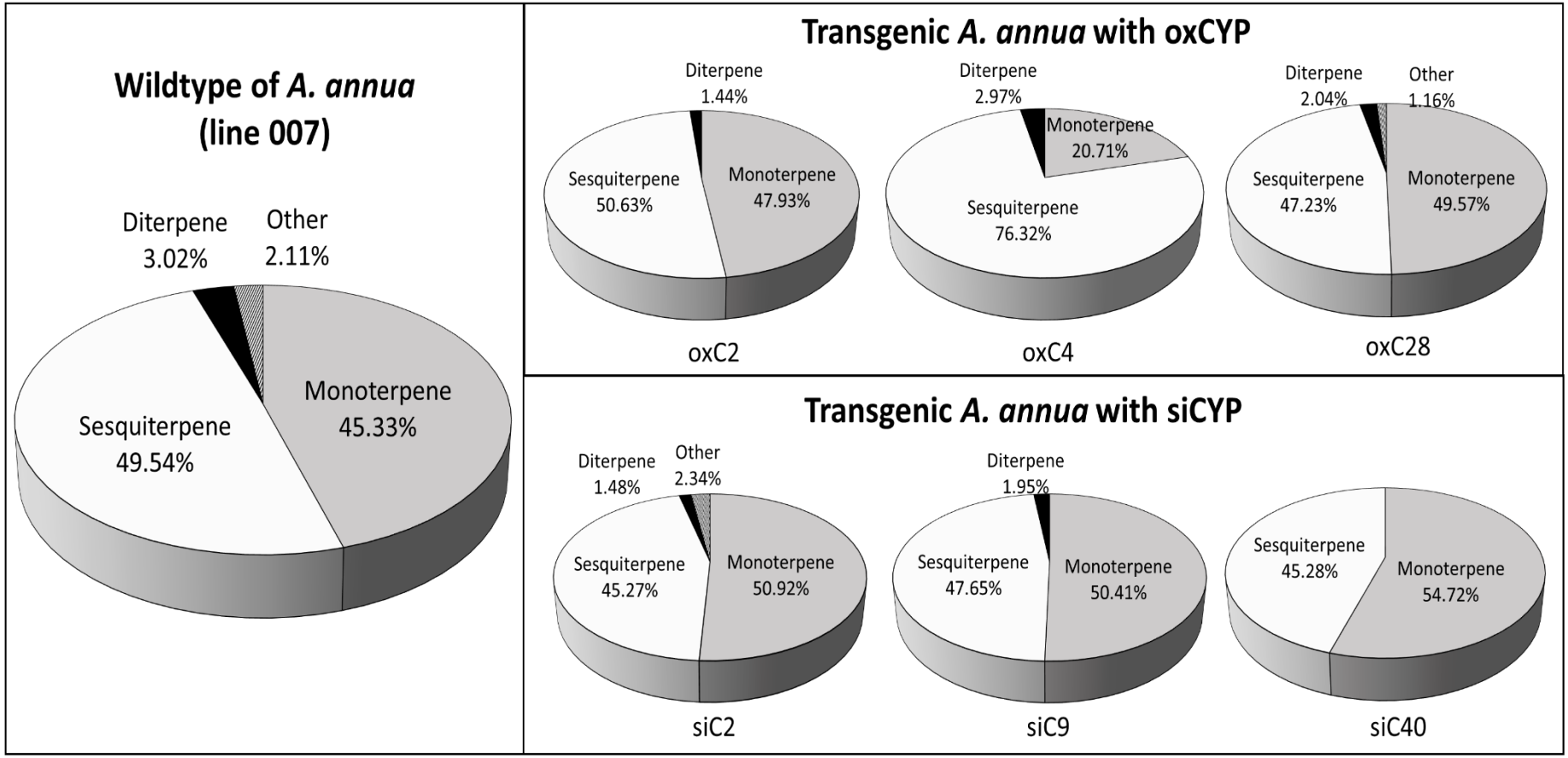
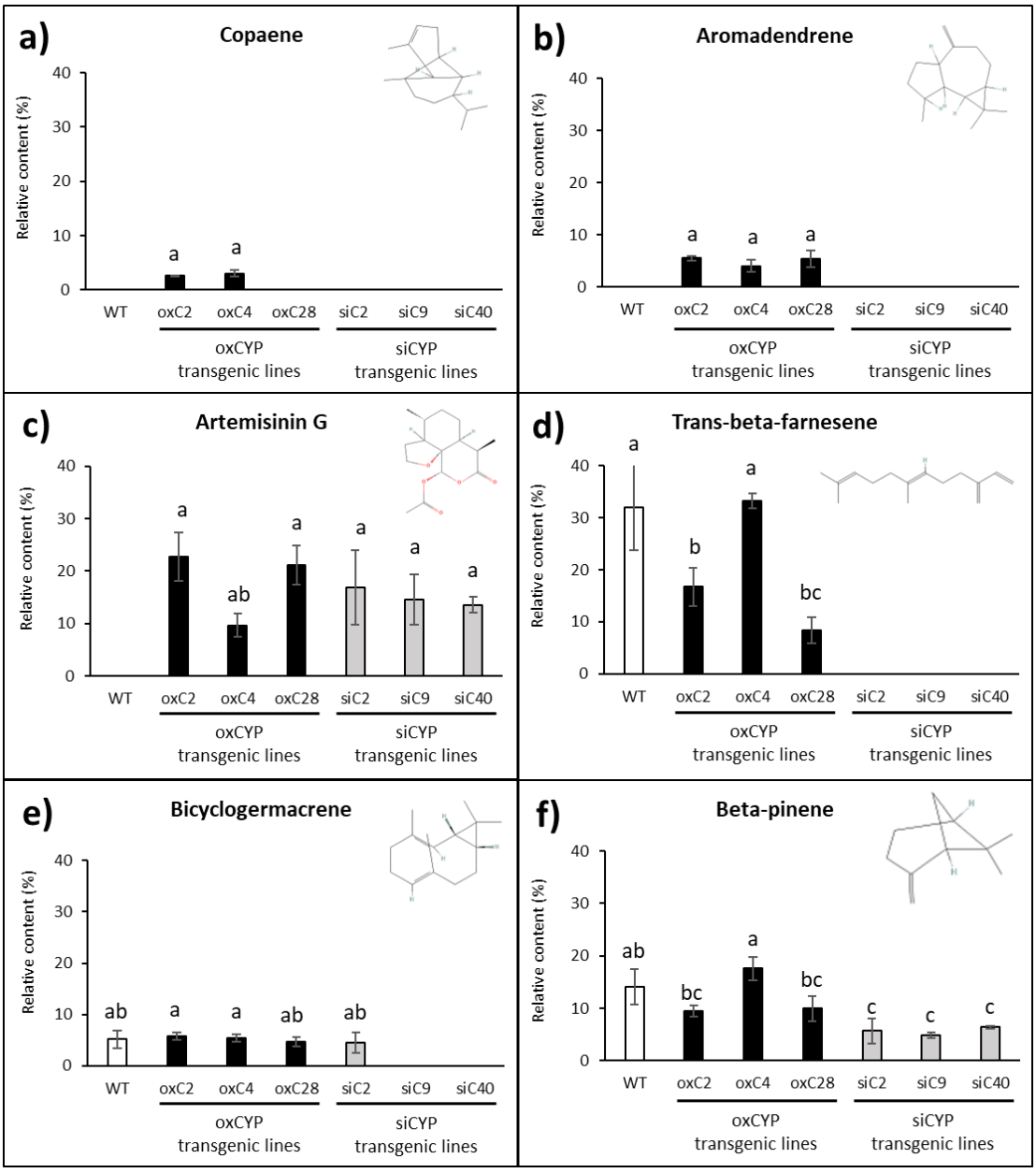


Table 3.5 Phytochemical profile of dichloromethane extracts from leaves of wild type and transgenic *A. annua* with oxCYP or siCYP expression after 2 weeks grown in plant factory with artificial lighting

Compound types	Compound name	Qual	Relative content (%)						
			WT	oxC2	oxC4	oxC28	siC2	siC9	siC40
Monoterpene (C10)	2-Pinene	95	3.96	4.55	-	4.64	3.58	-	-
	Camphene	98	11.27	16.91	-	16.66	11.79	10.48	13.82
	Beta-pinene	97	14.12	9.48	17.59	9.95	5.68	4.90	6.43
	Eucalyptol	99	23.21	19.90	24.11	14.62	13.71	11.91	11.05
	Camphor	98	66.16	128.78	-	122.09	97.24	72.54	97.41
	Endo-borneol	90	14.76	13.19	-	10.59	10.42	8.51	-
Sesquiterpene (C15)	Copaene	96	-	2.65	3.03	-	-	-	-
	Caryophyllene	99	15.66	21.53	10.58	15.03	15.35	15.90	14.42
	Trans-beta-farnesene	97	32.03	16.72	33.17	8.33	-	-	-
	Beta-selinene	98	16.93	8.14	13.18	9.46	6.92	5.64	5.58
	Germacrene D	98	45.92	59.45	47.51	46.32	35.97	41.17	40.91
	Aromadendrene	90	-	5.51	4.05	5.47	-	-	-
	Bicyclogermacrene	95	5.16	5.85	5.46	4.72	4.53	-	-
	Alpha-himachalene	81	4.24	-	3.58	4.20	-	-	-
	Dihydro-epi-deoxyarteannuin B	90	17.29	29.26	15.62	28.26	21.09	13.89	16.80
	Arteannuin I	90	-	15.83	-	12.49	11.84	-	-
	Deoxyqinghaosu	81	8.66	15.96	7.87	14.68	14.05	11.25	15.28
	Artemisinin G	83	-	22.74	9.65	21.17	16.89	14.56	13.54
Diterpene (C20)	Neophytadiene	99	8.89	5.78	5.99	7.33	4.13	4.19	-
Other	Cyclohexanone, 2,3,3-trimethyl-2-(3-methyl-1,3-butadienyl)-, (E)-	93	-	-	-	4.18	6.53	-	-
	Octadecanal	93	6.23	-	-	-	-	-	-

Wild type (WT), transgenic *A. annua* with oxCYP and siCYP expressions were examined in this study. The metabolites were determined by GC-MS and quantified by comparing with the internal standard (methyl heptadecanoate, C17). Only compounds with more than 80% match quality to the Wiley 10 no. 14 database were shown.

Figure 3.10 Variations of terpenoid contents in wild type and transgenic *A. annua* with oxCYP or siCYP expression



Different letters in each group of bars indicate a highly significant difference at $p \leq 0.01$ analyzed by Tukey's HSD test. Error bars represent standard deviation.

Chapter 4 General discussions and conclusion

Results obtained from this present study demonstrated the role of gene regulations on phenotypic responses of model plants. Heterologous and homologous expressions were conducted in two different model systems to examine morphological and metabolic changes. Dicotyledon gene involve in regulation of developmental transition and floral induction of *Arabidopsis* was ectopically expressed in monocot *Tricyrtis* sp. On the other hand, homologous expressions of genes involved in plant metabolite production were performed in dicotyledon medicinal plant, *Artemisia annua* L., using both overexpression and suppression approaches.

In Chapter 2, successful production of dwarf and early flowering transgenic plants in the monocot liliaceous ornamental plant *Tricyrtis* sp. was demonstrated by ectopic expression of floral homeotic gene LEAFY from the dicot cruciferous plant *A. thaliana* (*AtLFY*). LFY encodes a plant-specific transcription factor that regulates the transition from vegetative to reproductive stage in *A. thaliana* (Schultz and Haughn 1991; Weigel et al. 1992; Parcy et al. 1998). LFY has been ectopically expressed in several model plants and showed its crucial role in induction of dwarfism and early flowering (Liu et al. 2017; Weigel and Nilsson 1995; He et al. 2000), which are attractive traits for ornamental plants. To investigate the possibility of using *LFY* for ornamental plant molecular breeding, *AtLFY* was ectopically expressed in the liliaceous ornamental plant *Tricyrtis* sp. Nine independent transgenic plants were obtained from *Agrobacterium*-mediated gene transformation with *AtLFY*. All of them exhibited dwarf phenotypes when compared to the vector control. According to the degree of dwarfism, these transgenic plants were classified into three types: an extremely dwarf phenotype with smaller leaves (Type I); moderately dwarf phenotypes (Type II); and slightly dwarf phenotypes (Type III). Type III transgenic plants and vector control plants produced 1-4 apical flower buds, whereas Type I and Type II transgenic plants produced only one apical flower bud. Moreover, all of the transgenic plants produced flower buds 1–3 weeks earlier than the vector control. However, Type I and Type II transgenic plants produced non-fully opened flowers. Interestingly, level of *At*LFY expression was found to be correlated with the degree of dwarfism in this study, suggesting that the

morphological changes observed in the transgenic plants were caused by ectopic expression of *AtLFY*. In addition to dwarf and early flowering, high levels of LFY also induced abnormal flowers in Type I and Type II transgenic plant. These could be explained by caused by a strong activity of the CaMV35S promotor (Otani et al. 2013). Therefore, efficient production of dwarf transgenic *Tricyrtis* sp. plants with a normal number of fully opened flowers may be possible by using weaker or tissue-specific promoters (Sakamoto et al. 2003). Lower levels of LFY ectopic expression, thus, may be beneficial in the production of dwarf and early flowering ornamental plants.

Although heterologous expression of *AtLFY* induced morphological changes in transgenic *Tricyrtis* sp, the phenotypic response varied depending on gene expression levels, which were also affected by either genetic or environmental factors. To investigate the phenotypic responses in homologous host, manipulation of genes involved in the metabolic pathway was performed in medicinal plants.

Chapter 3 described homologous expressions of artemisinin biosynthetic gene in medicinal plant, *Artemisia annua* L, which produces artemisinin and various useful compounds (Bhakuni et al. 2001; Czechowski et al. 2018). Synergistic role of some terpenoid compounds and artemisinin was reported to promote bioactivity of *A. annua* extracts (Karaket et al. 2014). Although homologous overexpression of genes in artemisinin biosynthesis has been extensively studied (Banyai et al. 2010; Nafis et al. 2011; Ma et al. 2015; Shi et al. 2017), most reports emphasized on production of high artemisinin-producing lines. The role of gene in other biochemical production remains limited. In order to investigate the impact of key enzyme genes on biochemical production, three genes at intermediate formation and cyclization of artemisinin backbones (*FPS, ADS* and *CYP71AV1*) were examined. The experiments were conducted in both overexpression and suppression approaches. Unfortunately, only RNAi vector with *CYP71AV1* was successfully produced. The role of *CYP71AV1* in the biosynthesis of *A. annua* metabolites was further investigated. The siCYP plasmid was subsequently expressed in *A. annua* and yielded three independent transgenic plants. The siCYP transgenic lines were tested for metabolic fingerprints in comparison with oxCYP transgenic lines (from the previous study) and wild type. The results revealed 21 volatile compounds with more than 80% match quality to the mass spectral library.

A total of 19 constituents were determined to be terpenes, including 6 monoterpenes, 12 sesquiterpenes, and 1 diterpene. Generally, amount of metabolites in *A. annua* varies greatly depending on cultivar and developmental stage (Ferreira et al. 2005; Rana et al. 2013). High concentrations of terpenes, flavonoids, coumarin, and phenolic acids are found in apical meristem, but artemisinin is usually accumulated at floral bud formation (Towler and Weathers, 2015). The production of these compounds seems to be correlated with glandular trichomes (GSTs) (Maes et al. 2011). However, the plant materials in this study were harvested when GSTs were not fully developed, at a relatively young stage. Consequently, no complex structural compounds were found in this study. Although different plant lines produced biochemicals in various amounts and compositions, siCYP transgenic lines tended to produce less number of sesquiterpenes. Copaene, trans-β-farnesene and aromadendrene, which exerted several pharmaceutical properties, were absent in siCYP transgenic lines. In contrast, some useful compounds were present or elevated in transgenic *A. annua* with oxCYP. Surprisingly, four artemisinin derivatives were observed in this study. There were no obvious differences between transgenic lines, but these compounds were absent or expressed at a lower level in wild type, indicating alteration of *CYP71AV1* either by oxCYP or siCYP affected the production of artemisinin derivatives. In addition to the construct expressions, different insertion sites, copy number and interference from epigenetic processes may also cause variations in gene expression, resulting in diverse proportions of terpenoid types and content among transgenic lines. Phenotypic analysis was examined at immature vegetative stage of T_0 transgenic plants, where transgene instability may occur. Further studies will conduct in T_1 transgenic plant and will analyze gene expression and phenotypic parameter at different developmental stages.

In summary, this present study demonstrated expressions of dicotyledonous genes in original host or different species. Dwarf and early flowers have been successfully induced by heterologous expression of *Arabidopsis* floral homeotic gene (*AtLFY*) in transgenic *Tricyrtis* sp. plants. The results provide a possibility to use molecular breeding for producing dwarf and early flowering plants, which will be advantageous for their attractive characteristics. Variations of metabolites in *A. annua* were achieved by homologous expression of artemisinin biosynthetic gene either by overexpression or

suppression. This enables us to better understand the role of gene expression in metabolic pathway and allows us to further implement this knowledge to induce novel compounds or high-producing lines for pharmaceutical application.

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Appendix

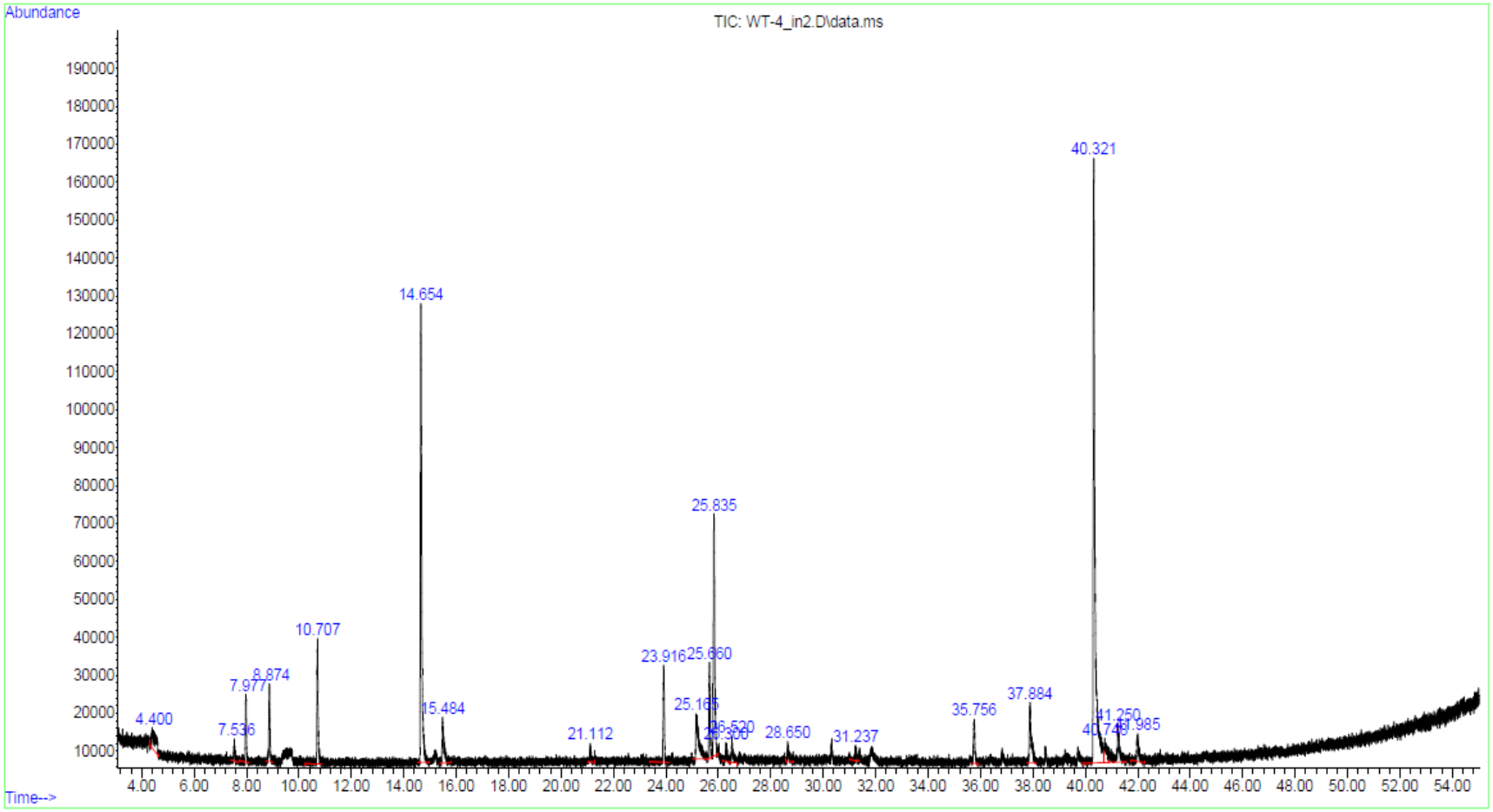

Figure 1 GC-MS chromatogram of volartile compounds detected in leaves of wild type.

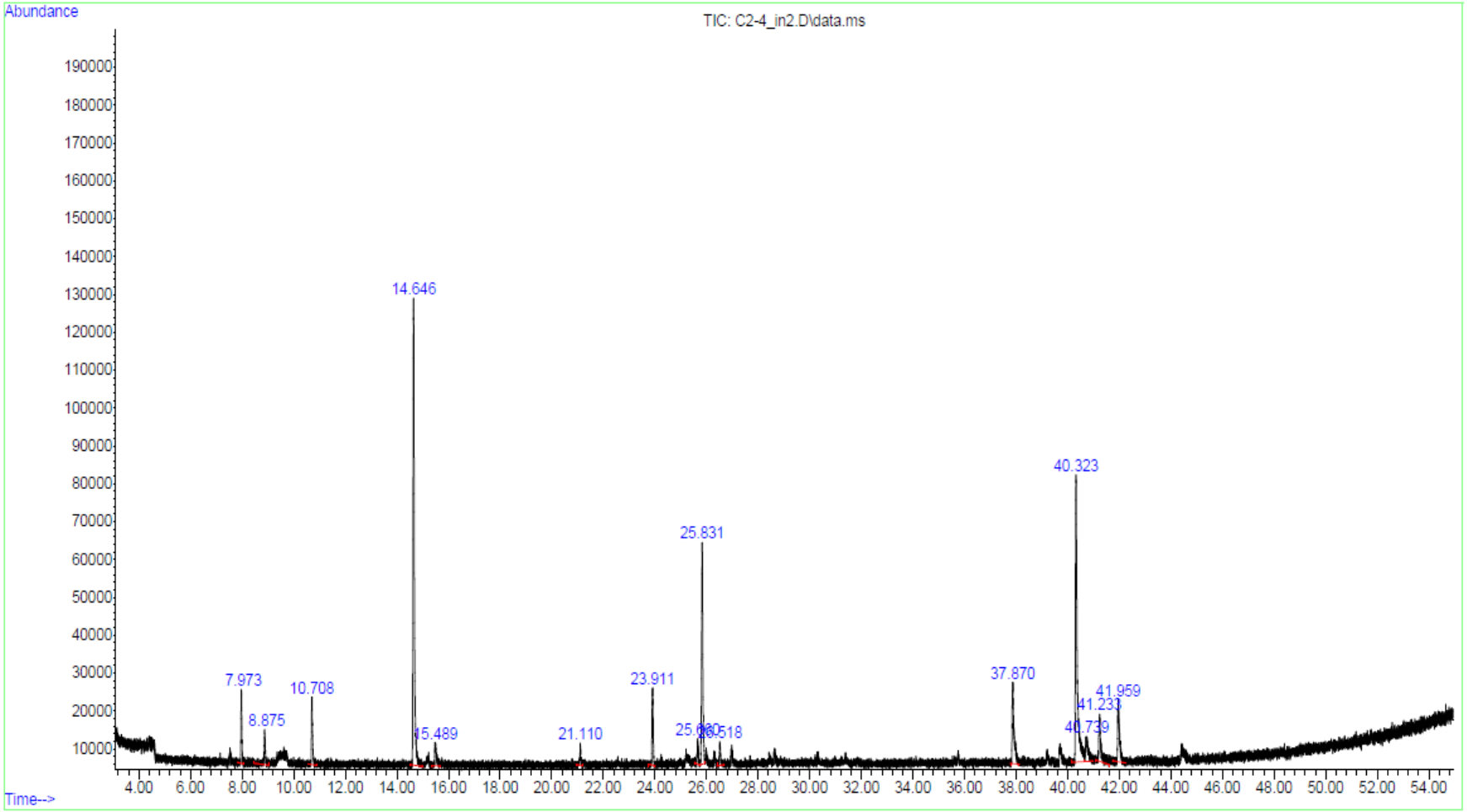

Figure 2 GC-MS chromatogram of volartile compounds detected in leaves of transgenic oxC2 plant

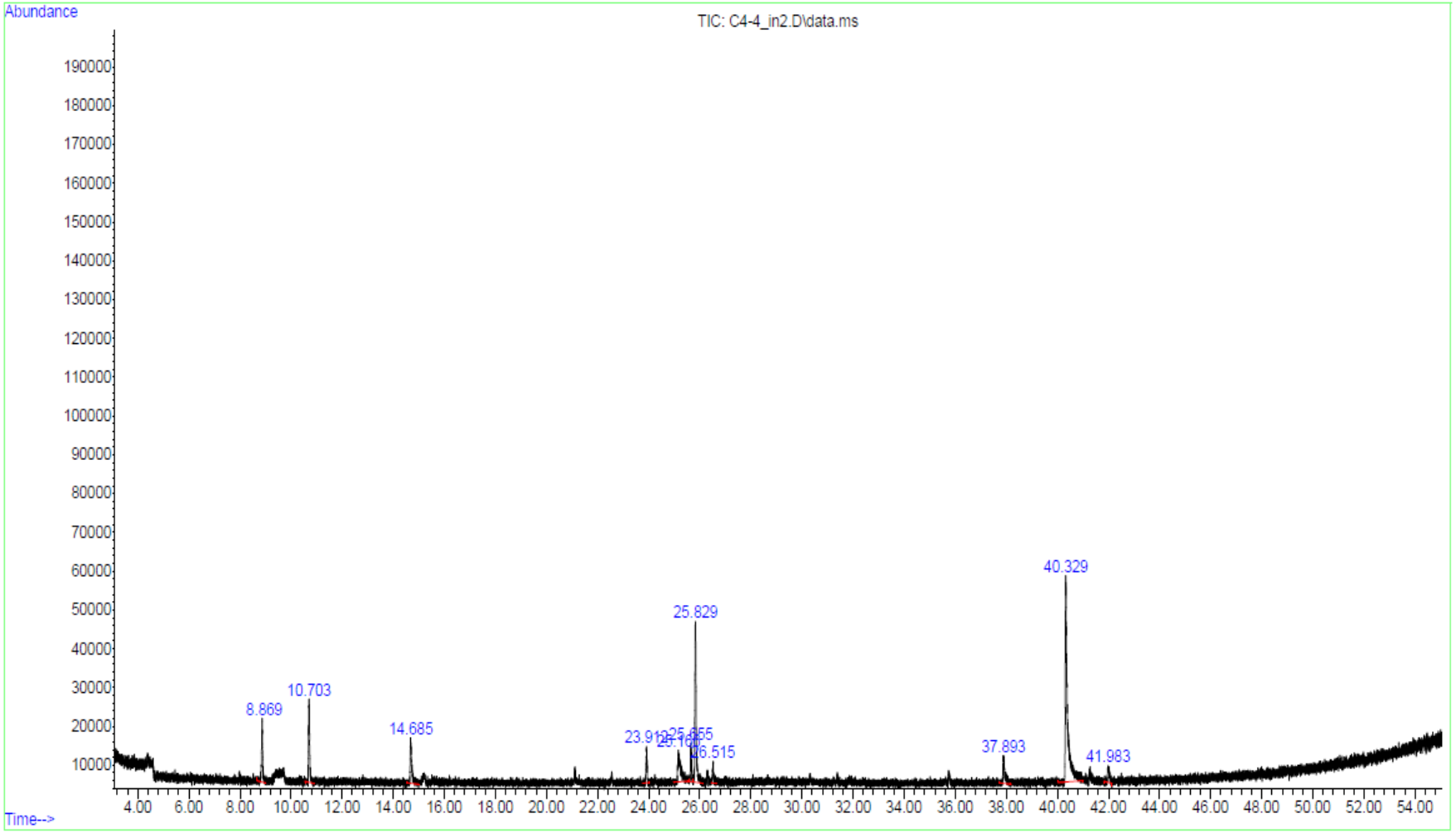

Figure 3 GC-MS chromatogram of volartile compounds detected in leaves of transgenic oxC4 plant

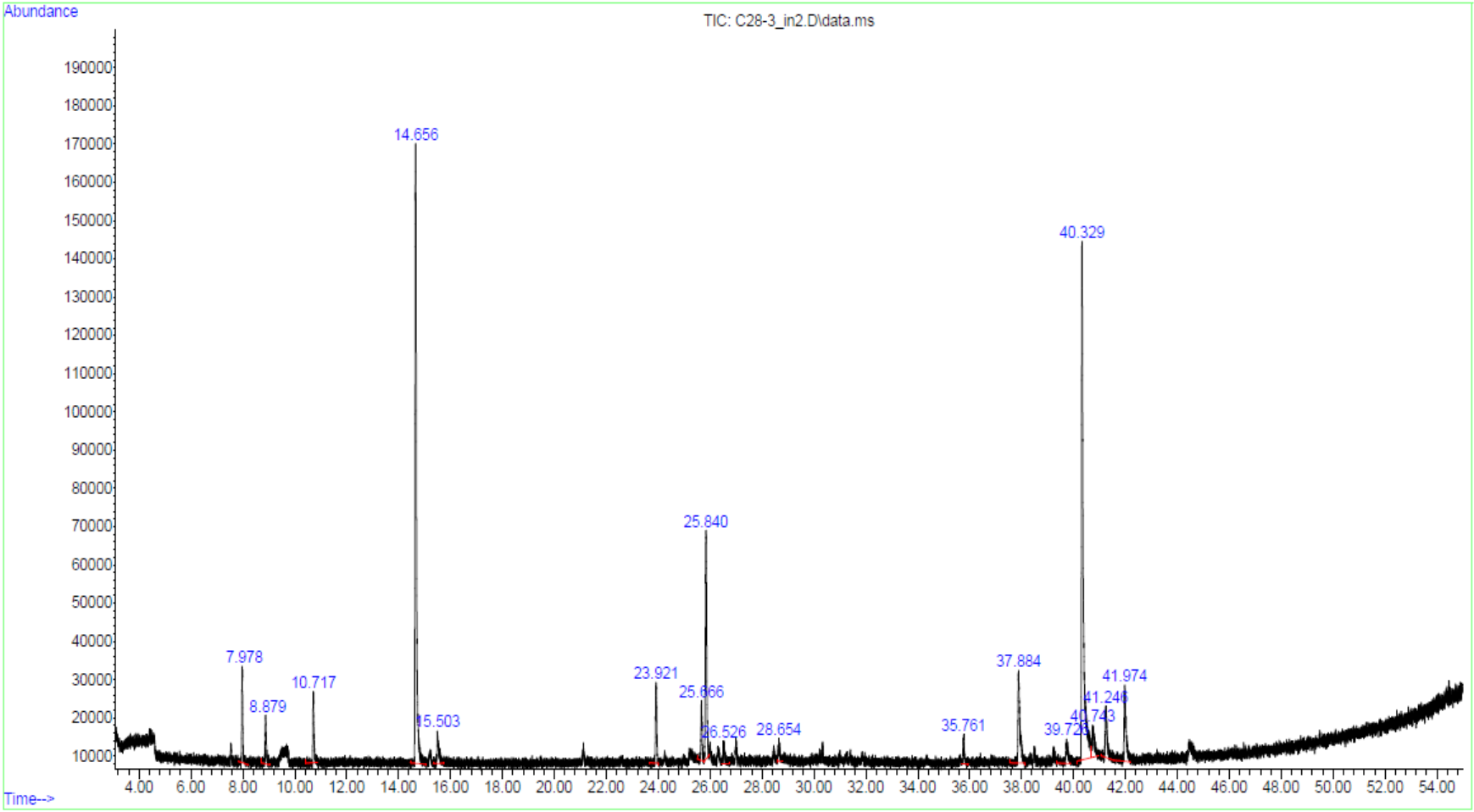

Figure 4 GC-MS chromatogram of volartile compounds detected in leaves of transgenic oxC28 plant

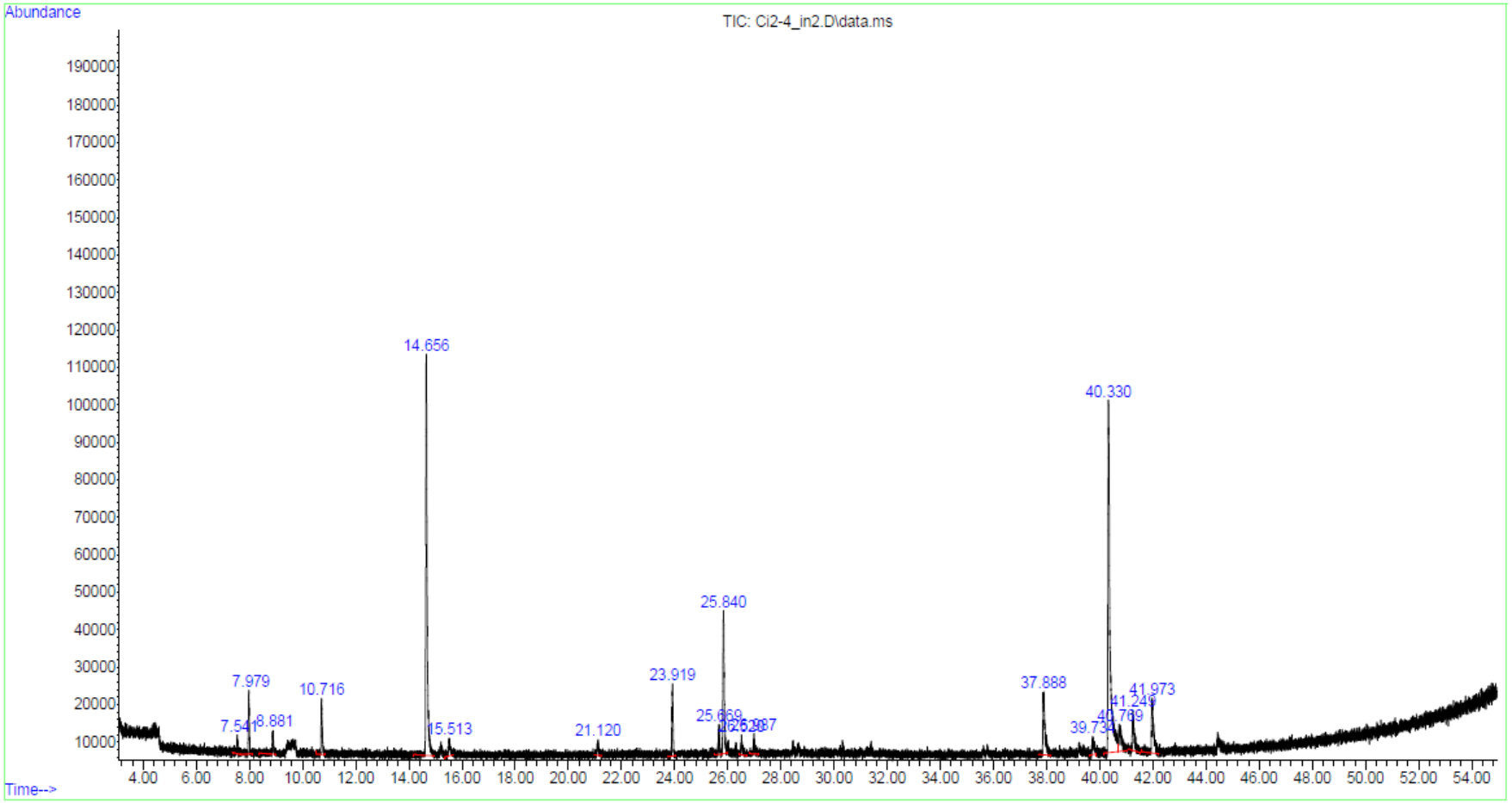

Figure 5 GC-MS chromatogram of volartile compounds detected in leaves of transgenic siC2 plant

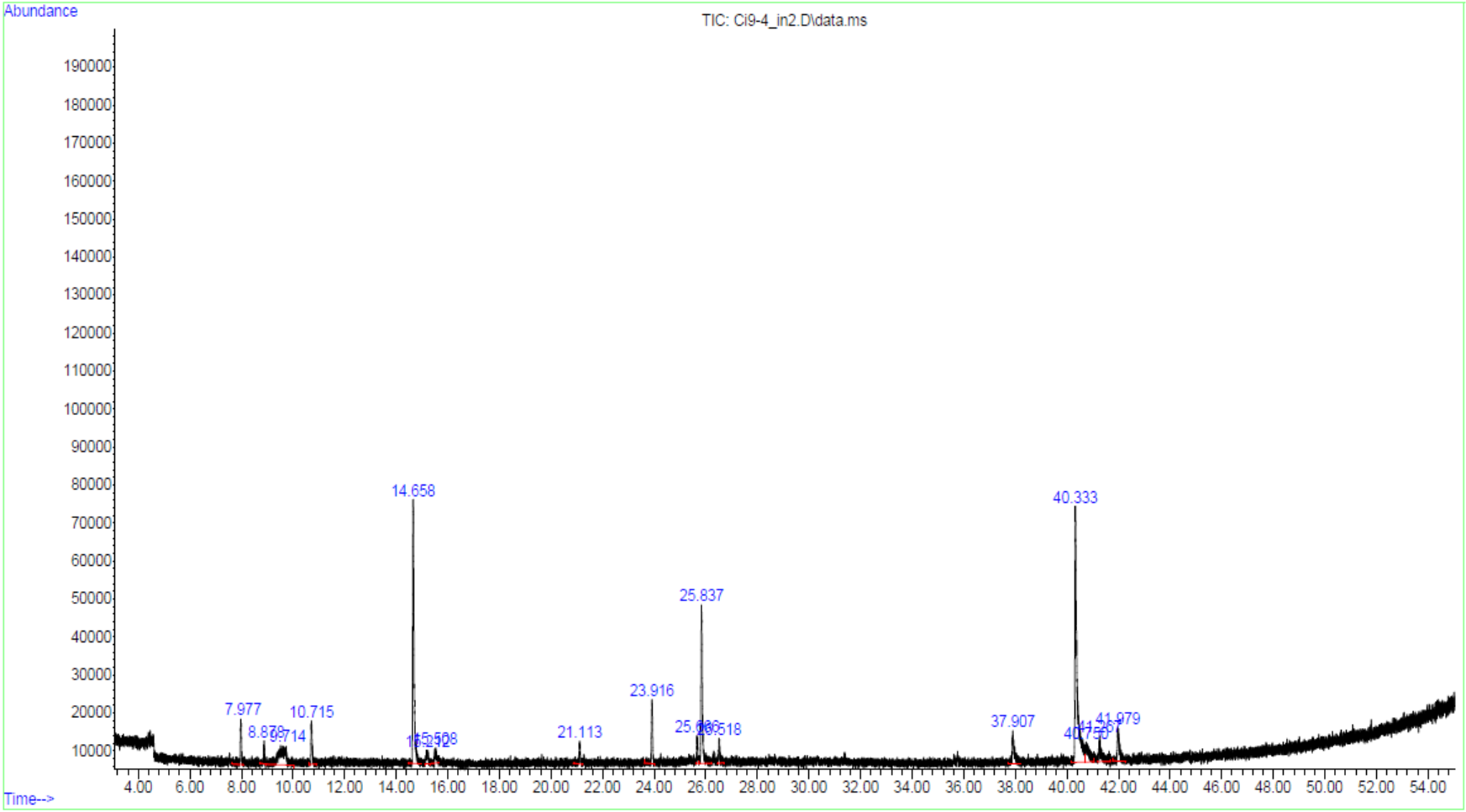

Figure 6 GC-MS chromatogram of volartile compounds detected in leaves of transgenic siC9 plant

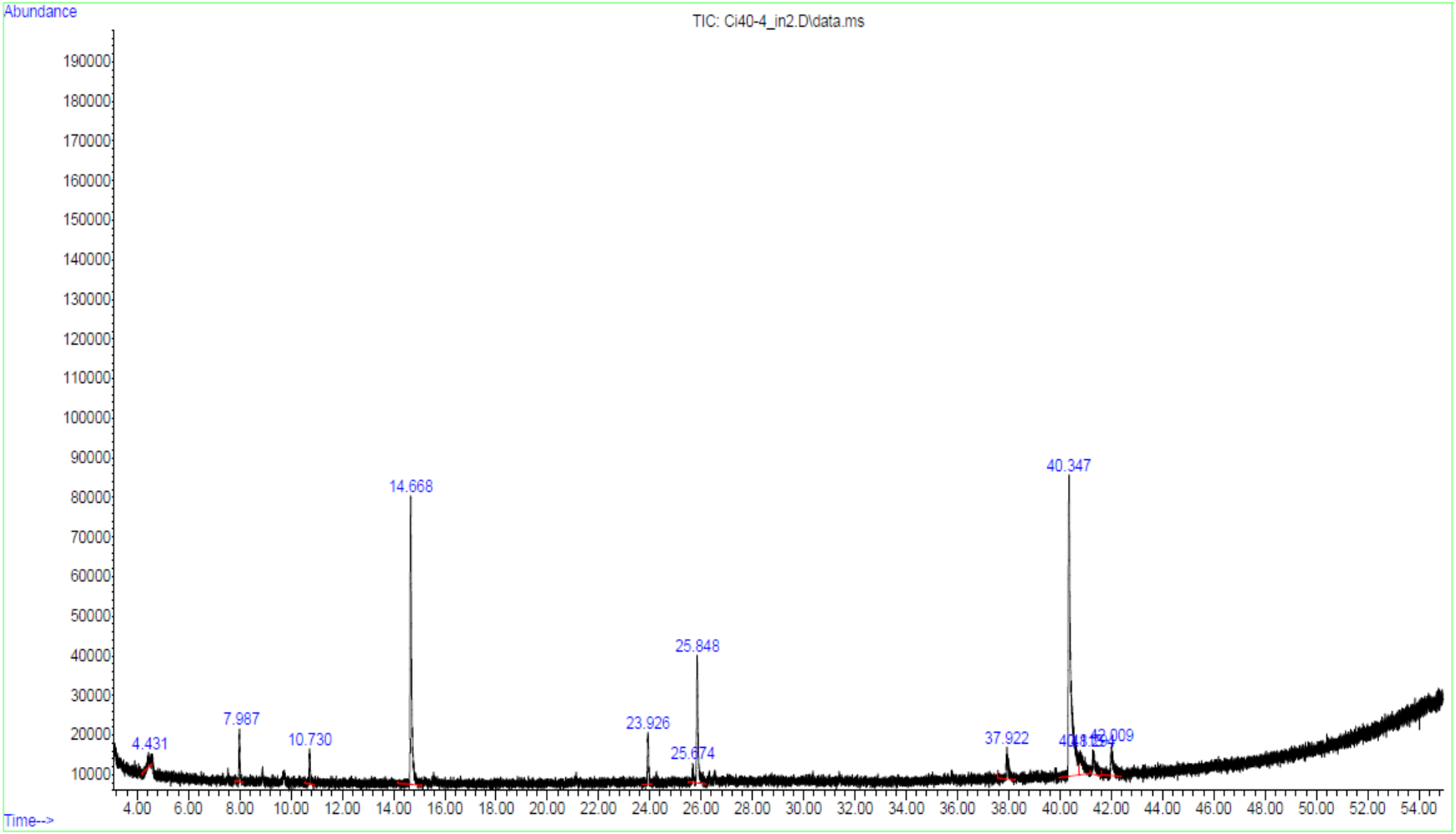

Figure 7 GC-MS chromatogram of volartile compounds detected in leaves of transgenic siC40 plant