A Dissertation for the Degree of Doctor of Philosophy

Studies on *Ginkgo biloba* (*E*)-4-Hydroxy-3-methylbut-2-enyl Diphosphate Reductase Gene and Promoter

은행 IDS 유전자와 프로모터의 기능 연구

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School of Agricultural Biotechnology

Seoul National University

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2013년 1월

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Adviser: Soo-Un Kim

A dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy to the Faculty of the School of Agricultural Biotechnology Seoul National University

By

Min-Kyoung Kang

Date Approved: _____

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OVERALL ABSTRACT

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Major in Applied Life Chemistry

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Isoprenoids, also known as terpenoids, are derived from the five-carbon building units isopentenyl diphophate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Even though they are synthesized in all living organisms, plants have more diverse and abundant isoprenoid compounds compared to others. Plants have two distinct isoprenoid biosynthetic pathways, 2-C-methyl-D-erythritol 4-phosphate (MEP) and mevalonic acid (MVA) pathways. In this study, plant IDS, the terminal enzyme in MEP pathway, is focused. *Ginkgo biloba*, one of the gymnosperm tree known as 'living fossil', has three copies of IDS genes. They are divided into two classes: GbIDS1 to class 1, and GbIDS2 and 2-1 to class 2. Each enzyme class is known to separately participate in primary and secondary metabolisms. In this research, promoter analysis and overexpression study of *GbIDS*s were performed respectively in Arabidopsis and poplar. The *GbIDS1* and *GbIDS2* promoters were fused with GUS protein and then introduced into

Arabidopsis. *GbIDS1pro*::*GUS* transformant showed GUS expression in most organs except for roots, petals, and stamina, whereas *GbIDS2pro*::*GUS* was expressed only in the young leaves, internodes where the flower and shoot branched, and notably in primary root junction. This pattern of GUS expression correlated with high transcript level of *GbIDS2* in Ginkgo roots compared to that of *GbIDS*. Methyl jasmonate (MeJA) treatment resulted in down-regulated *GbIDS1pro* activity in Arabidopsis leaves and upregulated *GbIDS2pro* activity in roots. The similar patterns of GUS activity in *GbIDS2pro:: GUS* Arabidopsis roots were also seen upon treatments of gibberellins (GA), abscisic acid (ABA), and indole butyric acid (IBA). Each of the *GbIDS1* and *GbIDS2* overexpression construct was introduced into poplars. Ten *GbIDS1* overexpression lines were obtained while no transformants were made with GbIDS2. GbIDS1 transgenic poplars were taller than wild-type (WT) BH poplars by 25% and have 2 more leaves in indoor condition 7 weeks after potting in soil. Twenty five weeks after potting in outdoor nursery, GbIDS1 plants in pot gained height by 7% compared to BH, and showed delayed winter bud formation. In addition, overexpression of *GbIDS1* gene led increase of chlorophyll and carotenoid contents by approximately 20% in transgenic poplars compared to WT poplars. Chlorophyll-related genes, *CHS* (chlorophyll synthase) and *CAO* (chlorophyll a oxidase) transcript levels were higher in transgenic poplars by 30% and 50% respectively. Transcript level analyses of GA biosynthetic genes, *KS* (kaurene synthase), *GA20ox* (gibberellin 20 oxidase), and *GA2ox* (gibberellin 2 oxidase), were performed in poplar. In this analysis, transcript levels of bioactive GA synthesis gene, *KS* and *GA20ox,* were up-regulated while GA inactivation gene, *GA2ox*, was down-regulated in transgenic poplars. In spite of signal peptide deletion, tGbIDS2 (truncated GbIDS2

devoid of signal peptide) targeted to the chloroplast. In the heterozygote Arabidopsis plants, overexpression of *tGbIDS2* was previously reported to lead rapid growth and early flowering. However, in homozygote *tGbIDS2* overexpression transgenic Arabidopsis in the current research, there were no significant phenotype changes compared to the Col-0 wild type (WT). Besides, little changes were observed in chlorophyll and carotenoids contents in transgenic and Col-0 Arabidopsis plants. On the other hand, transcript levels of floral genes and *GA4* displayed differences between WT and transgenic Arabidopsis plants. *CO* (CONSTANTS) levels were up-regulated by 60% but *FLC* (FLOWERING LOCUS C), *SOC1* (SUPPRESSOR OF OVEREXPRESSION OF CO1), and *LFY* (LEAFY) were down-regulated by 26%, 32%, and 24%, respectively. Also transcript level of *GA4* gene increased by 56% in transgenic Arabidopsis, it infers decrease of GA amounts in transgenic Arabidopsis. Through these experiments, physiological differences of each GbIDS were examined, and applicability of GbIDSs for genetic engineering was evaluated.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
CAO	Chlorophyll a oxidase
CDP-ME	4-Diphosphocytidyl-2-*C*-methyl-D-erythritol
CDP-MEP	4-Diphosphocytidyl-2-*C*-methyl-D-erythritol-2 phosphate
CHS	Chlorophyll synthase
CMEK	4-(Cytidine 5′-diphospho)-2-*C*-methyl-D- erythritol kinase
CO	CONSTANTS
ent-CPS	*ent*-copalyl diphosphate synthase
CTP	Cytidine tiphosphate
DMAPP	Dimethylallyl diphosphate
DXP	1-Deoxy-D-xylulose 5-phosphate
DXR	1-Deoxy-D-xylulose 5-phosphate reductoisomerase
DXS	1-Deoxy-D-xylulose 5-phosphate synthase
FLC	FLOWERING LOCUS C
FT	FLOWERING LOCUS T
GA	Gibberellic acid
GA2ox	Gibberellin 2 oxidase
GA20ox	Gibberellin 20 oxidase
G-3-P	D-Glycealdehyde 3-phosphate
GGPP	Geranylgeranyl diphosphate
HDR	(*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase
HDS	(*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase

HMBPP	(*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate
HMGR	3-Hydroxy-3-methylglutaryl coenzyme A reductase
IBA	Indole butyric acid
IDS	Isopentenyl diphosphate synthase
IPP	Isopentenyl pyrophosphate
ISPS	Isoprene synthase
KS	Kaurene synthase
LFY	LEAFY
LPS	Levopimaradiene synthase
ME-cPP	2-*C*-Methyl-D-erythritol 2,4-cyclodiphosphate
MECS	2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase
MECT	2-*C*-Methyl-D-erythritol-4-phosphate cytidyltransferase
MeJA	Methyl jasmonate
MEP	2-*C*-Methyl-D-erythritol 4-phosphate
MVA	Mevalonate
ORF	Open reading frame
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real time-PCR
RT-PCR	Reverse transcriptase-PCR
SA	Salycilic acid
SOC1	SUPPRESSOR OF OVEREXPRESSION OF CO1
SPL	SQUAMOSA BINDING FACTOR-LIKE
TAIL-PCR	Thermal asymmetric interlaced-PCR

LITERATURE REVIEW

1. *Ginkgo biloba*

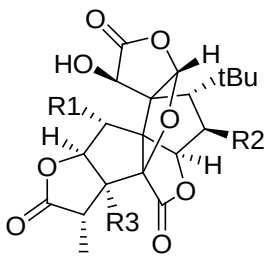
The *Ginkgo biloba* L. is a perennial deciduous tree belonging to gymnosperm. Ginkgo has no close living relatives classified in a separative division, the Ginkgophyta. Also the tree is dioecious with male and female flowers on separate plants. However, their sex can be distinguished after the tree is around 30 years old. The height of Ginkgo can reach over 30 meters and the tree survives more than 1000 years. *G. biloba* originated from China and its earliest documented site is the south region of the Yangtse River in the 11th century. It has been introduced to Europe and North America in the 18th century (Gertz and Kiefer, 2004).

The tree appeared in the Jurassic period 170 million years ago and on account of this fact is called the 'living fossil'. Long-period survival of the tree implies its resistance to bacteria, fungi, and insects and adjustability in the severe environment. Not only its long history but pharmacological uses highlights the importance of this tree (Singh *et al.*, 2008; Sierpina *et al.*, 2003). A lot of studies have discovered the medicinal efficacy of *G. biloba* extracts from leaves, roots, and barks. The standardized Ginkgo extracts EGb 761 is especially well known plant extracts containing flavonoids and terpenoids (24% flavone and 6% terpenoides). The EGb 761 has effects on cerebrovascular disease, Alzheimer's dementia, memory enhancement, intermittent claudication, tinnitus, and so on (Gertz and Kiefer, 2004; Smith

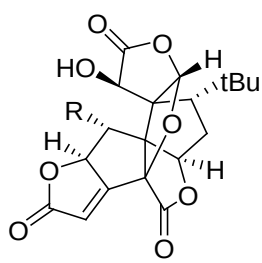
and Luo, 2004). Diverse numbers of secondary metabolites such as terpenoids, polyphenols, allyl phenols, organic acids, carbohydrates, fatty acids and lipids, inorganic salts, and amino acids were reported in *G. biloba*. Among the compounds, terpene trilactones and flavonoids are highly regarded the main bioactive elements (Mahadevan and Park, 2008).

2. Ginkgolide biosynthesis

Ginkgolides, highly modified diterpene lactones (Figure 1), possess high anti-platelet antagonist factor (PAF) activity (Mahadevan and Park, 2008) and insecticidal activity (Lee *et al.*, 2005). Biosynthesis of ginkgolides is initiateted by condensation of IPP and DMAPP, which are derived from MEP pathway. Those isoprene units are converted into diterpenoid precusor geranylgeranyl pyrophosphate (GGPP), and then levopimaradiene synthase (LPS) participates in the next step. The compound undergoes multistep reaction putatively by P450 enzymes to produce ginkgolides. Although studies on the ginkgolide biosynthesis have been performed (Figure 2), exact site of the ginkgolides biosynthesis has been under debate (Huh and Staba, 2007; Cartayrade *et al.*, 1997; Laurain *et al.*, 1997; Carrier *et al.*, 1998). However, recent studies give weight to the biosynthesis of ginkgolides in the roots and transportation to aerial parts (Kim *et al.*, 2008b; Kim *et al.*, 2012).



Ginkgolide A: R1=R2=H, R3=OH
Ginkgolide B: R1=R3=OH, R2=H
Ginkgolide C: R1=R2=R3=OH
Ginkgolide J: R1=H,R2=R3=OH
Ginkgolide M: R1=R2=OH, R3=H



Ginkgolide K: R=OH
Ginkgolide L: R=H

Figure 1. Structure of ginkgolides.

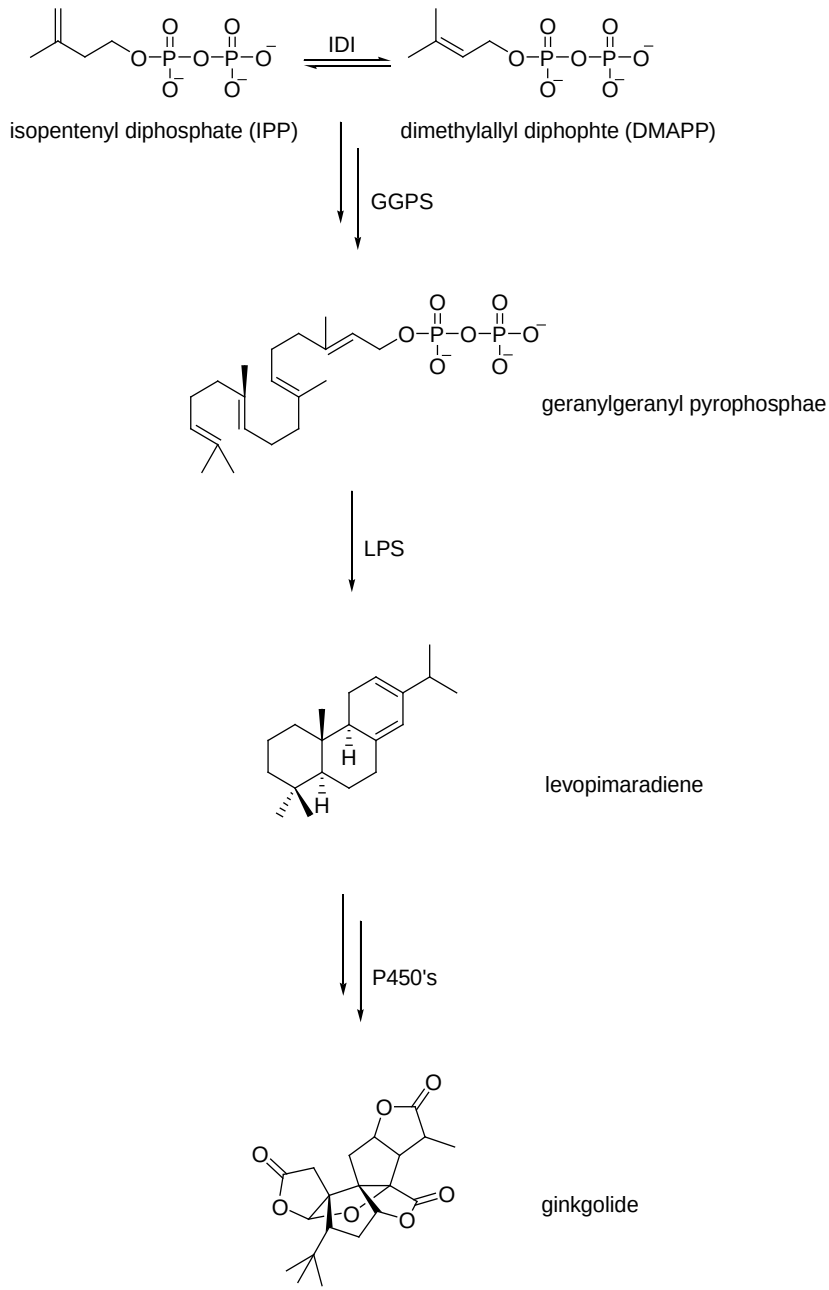


Figure 2. Ginkgolide biosynthesis.

3. Isoprenoid biosynthesis

Isoprenoids, also known as terpenoids, are the most structurally diverse group of natural products. Numbers of terpenois have various functions and also play crucial roles in living organisms. Primarily, they are involved in the membrane structure (hopanoids and sterols), redox reactions (ubiquinone, menaquinone, plastoquinone, and phylloquinone), light harvesting and photoprotection (carotenoids and chlorophylls), and regulation of growth and development (steroid hormones, gibberellins, and cytokines). In addition, their secondary metabolites participate in protection against herbivores and pathogens, attraction of pollinators and seed-dispersing animals, and allelopathy.

Although they have complicated structure and functions, terpenoids are biosynthetically polymers of simple five-carbon isoprene units, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Both IPP and DMAPP are synthesized by two different pathways, mevalonic acid (MVA) and 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathways. Every living organisms uses one or two of the isoprenoid pathways. Archaea, fungi, and animals use MVA pathway and some protozoa and most eubacteria utilize MEP pathway to produce terpenoids. In contrast to other living organisms, plants have two isoprenoid pathways in separated compartments, MVA pathway in cytosol and MEP pathway in plastid (Figure 3). Both pathways differ in not only site of operation and raw materials but also post-pathway end products. In general, MVA pathway only supplies IPP, which

is subsequently converted into DMAPP by isopentenyl diphosphate isomerase (IDI) for sesqui- and triterpenes, whereas plastidic MEP pathway provides IPP and DMAPP for mono-, di-, and triterpenes (Lange *et al.*, 2000).

4. MEP pathway in plants

The plants have two distinct biosynthetic isoprenoid pathways, MVA pathway in cytosol and plastidial MEP pathway. And the existence of MEP pathway was first verified by labeling experiments in bacteria (Rohmer *et al.*, 1993). The isoprenoids produced *via* MEP pathway are considered as typical and exclusive plastidial isoprenoids. Especially, the secondary isoprenoid metabolites have been exploited as commercial products such as drugs, polymer, flavors, and so on. Even though the MEP pathway has great potentials for engineering to produce secondary isoprenoid metabolites, enough information about the enzymes participating in this pathway is not available until recently.

In MEP pathway, seven enzymes are involved to produce the precursors of the MEP pathway, IPP and DMAPP (Figure 4). The first step of the MEP pathway is catalyzed by DXP synthase (DXS) (Estévez *et al.*, 2001). In the next step, DXP is reductively isomerized to produce 2-*C*-methyl-D-erythritol 4-phosphate (MEP) by DXP reductoisomerase (DXR) (Carretero-Paulet *et al.*, 2002). MEP cytidyltransferase (MCT) converts MEP into 4-(cytidine 5’-diphospho)-2-C-methyl-D-erythritol (CDP-ME) in the presence

of CTP by transferring a cytidilyl moiety to MEP. Subsequently, CDP-ME is phosphorylated into 4-(cytidine 5'-diphospho)-2-*C*-methyl-D-erythritol 2-phosphate (CDP-MEP) by CDP-ME kinase (CMK) in the presence of ATP. CDP-MEP is then converted into 2-*C*- methyl-D-erythritol 2,4-cyclodiphosphate (ME-cPP) by ME-cPP synthase (MCS) through the elimination of CMP. In the last two steps, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) is synthesized and reduced by HMBPP synthase (HDS) and HMBPP reductase (IDS), respectively, in the presence of NADH, FAD, and a divalent cation such as Co^{2+}.

Interestingly, the genes encoding key enzymes in the MEP pathway have been revealed in many plants as multi copy for distinguished spatial and temporal expression (Cordoba *et al.*, 2009; Jung *et al.*, 2008; Kim *et al.*, 2009; Kim *et al.*, 2006c; Phillips *et al.*, 2007; Wiberley *et al.*, 2009; Seetang-Nun *et al.*, 2008).

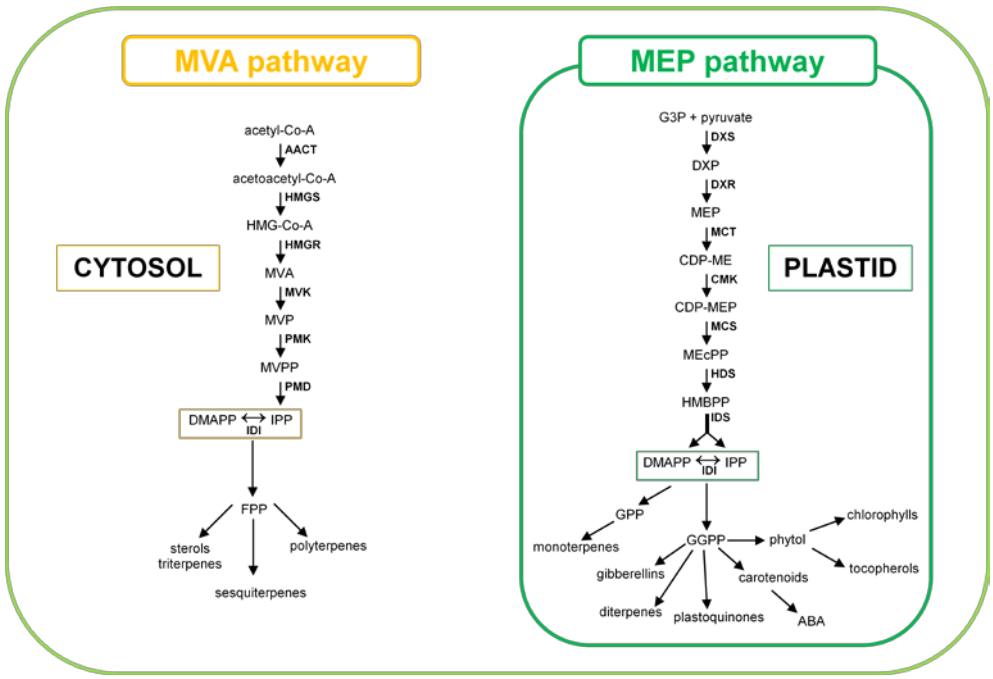


Figure 3. Isoprenoid biosynthesis in plants.

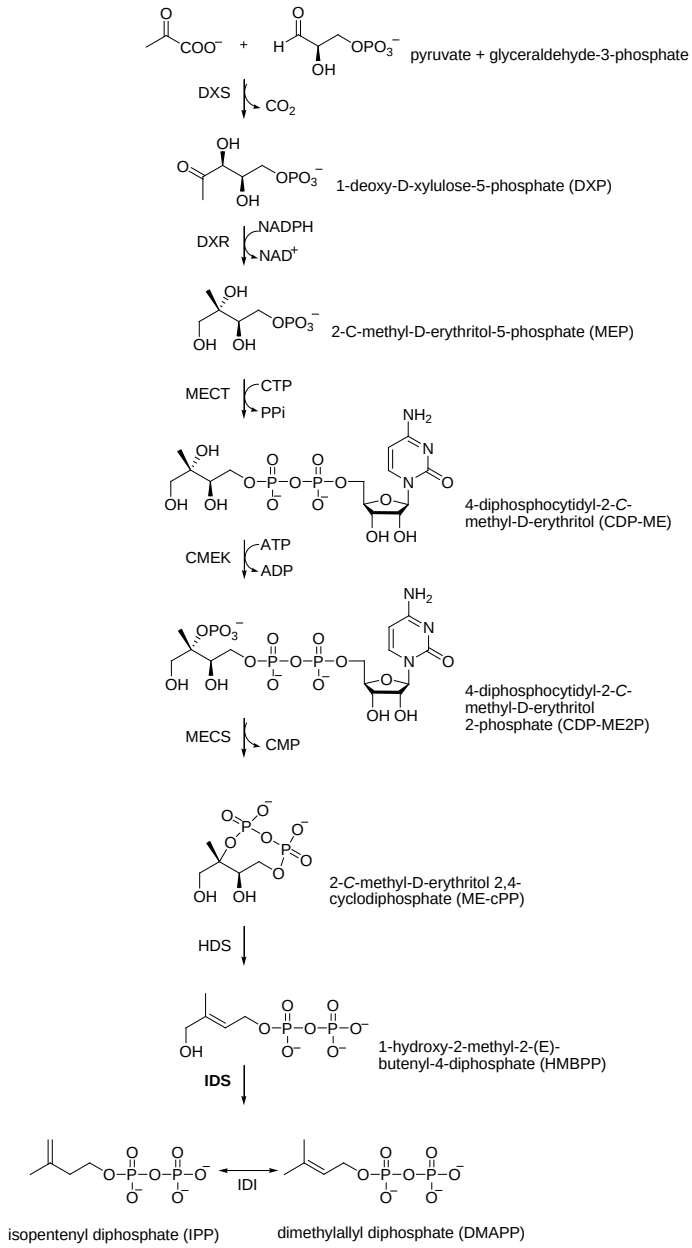


Figure 4. MEP pathway.

5. (*E*)-4-Hydroxy-3-methylbut-2-enyl Diphosphate Reductase/Isopentenyl Diphosphate Synthase (HDR/IDS) in plants

The last enzyme in the MEP pathway, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase/isopentenyl diphosphate synthase (HDR/IDS) produces IPP and DMAPP concomitantly (Lange *et al.*, 2000). In addition, IDSs from gymnosperms such as *Ginkgo biloba*, *Pinus taeda* (Kim *et al.*, 2008b), *Pinus densiflora* (Enjuto *et al.*, 1995), and *Cycas revoluta* (Kim *et al.*, 2008b) are encoded by isogenes. Through the phylogenetic analysis and molecular works, Kim et al. (Kim *et al.*, 2008b) classified gymnosperm IDSs into two subclades: *IDS1* and *IDS2* (Figure 5). *Oryza sativa* also harbors two copies of *IDS* (Jung *et al.*, 2008). As expected from their multi-copy nature, IDS genes in gymnosperms exhibited tissue-specific transcription pattern and showed different responses upon MeJA treatment (Kim *et al.*, 2008b; Kim *et al.*, 2009). However, physiological role and regulatory mechanism of each *IDS* isogene are yet to be elucidated. Ginkgo harbors 3 copies of *IDS* gene, designated as *GbIDS1*, *GbIDS2*, and *GbIDS2-1*. GbIDS1 and GbIDS2 were suggested to function in primary and secondary metabolisms, respectively, based on occurrence of isogene transcript among particular Ginkgo organs (Kim *et al.*, 2008b).

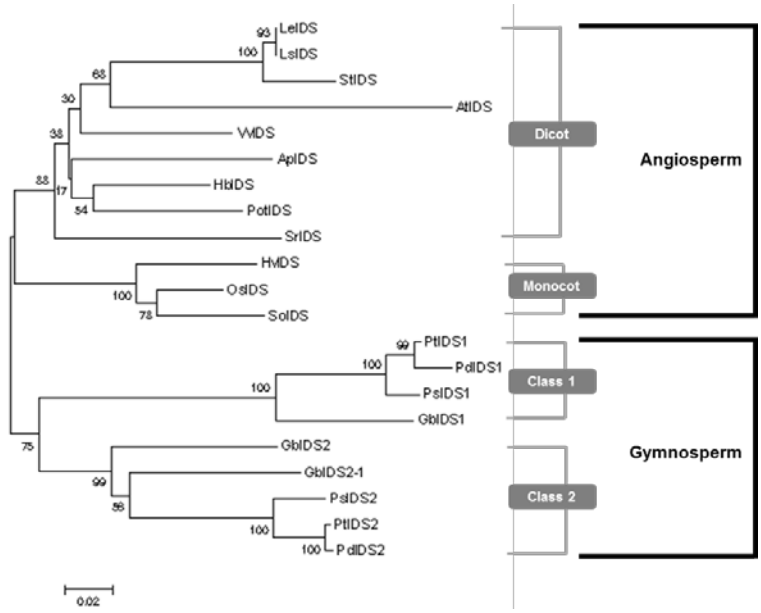


Figure 5. Phylogenetic tree of plant IDSs. AtIDS (*Arabidopsis thaliana*, AAW82381), OsIDS (*Oryza sativa*, AAT77894), ApIDS (*Anthemis palaestina*, AAG21984), StIDS (*Symphytum tuberosum*, ABB55395), SrIDS (*Stevia rebaudiana*, ABB88836), LeIDS (*Lycopersicon esculentum*, TC124188), VvIDS (*Vitis vnifera*, TC32365), LsIDS (*L. sativa*, TC12240), HvIDS (*Hordeum vulgare*, TC109922), SoIDS (*Saccharum officinarum*, TC14752), HbIDS (*Hevea brasiliensis*, AB294708), PotIDS (*Populus trichocarpa*, EU693025), GbIDS1 (*Ginkgo biloba*, DQ251631), GbIDS2-1 (*G. biloba*, DQ251632), GbIDS2 (*G. biloba*, DQ252633), PtIDS1 (*Pinus taeda*, EF095154), PtIDS2 (*P. taeda*, EF095155), PdIDS1 (*Pinus densiflora*, EU439296), and PdIDS2 (*P. densiflora*, EU439297). The TC numbers were obtained from The Institute for Genomic Research (TIGR) through the TIGR Gene Indices Clustering Tools (http://www.tigr.org./tdb/tgi/software/). The phylogenetic tree was constructed using neighbor joining algorithm in MEGA 5.0 software.

6. GA biosynthesis

Gibberellins (GAs) are a group of diterpenoid carboxylic acids synthesized from GGPP, which is produced *via* MEP pathway. Bioactive GAs regulate plant growth and development, including seed germination, stem elongation, leaf expansion, and flower and seed development (Hedden, 2012). GA biosynthesis process is consisted of three stages, the hydrocarbon *ent*-kaurene formation, oxidation by cytochrome P450 monooxygenases (P450s) to GA12, and the active hormone formation by 2-oxoglutarate-dependent dioxygenases (2-ODDs) (Figure 6). The formations of *ent*-kaurene from GGPP are catalyzed by *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS). In higher plants, biosynthesis of active GAs is controlled by the balance between their rates of biosynthesis and deactivation. The GA20ox and GA3ox genes act as key enzymes for bioactive GAs synthesis, whereas GA2ox is the major GA inactivation enzyme (Yamaguchi, 2008).

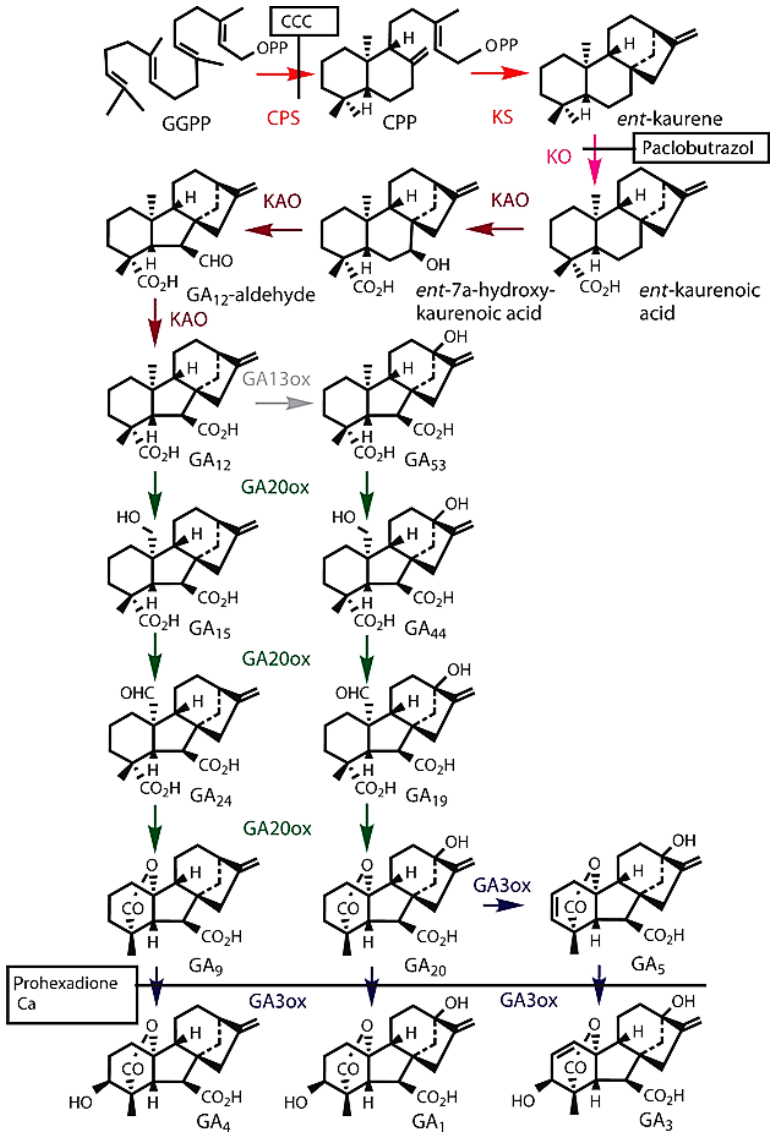


Figure 6. Gibberellin biosynthetic pathway (Hedden, 2012).

7. Flowering in Arabidopsis

In plants, appropriate timing of flowering is vital for reproductive success. The four major pathways are known to be involved in regulatory network: long-day, autonomous, vernalization (a long period of cold for flowering), and gibberellin-dependent pathways (Figure 7) (Roux *et al.*, 2006). These four pathways normally regulate flowering pathway integrators *FT*, *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*), and *LEAFY* (*LFY*) and the flowering time is controlled by the expression level of these integrators (Blazquez and Weigel, 2000; Onouchi *et al.*, 2000; Lee and Lee, 2010). Those integrators are regulated is by two antagonistic flowering regulators, *CONSTANS* (*CO*), encoding a zinc finger protein, and *FLOWERING LOCUS C* (*FLC*), encoding a MADS box transcription factor (Putterill *et al.*, 1995; Samach *et al.*, 2000). *CO*, mediating the long-day pathway, acts as a positive regulator, whereas *FLC*, mediating the autonomous/vernalization pathway, acts as a negative regulator of flowering. SOC1, encoding a MADS box transcription factor, integrates multiple flowering signals derived from photoperiod, temperature, hormone, and age-related signals (Figure 8). Among of four factors of SOC1 activity, SPL (SQUAMOSA BINDING FACTOR-LIKE) transcription factors are involved in age-dependent regulation. SPL transcription factors participate in the phase transitions, in this process they are post-transcriptionally silenced by miR156 so that juvenile growth is promoted. The SPLs act as a positive flowering regulator whereas overexpression of miR156 led the late

flowering by repressing the SPL activity (Albani and Coupland, 2010). One of the floral integrator, LFY is activated by SOC1 and AGL24, which directly bind to the LFY promoter (Lee *et al.*, 2008).

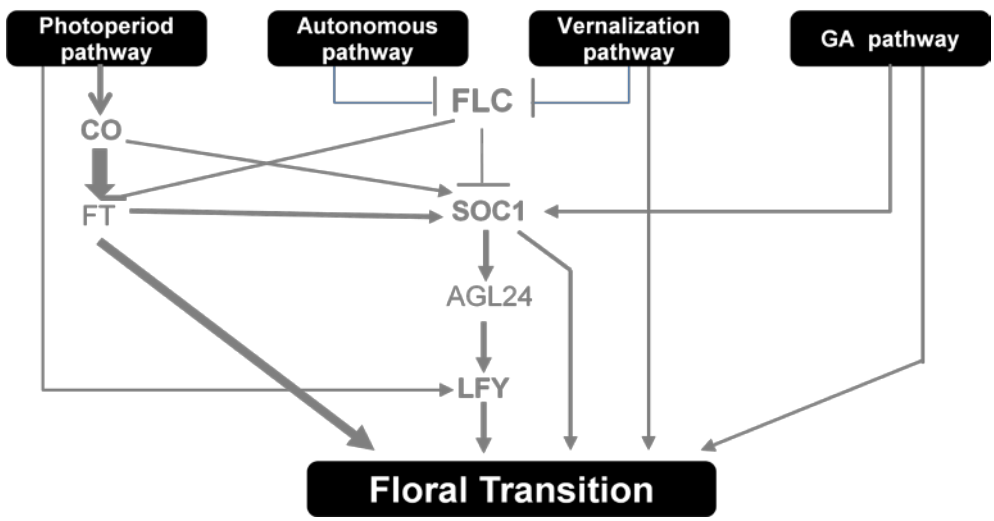


Figure 7. Interaction of flowering time genetic pathways.

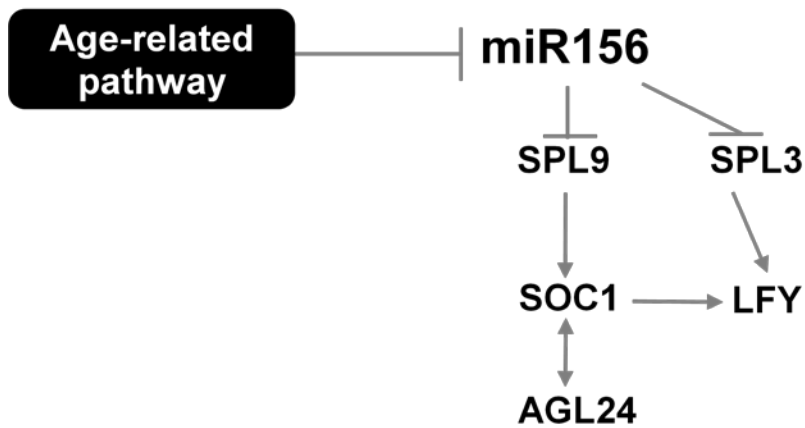


Figure 8. A model for the regulation of flowering by miR156 in Arabidopsis.

PART I

Distinct Expression Patterns of Two *Ginkgo biloba* (*E*)-4-Hydroxy-3-methylbut-2-enyl Diphosphate Reductase/Isopentenyl Diphosphate Synthase (HDR/IDS) Promoters in Arabidopsis Model

1.1. ABSTRACT

(*E*)-4-Hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR) or isopentenyl diphosphate synthase (IDS) is an enzyme at the final step of the MEP pathway. The multi-copy nature of *IDS* gene in a gymnosperm *Ginkgo biloba* is known. To evaluate the function of each isogene, the roles of the promoters were examined in Arabidopsis model. Among the promoters of *GbIDS* series, about 1.3 kb of *GbIDS1pro* and 1.5 kb of *GbIDS2pro* were cloned and fused with *GUS*. The *GbIDS1pro*::*GUS* was introduced into Arabidopsis to show GUS expression in most organs except for roots, petals, and stamina, whereas the *GbIDS2pro*::*GUS* was expressed only in the young leaves, internodes where the flower and shoot branched, and notably in primary root junction. This pattern of GUS expression correlated with high transcript level of *GbIDS2* compared to that of *GbIDS1* in Ginkgo roots. Methyl jasmonate (MeJA) treatment resulted in down-regulated *GbIDS1pro* activity in Arabidopsis leaves and upregulated *GbIDS2pro* activity in roots. The same pattern of gene regulation in roots was also seen upon treatments of gibberellins (GA), abscisic acid (ABA), and indole butyric acid (IBA).

Keywords

Ginkgo biloba, Ginkgolides, Isoprenoid, (*E*)-4-Hydroxy-3-methylbut-2-enyl Diphosphate Reductase/Isopentenyl Diphosphate Synthase (HDR/IDS), Promoter, β-Galactosidase (GUS)

1.2. INTRODUCTION

Ginkgo biloba L., a 'living fossil' that survived from Jurassic era, is a perennial deciduous and dioecious tree with broad range of uses. It contains valuable compounds such as ginkgolides and flavonoids with pronounced pharmacological and economic importance (Singh *et al.*, 2008; Sierpina *et al.*, 2003). Ginkgolides, highly modified diterpene lactones, possess high anti-platelet antagonist factor activity (Mahadevan and Park, 2008) and insecticidal activity (Lee *et al.*, 2005). Although studies on the ginkgolide biosynthesis have been performed, exact site of the ginkgolides biosynthesis has been under debate (Roux *et al.*, 2006; Huh and Staba, 2007; Laurain *et al.*, 1997; Rohmer *et al.*, 1993). However, recent studies give weight to the biosynthesis of ginkgolides in the roots and transportation to aerial parts (Kim *et al.*, 2012; Kim *et al.*, 2008b).

Terpenoids are the most structurally diverse group of plant natural products. Although they have complicated structure and functions, terpenoids are biosynthetically polymers of simple five-carbon isoprene units, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). The plants have two isoprenoid pathways, differing how the five-carbon units are generated: classical mevalonic acid (MVA) pathway and recently elucidated 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway. Both pathways differ in not only site of operation and raw materials but also post-pathway end products; in general, MVA pathway

operating in cytosol supplies IPP, which is subsequently converted into DMAPP by isopentenyl diphosphate isomerase (IDI) for sesqui- and triterpenes, whereas plastidic MEP pathway provides IPP and DMAPP for mono-, di-, and triterpenes (Lange *et al.*, 2000).

The seven enzymes operating in the MEP pathway in *G. biloba* were all characterized (Kim *et al.*, 2008b; Kim *et al.*, 2005b; Kim *et al.*, 2006c; Kim *et al.*, 2006b; Kim *et al.*, 2008a; Kim *et al.*, 2006a; Kim and Kim, 2010). The last enzyme in the MEP pathway, (*E*)-4-hydroxy-3-mehtylbut-2-enyl diphosphate reductase/isopentenyl diphosphate synthase (HDR/IDS) produces IPP and DMAPP concomitantly (Lange *et al.*, 2000) and was reported to have multi-copy gene in gymnosperms such as *Ginkgo biloba, Pinus taeda* (Kim *et al.*, 2008b), *Pinus densiflora* (Kim *et al.*, 2009), and *Cycas revoluta* (Kim *et al.*, 2008b). Kim et al. classified gymnosperm HDR/IDSs into two clades (Sierpina *et al.*, 2003): *IDS1* and *IDS2*. *Oryza sativa* also harbors two copies of *IDS* (Jung *et al.*, 2008). As expected from their multi-copy nature, *IDS* genes in gymnosperms exhibited tissue-specific transcription pattern and showed different responses upon MeJA treatment (Kim *et al.*, 2008b; Kim *et al.*, 2009).

However, physiological role and regulatory mechanism of each *IDS* isogene are yet to be elucidated. Ginkgo harbors 3 copies of *IDS* gene, designated as *GbIDS1, GbIDS2*, and *GbIDS2-1*. GbIDS1 and GbIDS2 were suggested to function in primary and secondary metabolisms, respectively, based on occurrence of isogene transcript among particular Ginkgo organs

(Kim *et al.*, 2008b). To provide decisive evidence of organ-specificity and to suggest physiological role of each isogene, we here report isolation of 1.3 kb of *GbIDS1* and 1.5 kb of *GbIDS2* from 5'-upstream regions and the promoter-driven GUS expression in Arabidopsis model, because reliable transformation of Ginkgo is yet not available. In addition, correlation of the promoter experiment in the model plant with *IDS* transcript levels in Ginkgo organs further substantiated putative role of each isogenic *HDR/IDS*.

1.3. MATERIALS AND METHODS

1.3.1. Plants and growth condition

Arabidopsis thaliana (Columbia ecotype) seeds were sown on the 1/2 Murashige and Skoog (MS) agar medium and stored at 4ºC in the dark. After 3 days of vernalization, the plates were transferred into a 23ºC growth chamber with a 16/8h photoperiod. Dehulled *G. biloba* seeds were purchased from Sillim Market (Seoul, Korea) and sown in vermiculite. The growth conditions for the ginkgo seedlings were same as *A. thaliana* as described above and the plant was maintained for 2 months or until 5-leaf stage before extraction of RNA (Figure 1.1). *G. biloba* male and female flowers were collected from about 40 year-old trees at Seoul National University campus in Seoul, Korea.

Figure 1.1. 2-month-old Ginkgo seedlings.

1.3.2. Isolation and analysis of *GbIDS1* and *GbIDS2* promoters

The promoters of *GbIDS1* and *GbIDS2* were isolated from the genomic DNA through the thermal asymmetric interlaced PCR (TAIL-PCR) procedure as described by Liu and Huang with some modifications (Liu and Huang, 1998). The detailed program and primers of TAIL-PCR are listed in Tables 1.1 and 1.2. The isolated promoter sequences were analyzed using the Signal Scan Program PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) and PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) database.

Table 1.1. Primers used in TAIL-PCR.

Primer		Sequence
gene-specific primer	GbIDS1-1	5’-AGGTTCTGAGCTGCATACCACAGCA-3'
	GbIDS1-2	5’-CTGTTCGCAGTCCACATGGCAA-3'
	GbIDS1-3	5’-GGCCTTAGTATCAAACTCCTCTGCATCTG-3
	GbIDS2-1	5’-GTGAATGTGAAGCAAGGATGCCTGAT-3'
	GbIDS2-2	5’-CCATGGCACCGTGTATTACATACTCTCC-3'
	GbIDS2-3	5’-AAGTCTCCTTCTTGTGGCCGAACC-3'
promoter region specific primer	GbIDS1pro-1	5’-GCCACTTGGCGAGCTCTCCAA-3'
	GbIDS1pro-2	5’-CCATAATGCAAGATAGGCTCGGTCC-3'
	GbIDS1pro-3	5’-CAAATGTGTGCATTGGATGATGGAGAG-3’
	GbIDS2pro-1	5’-TTGTATACGGATACGTGTAGATAGTGGCG-3'
	GbIDS2pro-2	5’-GTGTGGTGCATAGTGTATACTCGTGGTAT-3'
	GbIDS2pro-3	5’-CGAAGTGACCAAGCTCGGAGCATC-3'
promoter primer	GbIDS1pro forward	5’-AAGCTTAGACAAATCTGCGTTGTT CA-3'
	GbIDS1pro reverse	5’-TCTAGACCAACCGGTTTATAGGAGGA-3'
	GbIDS2pro forward	5’-AAGCTTCATAAATAGTTAAAAACACCTCAA-3'
	GbIDS2pro reverse	5’-TCTAGAGGTTTCAGACAAACTAAACCCAA-3'
AD primer	(AD1)	5’-WGCNAGTNAGWANAAG-3'
	(AD2)	5’-AWGCANGNCWGANATA-3'
	(AD3)	5’-NGTCGASWGANAWGAA-3'
	(AD4)	5’-AGWGNAGWANCAWAGG-3'
	(AD5)	5’-GTNCGASWCANAWGTT-3'
	(AD6)	5’-TGWGNAGWANCASAGA-3'
	(AD7)	5’-WGTGNAGWANCANAGA-3'
	(AD8)	5’-NTCGASTWTSGWGTT-3'

Table 1.2. TAIL-PCR condition.

Reaction	Cycle no.	Thermal cycle condition
Primary	1	94°C (5 min)
	2-6	94°C (30 s), 62°C (1 min), 72°C (2.30 min)
	7	94°C (30 s), 25°C (3 min), raping to 72°C over 3 min, 72°C (2.30 min)
	8-22	94 °C (30 s), 65 °C (1 min), 72 °C (2.30 min) 94 °C (30 s), 65 °C (1 min), 72 °C (2.30 min) 94 °C (30 s), 44 °C (1 min), 72 °C (2.30 min)
	23	72 °C (5 min)
Secondary	1-12	94 °C (30 s), 65 °C (1 min), 72 °C (2 min) 94 °C (30 s), 65 °C (1 min), 72 °C (2 min) 94 °C (30 s), 45 °C (1 min), 72 °C (2 min)
	13	72 °C (5 min)
Tertiary	1-20	94 °C (30 s), 45 °C (1 min), 72 °C (2 min)
	21	72 °C (5 min)

1.3.3. Construction of plasmids and Arabidopsis transformation

Each cloned *GbIDS1* and *GbIDS2* promoters was inserted into the pBI121 vector in place of the 35S promoter (Figure 1.2). The construct was introduced into the *Agrobacterium tumefaciens* C58C1 strain using the freeze-thaw method(Weigel and Glazebrook, 2006). The transformation of *A.thaliana* was performed using floral dip method (Clough and Bent, 1998) and the transformants were selected on the 1/2 MS medium containing 50mg/L kanamycin. For the promoter function study, T3 generation *A.thaliana* transgenic plants were used.

1.3.4. Histochemical GUS assay

Transgenic Arabidopsis plants at each developmental stage were stained with 0.1% X-Gluc solution, overnight at 37ºC according to the method of Jefferson (Jefferson *et al.*, 1987). To examine the spatial patterns of GUS expression in detail, the leaf, stem, inflorescence node and roots were embedded using Spur's resin kit and sectioned on an ultramicrotome (MTX, RMC Products, USA), and were inspected under a light microscope (Axiophot, Zeiss, Germany).

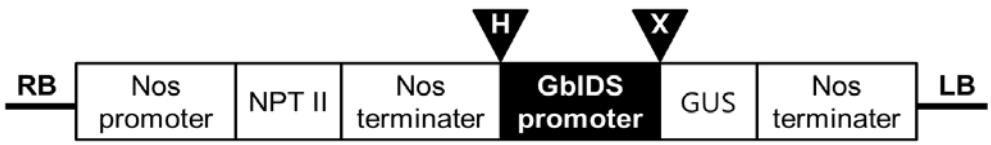


Figure 1.2. Construction of the *GbIDS1pro::GUS* and *GbIDS2pro::GUS*.

H: *Hin*dIII, X: *Xba*I

1.3.5. GUS activity assay

Each part of the transgenic *A.thaliana* plant was ground in liquid nitrogen and proteins were extracted with GUS extraction buffer (50mM phosphate buffer pH7.0, 10mM EDTA, 0.1% lauryl sarcosine, and 0.1% Triton X-100). The protein was determined by using Bradford assay, and GUS activity was measured on a fluorescence microplate reader (SPECTRAmax Gemini XS, Molecular Devices, USA) with excitation at 365 nm and emission at 455 nm. The activity as obtained in this experiment was subject to statistical analysis. Student's t-test was performed by using SAS (version 9.1). Statistical significances were denoted as * ($p < 0.05$) and ** ($p < 0.01$).

1.3.6. RNA preparation and reverse transcription

Total RNA from *G.biloba* seedlings and trees were extracted using the CTAB method (Chang *et al.*, 1993). And the concentration of each sample was measured by Nano Drop spectrometer (ACT Gene ASP-3700, USA). The one microgram of RNA was used to synthesize the first strand cDNA using the cDNA synthesis kit (Philekorea Technology, Korea) according to the manufacture's protocol.

1.3.7. Hormone treatments

Water solutions of 100 μM methyl jasmonate (MeJA), gibberellin (GA), indole butyric acid (IBA), abscisic acid (ABA), and 1 mM salycilic acid (SA) was sprayed onto Arabidopsis until droplet ran off from the plant. The

leaves and roots of the *A. thaliana* were collected 24 h after the treatment. For the quantitative real-time PCR (qRT-PCR), *G. biloba* seedlings were applied with MeJA (100 μM) and SA (1 mM) and collected as mentioned above.

1.3.8. RT-PCR and qRT-PCR

Reverse transcription-PCR (RT-PCR) analysis was performed for 30 cycles, each cycle consisted of 98ºC for 1 min, 55ºC for 5 s, 72ºC for 1 min using PrimeSTAR HS DNA Polymerase (Takara Bio, Ohtsu, Japan) and 50 ng of cDNA as a template. *GbIDS1* and *GbIDS2* transcript levels of the MeJA- and SA-treated ginkgo seedlings were determined by using qRT-PCR on Rotor-Gene 2000 (Corbett Research, Mortlake, NSW, Australia), using 50 ng of the cDNA with a QuantiMix SYBR Kit (Philekorea Technology). The *G. biloba* 18S gene served as an endogenous reference. The primers used for the PCRs were listed in Table 1.3.

Table 1.3. Primers of *G.biloba IDS* and *18S* genes.

Primer	**Forward (5'→3')**	**Reverse (5'→3')**
GbIDS1	TTTGCTGTGGTATGCAGCTC	TGCCTCACTTTCAGCCTTCT
GbIDS2	AGGGGGATTACACCTCCATC	ATCCATTAAGCTTGCCATCG
18S	ATGATAACTCGACGGATCGC	CTTGGATGTGGTAGCCGTTT

1.4. RESULTS

1.4.1. Isolation of *GbIDS1* and *GbIDS2* promoters

The promoter regions of *GbIDS1* (1269 bp, Genbank JX444962) and *GbIDS2* (1480 bp, JX444961) were isolated from the *G. biloba* genomic DNA (Figures 1.3 and 1.4). The predicted *cis*-acting regulatory elements from the *GbIDS* promoter sequences contained candidate sequences towards various biotic and abiotic stresses as listed in Tables 1.4 and 1.5. Putative TATA box of *GbIDS1pro* and *GbIDS2pro*, placed at −108 bp and −73 bp, respectively, and other regulatory elements predicted by PlantCARE analysis are listed in Figures 1.3 and 1.4.

Figure 1.3. Complete sequence of the *GbIDS1* promoter region. The *cis*-acting elements in the *GbIDS1* promoter are underlined. Sequence analysis was performed by Plant CARE.

Figure 1.4. Complete sequence of the *GbIDS2* promoter region. The *cis*-acting elements in the *GbIDS2* promoter are underlined. Sequence analysis was performed by Plant CARE.

Table 1.4. The putative cis-acting elements in the *GbIDS1* promoter sequence. Analysis was performed by PLACE.

Function	Element	Elem. Seq.	Freq.
ABA	ABRELATERD1	ACGTG	1
	LTRECOREATCOR15	CCGAC	1
	MYB1AT	WAACCA	5
	MYB2CONSENSUSAT	YAACKG	1
	MYCATRD22	CACATG	2
	OSE2ROOTNODULE	CTCTT	2
GA	GAREAT	TAACAAR	5
	MYBGAHV	TAACAAA	4
	TATCCACHVAL21	TATCCAC	1
	TATCCAOSAMY	TATCCA	4
	WRKY71OS	TGAC	5
	PYRIMIDINEBOXOSRAMY1A	CCTTTT	1
JA	T/GBOXATPIN2	AACGTG	1
Ethylene	ERELEE4	AWTTCAAA	1
SA	GT1CONSENSUS	GRWAAW	10
	WBOXATNPR1	TTGAC	2
Wounding	WBOXNTERF3	TGACY	4
	T/GBOXATPIN2	AACGTG	1
Disease resistance	WBOXATNPR1	TTGAC	2
	GT1GMSCAM4	GAAAAA	1
Water stress	MYCCONSENSUSAT	CANNTG	22
	MYCATRD22	CACATG	2
	MYCATERD1	CATGTG	2
	MYBCORE	CNGTTR	3
	MYB2CONSENSUSAT	YAACKG	1
	MYB1AT	WAACCA	5
	LTRECOREATCOR15	CCGAC	1
	DREDR1ATRD29AB	TACCGACAT	1
	DRECRTCOREAT	RCCGAC	1
	CBFHV	RYCGAC	1
Low temperature	LTREATLTI78	ACCGACA	1
	LTRECOREATCOR15	CCGAC	1
	DREDR1ATRD29AB	TACCGACAT	1
	CBFHV	RYCGAC	1

Salt	GT1GMSCAM4	GAAAAA	1
	DREDR1ATRD29AB	TACCGACAT	1
Heat shock	CCAATBOX1	CCAAT	8
Light	INRNTPSADB	YTCANTYY	1
	IBOXCORE	GATAA	4
	GT1CONSENSUS	GRWAAW	10
	DRECRTCOREAT	RCCGAC	1
	CIACADIANLELHC	CAANNNNATC	2
Etiolation	ABRELATERD1	ACGTG	1
	ACGTATERD1	ACGT	2

Table 1.5. The putative cis-acting elements in the *GbIDS2* promoter sequence. Analysis was performed by PLACE.

Functions	Element	Elem. Seq.	Freq.
ABA	LTRECOREATCOR15	CCGAC	1
	MYB1AT	WAACCA	2
	MYCATRD22	CACATG	1
	MYCCONSENSUSAT	CTCTT	12
	PYRIMIDINEBOXHVEPB1	TTTTTTCC	1
	RYREPEATBNNAPA	CATGCA	1
GA	CAREOSREP1	CAACTC	1
	GAREAT	TAACAAR	1
	MYBGAHV	TAACAAA	1
	PYRIMIDINEBOXHVEPB1	TTTTTTCC	1
	PYRIMIDINEBOXOSRAMY1A	CCTTTT	3
	WRKY71OS	TGAC	13
JA	T/GBOXATPIN2	AACGTG	1
SA	ELRECOREPCRP1	TTGACC	1
	GT1CONSENSUS	GRWAAW	12
	WBOXATNPR1	TTGAC	4
Auxin	CATATGGMSAUR	CATATG	4
	NTBBF1ARROLB	ACTTTA	5
Wounding	T/GBOXATPIN2	AACGTG	1
	WBOXNTERF3	TGACY	10
Disease resistance	SEBFCONSSTPR10A	YTGTCWC	1
	WBOXATNPR1	TTGAC	4
Water stress	ACGTATERD1	ACGT	4
	LTRECOREATCOR15	CCGAC	1
	MYB1AT	WAACCA	2
	MYCATERD1	CATGTG	1
	MYCATRD22	CACATG	1
Low temperature	LTRECOREATCOR15	CCGAC	1
Heat shock	CCAATBOX1	CCAAT	5
Light	-10PEHVPSBD	TATTCT	1
	GATABOX	GATA	13
	GT1CONSENSUS	GRWAAW	12
	IBOXCORE	GATAA	2

	INRNTPSADB	YTCANTYY	1
	SORLIP1AT	GCCAC	2
	SORLIP2AT	GGGCC	1
	TBOXATGAPB	ACTTTG	1
	ZDNAFORMINGATCAB1	ATACGTGT	1
Etiolation	ABRELATERD1	ACGTG	2
	ACGTATERD1	ACGT	4

1.4.2. Histochemical analysis of *GbIDSpro*-driven GUS expression in Arabidopsis

To ascertain the developmental-spatial expression patterns of the *GbIDS1* and *GbIDS2*, each construct of *GbIDS1pro::GUS* and *GbIDS2pro::GUS* was introduced into Arabidopsis. In the case of *GbIDS1* promoter, GUS expression was found in almost all developmental stages and tissue types except for roots, stamina, and petals (Figure 1.5). In contrast, *GbIDS2* promoter-driven GUS expression was very organ-specific; the expression was shown only in young leaves including meristem, internodes where the flower and shoot branched, and particularly junction of primary root (Figure 1.6). Furthermore, expression in root junction was detected only at specific growth stages; 5.10 (the first flower bud visible) to right before 6.00 (the first flower open) (Figure 1.6).

The sectioning of *GbIDS1pro::GUS*-expressed leaf, inflorescence, and internode stem provided information on the tissue distribution of IDS. In leaf, strong GUS expression in parenchyma cells were detected, in contrast to the very weak expression in the epidermal cells, if any (Figure 1.7). Terminal inflorescence stem had little expression pattern. In penultimate internode (Figure 1.7), whole tissue except for pith and xylem was stained. In the case of *GbIDS2pro::GUS* Arabidopsis, duct system was the main site of expression: the central vasculature in the root junction and the vascular bundle of flowering stem (Figure 1.8). Hormone treatment did not cause additional organ or tissue staining (Data not shown).

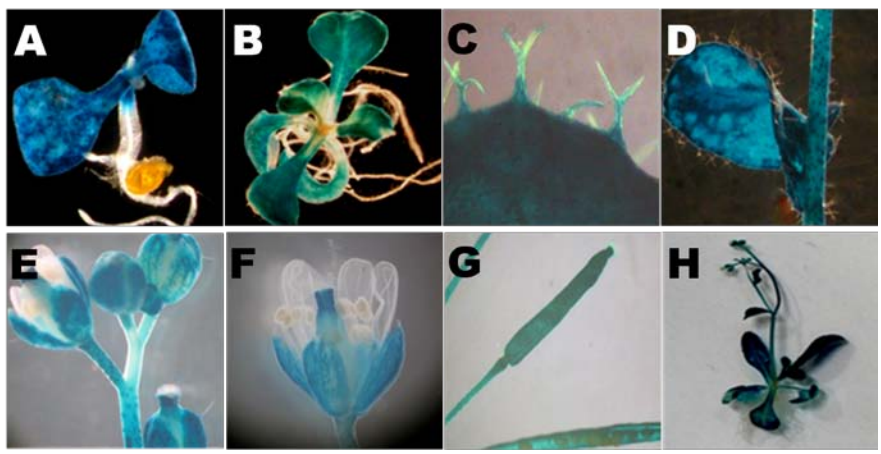


Figure 1.5. GUS staining in *GbIDS1pro::GUS* Arabidopsis. A: cotyledon fully opened, B: 1.04 stage Arabidopsis, C: trichomes on leaf, D: inflorescence stem and leaf, E: flower buds, F: flower, G: silique, H: 6-week-old plant.

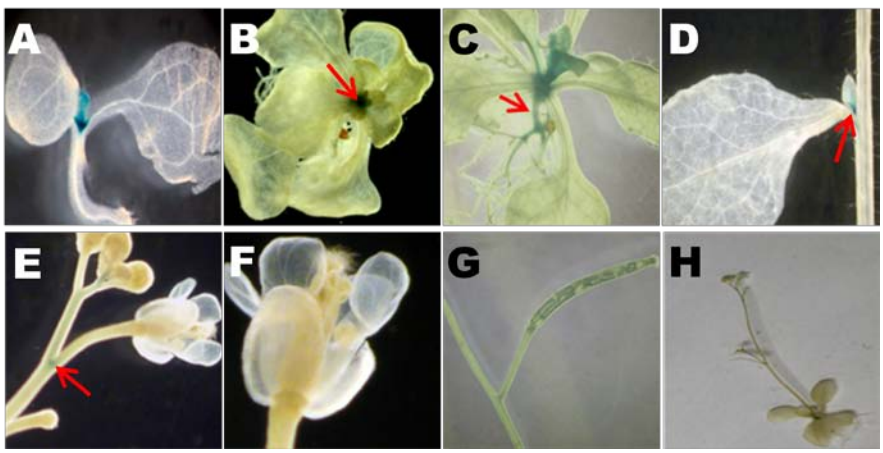


Figure 1.6. GUS staining in *GbIDS2pro::GUS* Arabidopsis. A: cotyledon fully opened, B: 1.04 stage Arabidopsis, C: roots, D: inflorescence stem and leaf, E: flower and buds in floral stem, F: flower, G: silique, H: 6-week-old plant. Red arrows indicate the site of visible GUS expression.

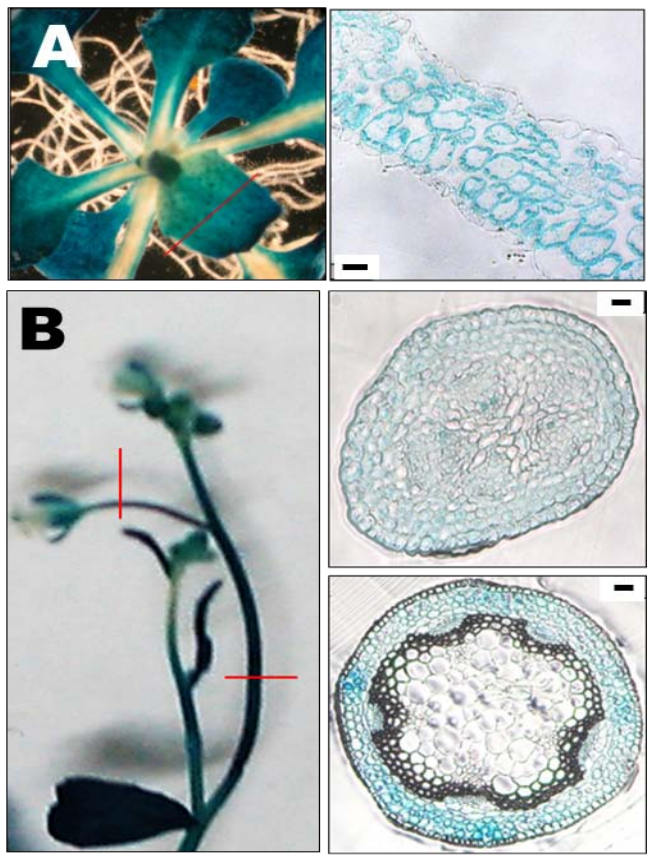


Figure 1.7. Tissue section of *GbIDS1pro::GUS* Arabidopsis with red line indicating site of sectioning. A: leaf at 1.08 stage (eight rosette leaves > 1mm in length), B: floral stem (upper) and internode stem (lower). Black bar represetns 20 μm.

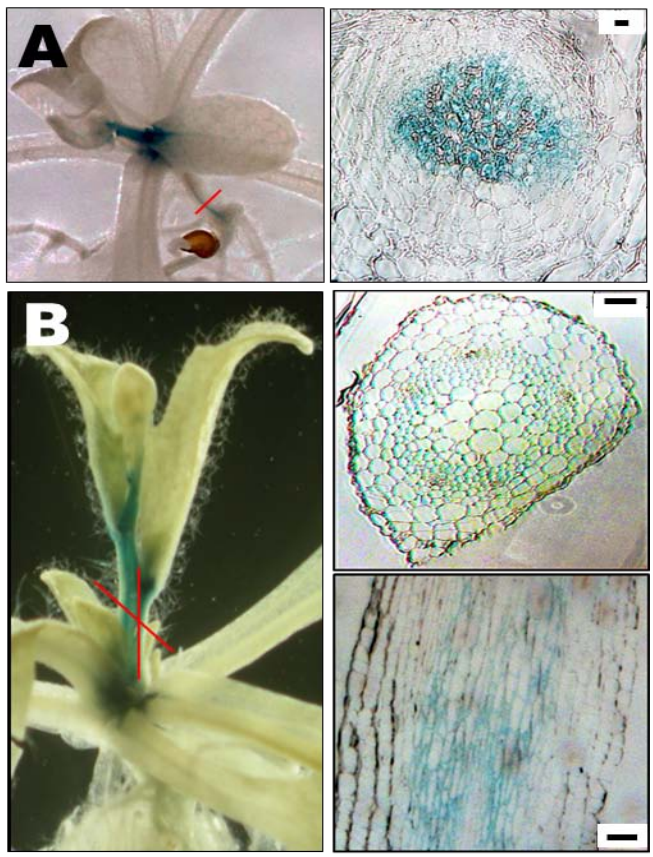


Figure 1.8. Tissue section of *GbIDS2pro::GUS* Arabidopsis with red line indicating site of sectioning. A: root, B: first internode cross (upper) and longitudinal (lower) sections. Black scale bar represents 20 μm.

1.4.3. Responsiveness of *GbIDS* promoters in Arabidopsis toward hormone treatments

GUS activity in Arabidopsis transformed with *IDSpro::GUS* was quantitatively assessed in each organ after hormone treatment. In particular, *GbIDS1pro*-driven GUS activity in the leaves and *GbIDS2pro*-driven GUS activity in roots are presented in Figures 1.5 and 1.6, because these two organs showed the most contrasting GUS staining patterns. The selection of the hormone was based on the putative hormone-related *cis*-acting elements in the *IDSpro* sequence analysises (Figures 1.3 and 1.4, Tables 1.4 and 1.5). The activity of GUS in the leaves and roots after the hormone treatment was measured in 5.10 stage plants and flowers and buds in stage 6.50 plants (50% of flower opened) (Figures 1.5 and 1.6). In the case of leaves, treatment of MeJA and GA resulted in pronounced decrease of GUS activity by 40 and 25%, respectively in *GbIDS1pro*-transformed Arabidopsis (Figure 1.9A). However, in the roots of *GbIDS2pro*::*GUS* plant, MeJA and GA significantly stimulated GUS activity by 80 and 140%, respectively (Figure 1.9A). GUS activity in ABA-treated plants also followed the same trend both in the leaves and root. However, SA treatments caused the opposite effect compared to MeJA and GA treatments by increasing the GbIDS1pro-related GUS activity in leaves by 10% and decreasing the GbIDS2pro-related GUS activity in roots by 20%. Therefore, behavior of *IDS1pro*-driven GUS activity and that of *IDS2pro*-driven activity upon MeJA and SA treatments in the roots and leaves were reciprocal. GUS activity in flowers

and buds did not show such distinctive pattern observed in leaves and roots (Figure 1.9B). In contrast to leaves and roots, GA and IBA treatments tended to increase GUS activity in flowers and buds, respectively, compared to MeJA and SA treatments (Figure 1.9B). MeJA and SA had little effect on GUS activity in flowers and buds.

1.4.4. Transcript distribution of *GbIDS1* and *GbIDS2* in Ginkgo

The organ-specific transcription of *GbIDS*s in Ginkgo was examined in detail by RT-PCR (Figure 1.10). In the case of *GbIDS1*, transcripts appeared in all organs examined except for root and cotyledon. However, *GbIDS2* transcripts were found in all organs including root and cotyledon, albeit in varying degree of transcript levels–the transcript level of *GbIDS2* in the leaves was relatively low compared to those of stem, root, as well as female and male flowers. In summary, *GbIDS2* level was lower than *GbIDS1* level in leaves. In contrast, *GbIDS2* levels were higher than those of GbIDS1 in male and female flowers. In root and cotyledon, only *GbIDS2* transcripts were seen.

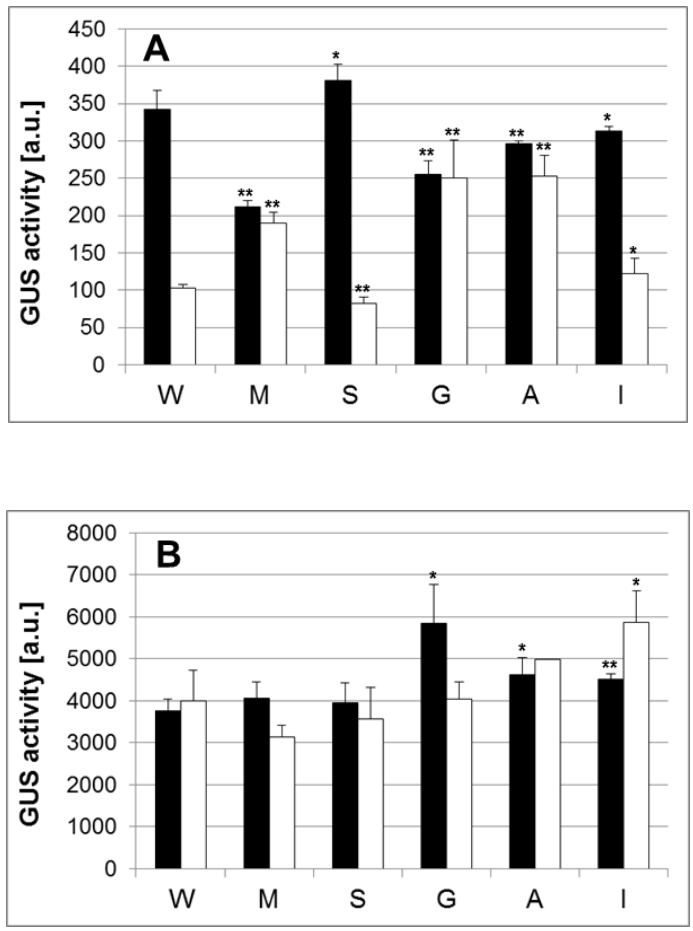


Figure 1.9. Fluorometric GUS assay of *GbIDSpro::GUS* Arabidopsis. A: *GbIDS1pro::GUS* Arabidopsis leaves and *GbIDS2pro::GUS* Arabidopsis roots (filled bar: leaves, empty bar: roots), B: *GbIDS1pro::GUS* flowers and buds (filled bar: flowers, empty bar: buds). W: water, M: MeJA, S: SA, A: ABA, I: a.u. denotes arbitraty unit. The asterisk symbol indicates **P < 0.01 and *P < 0.05 compared to the water treatment.

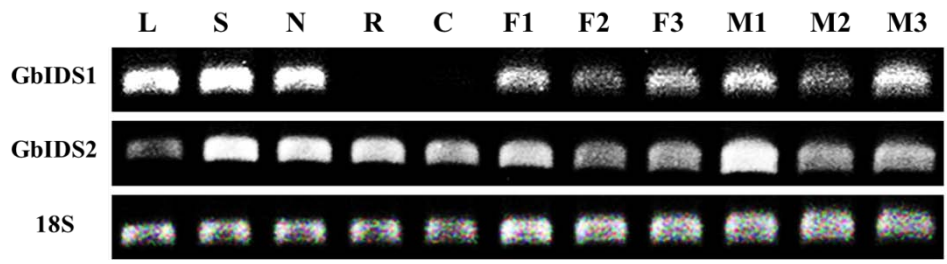


Figure 1.10. *GbIDS1* and *GbIDS2* expression in *Ginkgo biloba* as assessed by RT-PCR. L: leaf, S: stem, N: stem node, R: root, C: cotyledon, F1: female flower, F2: leaves around the female flower, F3: stem of F2, M1: male flower, M2: leaves around the male flower, M3: stem of M2.

1.4.5. *GbIDS1* and *GbIDS2* transcript level changes in Ginkgo by MeJA and SA treatments

To correlate the effects of MeJA and SA treatments in the transformed Arabidopsis with Ginkgo plant, *GbIDS* transcript levels in leaves and roots were measured after treating the elicitors to 2-month-old ginkgo seedlings. Initially, the resting *GbIDS2* transcript level was higher than the resting *GbIDS1* level by 100% in the leaves, whereas in the roots *GbIDS1* transcript level was almost negligible compared to *GbIDS2* level (Figure 1.11). MeJA treatment decreased *GbIDS1* transcript level in the leaves by 50%, whereas SA caused little change. However, upon MeJA treatment, *GbIDS2* transcript level in the leaves and roots increased by 150 and 40%, respectively (Figure 1.11).

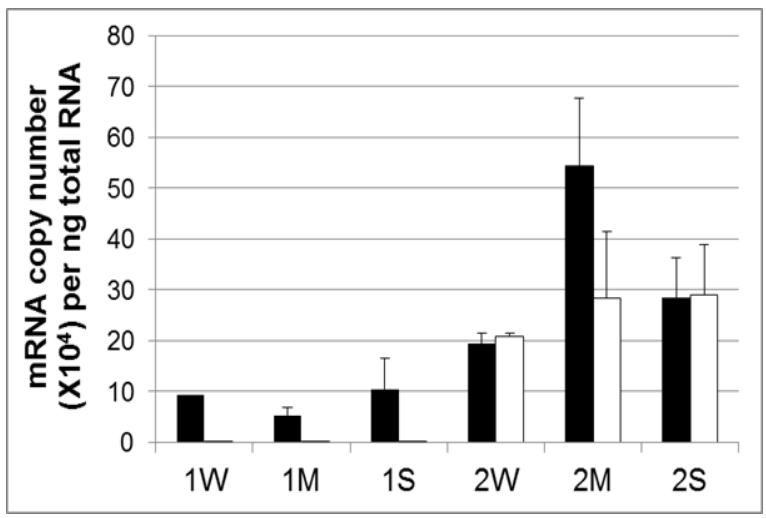


Figure 1.11. Transcript levels of *GbIDSs* in *G. biloba* seedlings after elicitor treatment. Filled bar: leaves, empty bar: roots, W: water, M: MeJA, S: SA, numbers represent GbIDS1 and GbIDS2.

1.5. DISCUSSION

Biosynthesis of isoprene unit in the classical MVA pathway is regulated in multiple levels. For example, 3-hydroxy-3-methylgluaryl-CoA reductase (HMGR), being the first committing enzyme in MVA pathway, is highly regulated from transcription to post-translation levels (Leivar *et al.*, 2011). In the case of Arabidopsis, HMGR gene is encoded as two copies: *HMG1* and *HMG2*. Transcripts of *HMG1S*, one of the alternative splicing forms of *HMG1*, appear in whole plant (Lumbreras *et al.*, 2003), whereas transcripts of *HMG2* and *HMG1L* transcripts are present in seedling, root, and inflorescence (Enjuto *et al.*, 1995). This observation is interpreted as that HMG1S is involved in housekeeping process, whereas HMGR2 has a more restricted function (Leivar *et al.*, 2011). Plants also harbor MEP pathway to synthesize isoprene units to build important primary metabolites such as carotenoids and chlorophylls as well as secondary metabolites such as ginkgolides in Ginkgo. The first enzyme in MEP pathway known to be regulated in transcription level is DXS (Walter *et al.*, 2002). In all plants so far examined, two to three copies of 1-deoxy-D-ribulose 5-phosphate synthase (DXS) gene (Walter *et al.*, 2002; Cordoba *et al.*, 2009) are known to exist. Among them, *DXS1* was suggested to provide isoprene units for housekeeping function, whereas *DXS2* for secondary metabolism (Kim *et al.*, 2006c; Walter *et al.*, 2002). It becomes clear that regulation of MEP pathway in gene level by differential expression of isozymes is not limited

to DXS; 1-deoxy-D-ribulose 5-phosphate reductase (DXR) (Seetang-Nun *et al.*, 2008), 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase (CMS) (Sando *et al.*, 2008), and 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS) in *Hevea brasilensis* (Sando *et al.*, 2008), 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase (CMK) in Ginkgo (Kim *et al.*, 2008a), (*E*)-4-hydroxy-3-mehylbut-2-enyl diphosphate synthase (HDS) in Populus (Wiberley *et al.*, 2009), and IDS in Ginkgo (Kim *et al.*, 2008b), Pinus (Liu and Huang, 1998; Kim *et al.*, 2009), and rice (Jung *et al.*, 2008). Similarly to DXS, Ginkgo and Pinus IDS1s are proposed to be involved in housekeeping function and IDS2s in secondary metabolism (Kim *et al.*, 2008b; Jefferson *et al.*, 1987). The present work on promoters of *GbIDS* genes was conducted to further confirm the role of each GbIDS isozyme in Ginkgo metabolism.

It was evident that each *GbIDS1pro* and *GbIDS2pro* drove GUS expression in different organs in Arabidopsis model. *GbIDS1pro* drove expression of GUS in the whole plant except in roots and stamina (Figure 1.5), whereas *GbIDS2pro*-driven GUS expression was confined only to root junction, young leaves, and shoot apices (Figure 1.6). These GUS expression patterns observed in Arabidopsis strongly correlated with RT-PCR data of Ginkgo organs - *GbIDS1* transcripts were abundant in most plant organs except for cotyledon and roots, whereas *GbIDS2* transcripts appeared in the roots and cotyledon (Figure 1.10). This RT-PCR data also collaborated with the afore-mentioned histological staining results (Figures

1.5 and 1.6) to indicate organ-specific manner of *IDS* gene transcription. One exception was *GbIDS2.* Its transcripts were found in male and female strobili, whereas no expression of GUS in flowering organs of *GbIDS2pro::GUS* plant was shown (Figures 1.5 and 1.6). MEP pathway enzymes are highly transcribed in plant ovary. For example, in flowers of *Salvia miltiorrhiza* and Arabidopsis, the DXR transcripts are most high in pistils among the flower parts (Carretero-Paulet *et al.*, 2002; Yang *et al.*, 2010). Therefore, MEP pathway is actively operating in pistils (Yang *et al.*, 2010). Then why *GbIDS2pro* did not function to drive GUS expression in Arabidopsis floral tissue is an open question.

The *GbIDS2pro*-directed GUS expression was tissue-specific: GUS staining appeared mostly in vasculature-related tissues in the root junction and young internode (Figure 1.8), presumably reflecting translocation of ginkgolide to aerial part of Ginkgo after biosynthesis (Cartayrade *et al.*, 1997). In the case of *Catharanthus roseus* and *Papaver somniferum*, the alkaloid-related MEP pathway genes are also present in vascular tissues (Facchini and De Luca, 2008; Oudin *et al.*, 2007). Therefore, it is highly possible that GbIDS2 is involved in the secondary metabolism, whereas GbIDS1 functions in the housekeeping process. The current observation is therefore in accordance with the suggestion of Kim et al. that GbIDS1 and GbIDS2 are related to the primary and the secondary metabolisms, respectively (Kim *et al.*, 2008b). The different roles among multicopy IDSs in the primary and the secondary metabolisms have been also proposed for *P.*

densiflora (Lichtenthaler, 1999) and *O. sativa* (Walter *et al.*, 2002).

GbIDS1pro-directed GUS expression occurred mainly in chloroplast of leaf parenchyma cells (Figure 1.7A) where photosynthesis actively takes place. Photosynthesis calls for active biosynthesis of light-harvesting pigments, chlorophyll and carotenoids, which are assembled from MEP pathway end products (Joyard *et al.*, 2009). Therefore, MEP pathway enzymes are known to be upregulated by light (Cordoba *et al.*, 2009). Kim et al. found that leaf *GbIDS1* transcript level is up-regulated by 4-fold upon illumination of etiolated Ginkgo embryo culture (Kim *et al.*, 2008b), whereas *GbIDS2* transcript level in the root decreased by 50% at the same time. The result strongly suggests that *GbIDS2* is not involved in biosynthesis of photosynthetic pigments. In the case of rice, only *OsIDS1* was shown to be light-responsive (Jung *et al.*, 2008). Taking these data together, it was strongly implicated that one of multicopy *IDS* in Ginkgo and rice is involved in photosynthesis, whereas another copy is involved in secondary metabolism.

Recently, Kim et al. studied promoter of Ginkgo levopimaradiene synthase (GbLPS), the enzyme catalyzing the cyclization of geranylgeranyl diphosphate as the first committed step in ginkgolide biosynthesis (Kim *et al.*, 2012). They found *GbLPSpro*-driven GUS expression in Arabidopsis occurs in roots, young leaves, and immature ovary and stamina. RT-PCR study confirmed the presence of *GbLPS* transcripts in roots and male strobili of Ginkgo. If *GbIDS2* is involved in ginkgolide biosynthesis in a more or

less organ-specific manner, the organ distribution of *GbIDS2* transcripts in Arabidopsis must have strong correlation with the distribution of *GbLPS* transcripts. The present experiment indicates that the distribution of *GbIDS2* transcripts indeed overlaps with that of *LPS* in roots or root junction, young leaves, and flowers. Therefore, involvement of *GbIDS2* in ginkgolide biosynthesis is again implicated. Cartayrade et al. first presented strong evidence that ginkgolide is synthesized in the roots and subsequently translocated to aerial part (Cartayrade *et al.*, 1997). GUS expression in root vasculature by both *GbLPSpro* (Kim *et al.*, 2012) and *GbIDS2pro* indirectly suggests such translocation is taking place. In *C. roseus*, genes involved in vinca alkaloid biosynthesis are also shown to be transcribed specifically in vascular cells to facilitate translocation after biosynthesis (Facchini and De Luca, 2008; Oudin *et al.*, 2007). However, GUS activity in meristem and young leaves, ovary, and stamina in *GbLPSpro*- and *GbIDS2pro*-transformed Arabidopsis' makes us to consider possible biosynthesis of ginkgolide in corresponding organs of Ginkgo plant. Carrier et al. also posited ginkgolide biosynthesis in aerial part of Ginkgo (Carrier *et al.*, 1998). Another possibility is that GbIDS2 in aerial part of Ginkgo is involved in biosynthesis of yet to be identified terpene.

Response of *GbIDS* promoters to hormones could provide clue to the function of the enzymes. MeJA- and SA-responsive sites are present in the promoter sequences of both GbIDSs (Figures 1.3 and 1.4, Tables 1.4 and 1.5). Production of MeJA and SA is involved in plant defense against biotic

stresses such as herbivore and pathogen attacks (Robert-Seilaniantz *et al.*, 2011). MeJA and SA interact antagonistically against each other to induce transcription of defense-related genes (Koornneef *et al.*, 2008). In this process, plant is costing its fitness to cope with the stress (Heil and Baldwin, 2002). Therefore, challenging Ginkgo with MeJA and SA would activate defense-related genes in plants concomitantly sacrificing housekeeping process. Indeed, in the present experiment, MeJA treatment to the transformed Arabidopsis significantly down-regulated *GbIDS1pro*-driven GUS activity in the leaves, while up-regulating *GbIDS2pro*-driven activity in the roots (Figure 1.9A). However, SA treatment induced opposite effects - GUS activity driven by *GbIDS1pro* in the leaves tended to increase, whereas that driven by *GbIDS2pro* in the root decreased. In Ginkgo seedlings, increase of *IDS2* transcript level, though not statistically significant, was observed by MeJA and SA treatments (Figure 1.11). In the case of *IDS1*, the transcript level was not much affected in both leaves and roots. Kim et al. previously observed 100 and 400% increase of *GbIDS2* transcript levels by MeJA treatment respectively in the radicle and leaves of Ginkgo embryo culture (Kim *et al.*, 2008b). Because MeJA is known to be involved mainly in defense against herbivores (McConn *et al.*, 1997), MeJA would mobilize *GbIDS* copy that is involved in biosynthesis of insecticidal ginkgolides. Therefore, the reciprocal changes among *GbIDSpro*-driven GUS activities and *GbIDS* transcript levels induced by MeJA and SA treatment again support that *GbIDS1* is related to housekeeping, whereas

GbIDS2 operates in secondary metabolism, most likely plant defense process. The multiple *IDS* genes in *P. densiflora* also behave similarly to Ginkgo *IDS* genes upon MeJA treatment (Kim *et al.*, 2009).

To sum up, functions of *GbIDS1* and *GbIDS2* were suggested through assessing the promoter-driven GUS expression in Arabidopsis model: *GbIDS1* in housekeeping and *GbIDS2* in ginkgolide biosynthesis. The behavior of the promoters in Arabidopsis and each gene transcripts in Ginkgo upon light and hormone treatments further supported the above suggestion.

1.6. ABSTRACT (IN KOREAN)

MEP 대사경로 마지막 단계 효소인 IDS/HDR는 겉씨 식물인 은행에는 세 벌로 존재한다고 알려져 있다. 본 연구에서는 은행 IDS들의 각각의 기능을 알아보기 위하여 은행의 프로모터 역할에 관하여 애기장대를 이용하여 실험해 보았다. 은행 IDS1의 프로모터 영역 1.3kb와 IDS2 프로모터 영역 1.5kb을 분리한 후, GUS 단백질이 들어 있는 벡터로 삽입하고 애기장대에 형질전환 시켰다. 은행의 IDS1 프로모터가 도입된 애기 장대에서는 뿌리, 꽃잎 그리고 수술을 제외한 식물체 거의 모든 조직과 기관에서 GUS 발현이 확인되었다. 반면, 은행의 IDS2 프로모터를 도입한 애기장대에서는 식물의 어린 잎과 분화 조직 그리고 뿌리의 특정 부분(원뿌리와 하배축 사이의 접합 영역)에서만 GUS가 발현 되었다. 이러한 GUS 발현 양상은 RT-PCR과 qRT-PCR을 통해 확인 된 은행의 *IDS1*과 *IDS2*의 전사 양상과도 상관관계를 가진다. 은행 IDS 프로모터가 도입된 애기장대 식물에 스트레스 유도인자인 Methyl jasmonate를 처리한 결과, 은행 IDS1 프로모터가 도입 된 식물체 잎에서는 GUS activity가 증가하였고 IDS2 프로모터가 도입 된 식물체의 뿌리에서는 activity가 감소하였다. 이와 같은 유전자 조절 양상은 IDS2 프로모터가 도입된 애기장대 뿌리에서 GA, ABA, 그리고 IBA를 처리하였을 때도 관찰되었다.

PART II

Functional Study of *Ginkgo biloba* (*E*)-4-Hydroxy-3-methylbut-2-enyl Diphosphate Reductase/Isopentenyl Diphosphate Synthase (HDR/IDS) Genes in Poplar

2.1. ABSTRACT

To assess the roles of *Ginkgo biloba* IDS multi-copy enzymes, overexpression of *GbIDS1* and *GbIDS2* in poplar was attempted. Ten lines of *GbIDS1* overexpression transgenic poplars were obtained while no transformants were made with *GbIDS2*. GbIDS1 accelerated plant growth and increased biomass of transgenic poplar plants. GbIDS1 transgenic poplars were taller than wild-type BH clone poplars by 25% and have 2 more leaves in indoor condition 7 weeks after potting in soil. In outdoor nursery, GbIDS1 overexpression poplar grew taller by 7%, and showed delayed winter bud formation. In addition, overexpression of *GbIDS1* gene led increase of chlorophyll and carotenoid contents by approximately 20% in transgenic poplars compared to BH poplars. Chlorophyll related genes, *CHS* (Chlorophyll synthase) and *CAO* (Chlorophyll a oxidase) transcript levels were higher in transgenic poplars by 30% and 50% respectively. Transcript levels of gibberellin biosynthetic genes, *KS* (kaurene synthase), *GA20ox* (gibberellin 20 oxidase), and *GA2ox* (gibberellin 2 oxidase) were assessed in BH and transgenic poplars. In this analysis, *KS* and *GA20ox* transcript levels were up-regulated by 170% and 85% respectively. However, *GA2ox* transcript level displayed 40% down-regulation in transgenic poplars. The results suggested that overexpression of *GbIDS1* gene was responsible for the increase of production of IPP and DMAPP, which affected the down-stream genes, ultimately led increase of biomass and terpenoids production.

Keywords

Ginkgo biloba, Poplar, Isoprenoids, MEP pathway, (*E*)-4-Hydroxy-3-methylbut-2-enyl Diphosphate Reductase/Isopentenyl Diphosphate Synthase (HDR/IDS), Chlorophyll, Carotenoid, Gibberellic acid (GA)

2.2. INTRODUCTION

Isoprenoids (also known as terpenoids) are broad range group of natural products and synthesized by all living organisms. In plants, they play vital roles such as photosynthesis (chlorophylls, carotenoids, and plastoquinone), respiration (ubiquinone), and growth and development (cytokinins, gibberellins, and abscisic acid). In addition, plants produce various secondary metabolites, which act plant defense against herbivores and pathogens. They have been used as industrial uses: flavors, drugs, and so on. Even though they have complex structures and diverse functions, terpenoids are derived by simple C5 carbon units, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Both terpenoid building blocks are synthesized by two distinct biosynthetic routes, mevalonate (MVA) and 2C-methyl-D-erythritol 4-phosphate (MEP) pathways. The latter, exists in plastid in plants and produces mono- (C10), di- (C20), and tetra-(C40) terpenoids. In the MEP pathway, IPP and DMAPP are synthesized by seven enzymes, and one of them, the (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase/isopentenyl diphosphate synthase (HDR/IDS) participates in the last step of the pathway is the target in this study. Recently, the importance of IDS gene has been reported in many researches (Botella-Pavía *et al.*, 2004; Kim *et al.*, 2009). However, little studies of IDS are available compared to the 1-deoxy-D-xylulose-5-phosphate synthase (DXS) (Estévez *et al.*, 2001; Lois *et al.*, 2001; Morris *et al.*, 2006; Mandel

et al., 1996) and 1-deoxy-D-xylulose-5-phosphate reductase (DXR) (Carretero-Paulet *et al.*, 2006; Hasunuma *et al.*, 2008; Xing *et al.*, 2010).

Ginkgo biloba L. is well known medicinal tree, which possesses terpenoids such as ginkgolides and bilobalide. This tree has multi-copy IDSs, a trait of gymnosperms such as *Pinus taeda*, *P. densiflora* and *Cycas revoluta* (Kim *et al.*, 2008b) (Kim *et al.*, 2009). Previous reports demonstrated cell- and tissue specific expression pattern of muticopy *IDS*s, and elucidated responses upon biotic and abiotic stresses of each IDS isozyme (Kim *et al.*, 2009; Kim *et al.*, 2008b; Kang *et al.*, 2013). However, functional study of plant IDSs by overexpressing *IDS* gene in plants has not been reported.

In this study, heterologous transformation system using poplar trees is applied, because direct transformation of Ginkgo tree is not available to date. Poplar has many advantages in genetic engineering, being model woody plant. Its modest genome size, rapid growth, and availability of the poplar genome sequence provide us opportunity to easily manipulate the gene in the genetic engineering (Tuskan *et al.*, 2006). Also noted is that poplar transformation and regeneration system have well been established by efforts of many researchers (Cseke *et al.*, 2007; Yevtushenko and Misra, 2010; Fillatti *et al.*, 1987). The present research could demonstrate roles of GbIDS as well as useful material for genetic engineering and metabolic study.

2.3. MATERIALS AND METHODS

2.3.1. Construction of plasmid

ORFs of the *G. biloba* IDS1 (GenBank accession No. DQ251631) and 2 (DQ252633) were amplified by PCR with *Taq* DNA polymerase (Promega, Madison, USA) and primers pair designed to include *Xba*I and *Bam*HI sites (Table 2.1). Then the PCR products were inserted into the pBI121 vector (Figure 2.1) and transferred into *Agrobacterium tumefaciens* LBA4404 strain using freeze-thaw method (Weigel and Glazebrook, 2006).

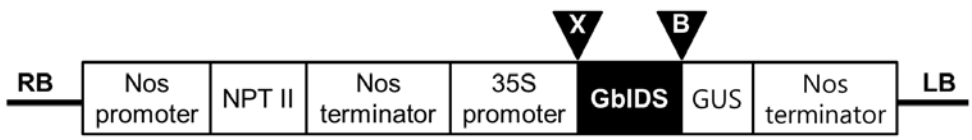


Figure 2.1. Construction of the T-DNA region of *GbIDS1* and *GbIDS2*.

X: *Xba*I, B: *Bam*HI

Table 2.1. Primers used for the overexpression constructs.

Primer	Forward (5’→3’)	Reverse (5’→3’)
GbIDS1	GCTCTAGAATGGCTTGCAGTT GTAGCTTTG	CGGGATCCTTATAGTGGCTGC AAGGCATC
GbIDS2	GCTCTAGAATGGCTCAAGCTT GTGCAGTATC	CGGGATCCCTACGCTACTTGC AAGGTCTCTTC

2.3.2. Poplar transformation and regeneration

The poplar used in this experiment was hybrid poplar clone (*Poplus alba* × *P. tremula* var. *glandulosa*, clone BH1). The clone was kindly provided by Drs. Choi Young-Im and Noh Eun-Woon at Department of Forest Genetic Resource, Korea Forest Research Institute (Suwon, Korea).

Poplar transformation was performed by co-cultivating the stem segments of the BH clone with *A. tumefaciens* LBA4404 carrying the *GbIDS1*and *GbIDS2* overexpression construct (Weigel and Glazebrook, 2006). The Agrobacterium strain was grown at 28ºC overnight in 50 ml LB medium. The bacteria were centrifuged at 1000 *g* for 10 min at 4ºC and resuspended in 20 ml of 0.85% NaCl solution. Acetosyringone was added into Agrobacterium suspension to the final concentration of 100 μM. Test tube-grown poplar stems were cut into 5-mm-long pieces, and then were soaked in the above suspension for 20 min. The stem cuttings were then transferred to 50 ml conical tube containing 20 ml of 0.85% NaCl solution. The tube was gently shaken to release bacteria. Then the segments were gently blotted on sterile papers and transferred to CIM medium (Table 2.2) and incubated at 26ºC in light for 2 days. The explants were then additionally cultured for 3 weeks in light on CIM2 (Table 2.2) and subcultured onto fresh CIM2 medium every 2 weeks. After 3-4 weeks on CIM2, transgenic calli were cultured on SIM (Table 2.2) for 3 weeks in light and subcultured every 2 weeks. Regenerated shoots were further screened on rooting medium, RIM (Table 2.2) containing 50 mg/L kanamycin. After 35-40 days on RIM,

the plants were transferred to the pots containing autoclaved vermiculite, covered with plastic wrap to maintain high humidity, and kept at 25ºC in a growth incubation room. The acclimated plants were finally moved to composite soil on May 17, 2012 after 6 weeks of acclimation.

2.3.3. Phenotypic assessment

Phenotype of wild-type BH clone and GbIDS1 transgenic poplars was checked regularly. The height and total number of leaves in BH clone and *GbIDS1* overexpression transgenic poplars were observed at 7 weeks after potting in composite soil. The pots were then removed to outdoor nursery and maintained for 10 weeks before assessing growth and winter bud formation.

Table 2.2. Composition of media for poplar regeneration.

Medium	Component	Use	Antobiotics
CIM	MS medium Sucrose Agarose 1.5% 2,4-D 10mM NAA 1.0mM BAP 0.1mM	Callus induction	Cefotaxim 100 mg/L Kanamycin 50 mg/L
CIM2	Same as CIM	Callus induction	None
SIM	WPM medium Zeatin 10 mM NAA 1.0 mM BAP 0.1 mM Sucrose Agarose 1.5%		Same as CIM
RIM	MS medium Sucrose Agarose 1.5%	Root induction	Same as CIM

2.3.4. Measurement of chlorophyll and carotenoid contents

Young leaves of poplar plants growing in test tubes were collected and weighed. The samples were ground in liquid nitrogen and added with 80% acetone, and then shaken in the dark for 15 min. The extracts were centrifuged at 4500 rpm for 15 min at 4℃. The supernatants were collected into new containers. Absorbance of the extract was measured at 470, 645, and 663 nm. The chlorophyll and carotenoid contents were calculated following the method of Ni et al. (Ni *et al.*, 2008).

Chl *a* (mg/g) = $(12.7 \times A_{663} - 2.69 \times A_{645}) \times V/(1000 \times W)$

Chl *b* (mg/g) = $(22.9 \times A_{645} - 4.86 \text{ x } A_{663}) \times V/(1000 \times W)$

Chl *a*+*b* (mg/g) = $(8.02 \times A_{663} + 20.20 \times A_{645}) \times V/(1000 \times W)$

Car (μg/ml) = $(1000 \times A_{470} - 1.82 \times \text{Chl a} - 85.02 \times \text{Chl b})/198$

V indicates volume of the extracts (ml) and W weight of fresh leaves (g).

2.3.5. RNA preparation and reverse transcription

Total RNAs were isolated from young leaves of poplars growing in test tubes. The plants were ground in liquid nitrogen and then the fine powders were transferred into the 1.5 ml Eppendorf tubes. Same volume of RNA extraction buffer (200 mM Tris-HCl, pH 8.0; 20 mM EDTA; 300 mM NaCl; 1% SDS) and water saturated phenol was added to the sample and vortexed. Then the mixtures were centrifuged for 3 min at 10000 *g* at room

temperature. The supernatants were extracted with 0.55 ml of chloroform, and then centrifuged again. Fifty five milliliters of sodium acetate solution (3 M, pH 5.3) and 1 ml absolute ethanol was added into the supernatant and chilled for 30 min at -20ºC. Then the samples were centrifuged for 15 min at 10000 *g* at 4ºC and the supernatants were removed. Three hundred microliters of 2 M LiCl was added to pellet and chilled on ice for 30 min. The samples were centrifuged again and the pellet was resuspended in 0.3 ml of ddH_2O. After 10 min of ice incubation, 3M sodium acetate (pH 5.3) and 0.3 ml of absolute ethanol were combined with the samples and chilled for 5 min at -20ºC. The samples were centrifuged at 10000 *g* at 4ºC for 10 min, supernatants were removed, and finally the pellets were dried. The total RNA was resuspended in 30 μl ddH_2O. The RNA concentration of each sample was measured on a micro-volume spectrometer (ASP-3700, ACTgene, USA). One microgram of RNA was used to synthesize the first strand cDNA using the cDNA synthesis kit (Philekorea Technology, Korea) according to the manufacture's protocol.

2.3.6. qRT-PCR analysis

Transcript levels of various genes in poplars were determined by using quantitative real-time polymerase chain reaction (qRT-PCR) on Rotor-Gene 2000 (Corbett Research, Mortlake, NSW, Australia), using 50 ng of the cDNA with a QuantiMix SYBR Kit (Philekorea Technology). The PCR condition was according to the manufacturer's recommendation. The primers used in this study are listed in Table 2.3.

2.3.7. Statistical analysis

The data as obtained in this experiment was subject to statistical analysis. Student's t-test was performed by using SAS (version 9.1). Statistical significances were denoted as * ($P \leq 0.05$) and ** ($P \leq 0.01$).

Table 2.3. Primers used for qRT-PCR of chlorophyll and GA related genes in poplar.

Primer	**Forward (5'→3')**	**Reverse (5'→3')**
CHS	TGGTCAAGCTTTCTTTGGGA	GCAGTTTCAGCACCAAAAGC
CAO	CATCTGGCCTCCAAGGGTAT	GGAAGGCAAGCAAACATGAA
KS	TCGATCGGCATTTTAGGAAG	AGCTCCCGAATCCTTCAAAT
GA20ox	TTGGGTGATTTCCTCTCTGG	TGGCAATTCGAAGAAGGTGT
GA2ox	ACACTTCTGGCCTGCAAATC	GCTGTTGGTCAAAACCCTGT
UBQ	CTCCAAAGTGAAAGGCCAGG ATG	ACTGTCAAAGCTCTTGGTGAG

2.4. RESULTS

2.4.1. Establishment of transgenic poplars

Total 47 transformants belong to10 lines of *GbIDS1* overexpression transgenic poplars were obtained through the transformation. Unlike GbIDS1, transgenic poplar plants of GbIDS2 could not be established because the shoots failed to develop roots..

2.4.2. RT-PCR analysis

To confirm the existence of *GbIDS1* transcripts in the transformants reverse-transcriptase polymerase chain reaction (RT-PCR) analysis was performed on RNA preparation from transgenic poplars. Band of about 1,450 bp, which was the expected size of GbIDS1 ORF, was amplified from each *GbIDS1* overexpression transgenic line poplars (Figure 2.2).

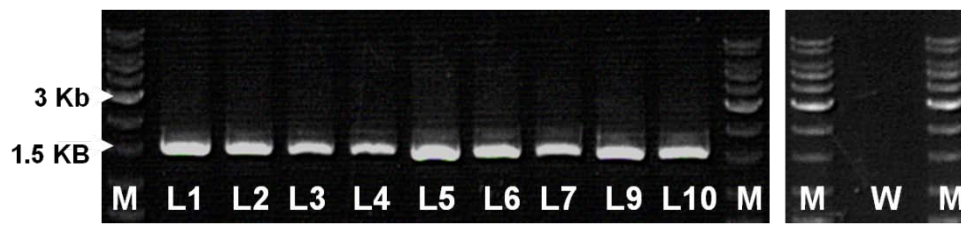


Figure 2.2. PCR bands of GbIDS1 ORF from BH clone and *GbIDS1* overexpression transgenic plants.
(M: marker, L1-L10: transgenic line poplars, W: BH clone)

2.4.3. Growth assessment of *GbIDS1* overexpression poplar

At 6 weeks after potting, it became evident that the growth performance of the transgenic poplars was better than wild type poplars (BH clone) (Figure 2.3). Transgenic poplars were approximately 25% taller than BH clone (Figure 2.4) and had 2 more leaves (Figure 2.5) at 7 weeks after potting. After 17 weeks after potting and 10 weeks in the outdoor nursery, winter bud formation was completed in BH clone poplars but not in GbIDS1 transgenic poplars (Figure 2.6). Height of GbIDS1 transgenic poplars was about 7% taller than BH clone poplars at 25 weeks after potting (Figure 2.7) when winter bud formation was complete in the transgenic plant. It was not possible to record leaf numbers this time because leaves of plants were severely damaged by herbivores.

Figure 2.3. BH clone and *GbIDS1* overexpression transgenic poplars 6 weeks after potting. The photos were taken on June 28, 2012. 'L' refers to line.

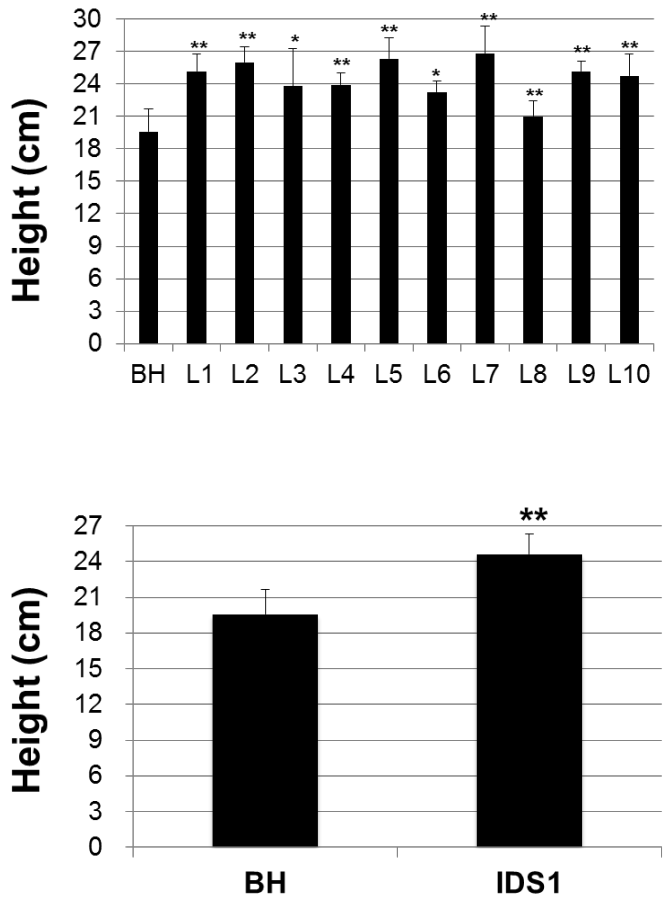


Figure 2.4. Height of BH clone and *GbIDS1* overexpression poplars 7 weeks after potting. It was measured on July 9th, 2012. The upper graph displays average height of BH clone and each transgenic line poplars. The lower one is average height of BH clone and combined IDS transgenic poplars. * ($P \leq 0.05$) and ** ($P \leq 0.01$), (*n* = minimum 3, maximum 5).

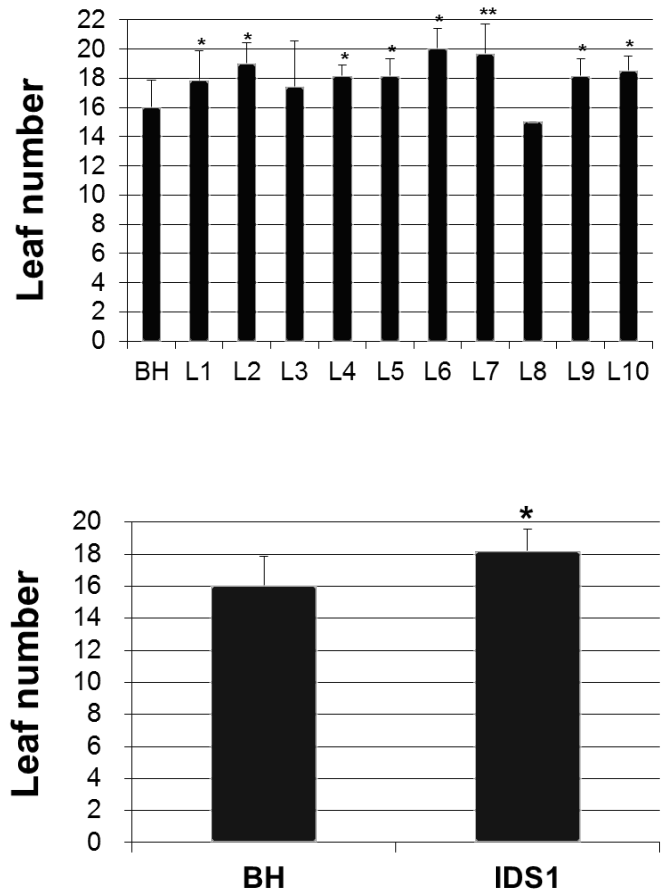


Figure 2.5. Leaf numbers of BH clone and *GbIDS1* overexpression poplars 7 weeks after potting. It was measured on July 9th, 2012. The upper graph displays average leaf numbers of BH clone and each transgenic line poplars. The lower one is average leaf numbers of BH clone and total IDS1 transgenic poplars. * ($P \leq 0.05$) and ** ($P \leq 0.01$), (*n* = minimum 3, maximum 5).

Figure 2.6. Winter bud formation of BH clone (up), *GbIDS1* overexpression poplars (down) 17 weeks after potting. Winter bud was evident in the WT plant on Sep 19, 2012, while *GbIDS1* overexpression line was still growing.

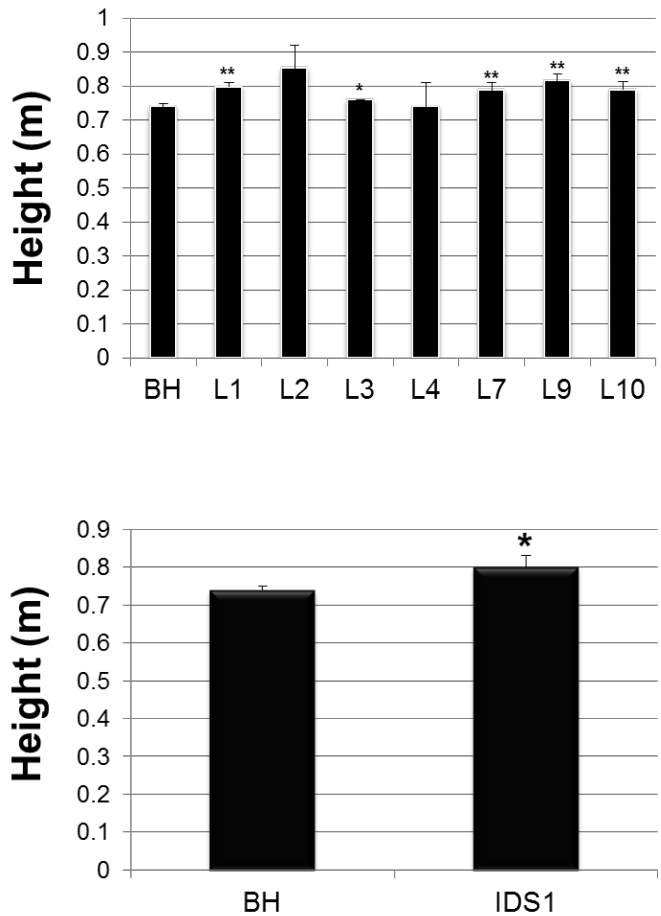


Figure 2.7. Height of BH clone and *GbIDS1* overexpression poplars 25 weeks after potting. It was measured on November 9th, 2012. The upper graph displays average height of BH clone and each transgenic line poplars. The lower one is average height of BH clone and total IDS transgenic poplars. * ($P \leq 0.05$) and ** ($P \leq 0.01$), (*n* = minimum 3, maximum 5).

2.4.4. Chlorophyll and carotenoid contents

The levels of chlorophylls and carotenoid were higher in *GbIDS1* overexpression transgenic poplars than BH clone poplars. The overexpression of *GbIDS1* in poplars increased chlorophyll a and b contents by 24% and 40% compared to WT respectively (Figure 2.8). Carotenoid contents were also approximately 21% higher in *GbIDS1* overexpression transgenic poplars than WT (Figure 2.9)

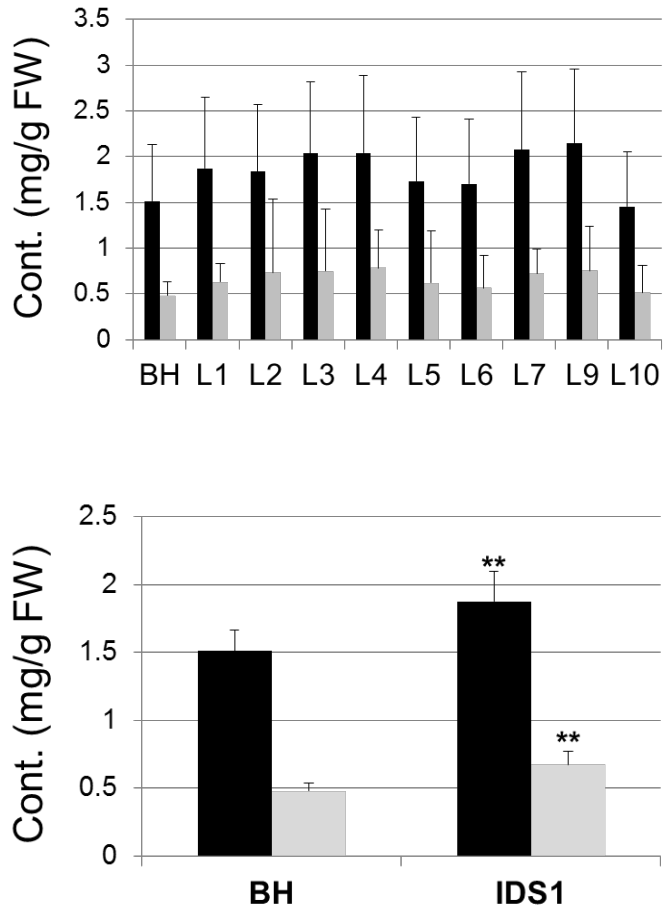


Figure 2.8. Chlorophyll contents of BH clone and *GbIDS1* overexpression poplars. Chl *a* (black) and Chl *b* (grey). * ($P \leq 0.05$) and ** ($P \leq 0.01$), (*n* = minimum 3, maximum 5).

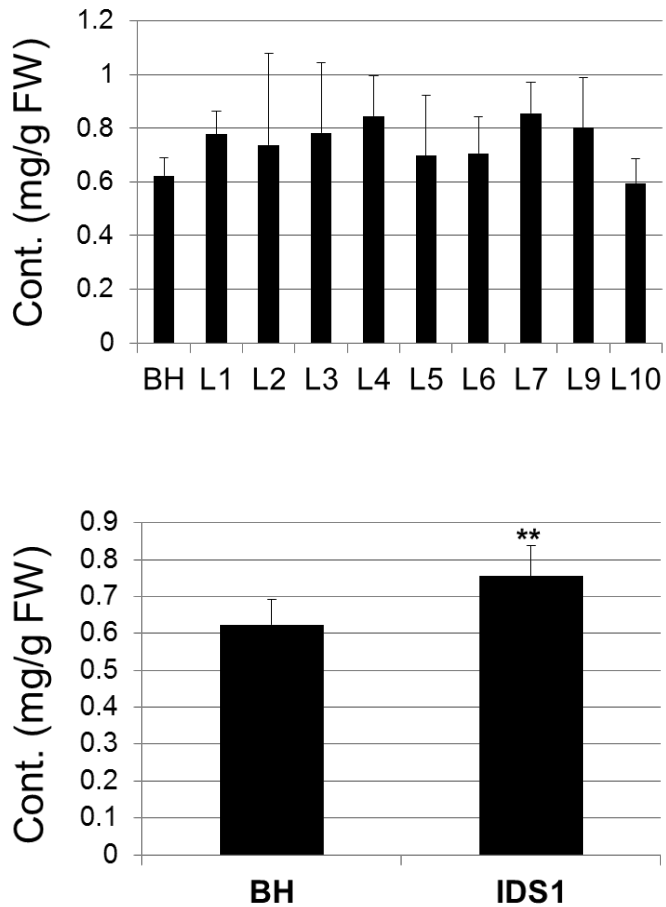


Figure 2.9. Carotenoid contents of BH clone and *GbIDS1* overexpression poplars. ** ($P \leq 0.01$), (*n* = minimum 3, maximum 5).

2.4.5. Transcript level of chlorophyll related genes

Attachment of phytol group, derived via MEP pathway, to haem moiety of chlorophyllide *a* by chlorophyll synthase (CHS) completes biosynthesis of chlorophyll *a*, and subsequent oxidation by chlorophyll *a* oxidase (CAO) results in formation of chlorophyll *b*. In the present study, transcript levels of these two critical chlorophyll biosynthesis genes in transgenic poplar were measured to assess effect of *IDS* overexpression on chlorophyll biosytnehsis. In GbIDS1 transgenic poplars, *CHS* transcript level was increased by 30% and *CAO* transcript by 50% compared with those of BH clone poplars (Figure 2.10).

2.4.6. Transcript level of GA related genes

GAs are group of diterpenoids and synthesized from GGPP *via* IPP. GA20 oxidase and GA2 oxidase control the level of bioactive GAs. The qRT-PCR analysis revealed that transgenic poplars had increased *KS* and *GA20ox* transcript levels by 170% and 85%, respectively, compared with those of BH clone poplars. However, transcript level of GA deactivation enzyme, GA2 oxidase, was decreased by 40% compared to WT (Figure 2.11)

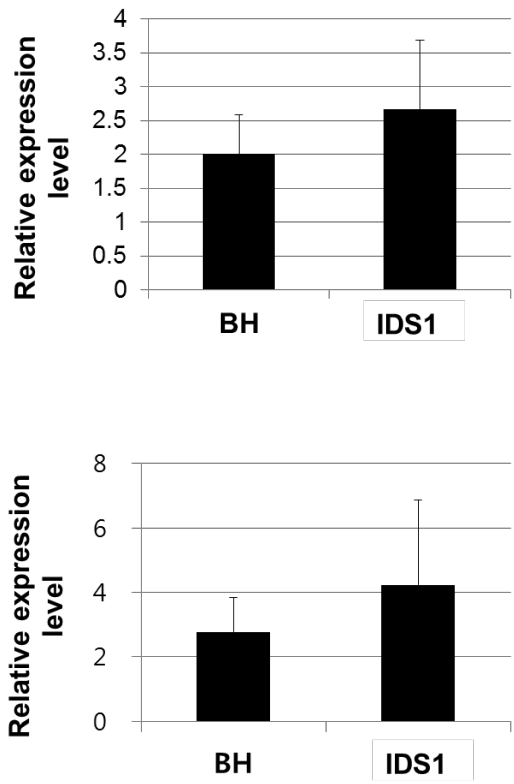


Figure 2.10. Transcript levels of chlorophyll related genes in BH clone and *GbIDS1* overexpression poplars. UP: *CHS*, and DOWN: *CAO*. (BH for $n = 3$, IDS1 for $n = 10$).

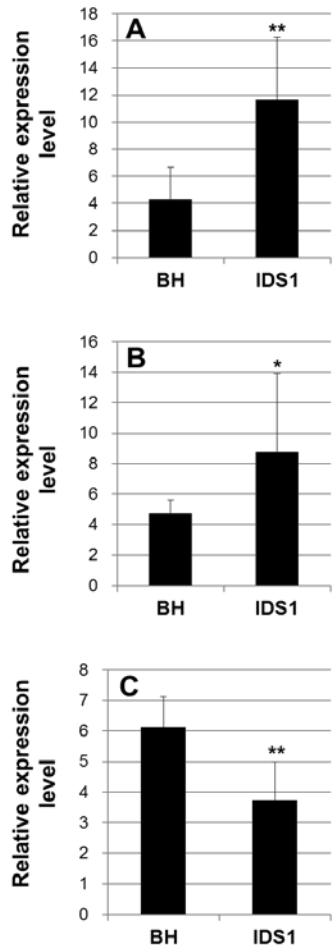


Figure 2.11. Transcript levels of GA related genes in BH clone and *GbIDS1* overexpression poplars. A: *KS*, B: *GA20ox*, and C: *GA2ox*.

* ($P \leq 0.05$) and ** ($P \leq 0.01$), (BH for $n = 3$, IDS1 for $n = 10$).

2.5. DISCUSSION

The first enzyme in MEP pathway, DXS, is known to be encoded by two to three copies of gene in the plants so far examined (Sando *et al.*, 2008; Seetang-Nun *et al.*, 2008; Kim *et al.*, 2006c; Kim *et al.*, 2009; Kim *et al.*, 2005a). In some plant species, genes encoding MEP pathway enzymes other than DXS also exist in multi-copies. One of the first known examples is IDS in Ginkgo and Pinus (Kim *et al.*, 2008b;Kim et al., 2009). It has been suggested that the one of isozymes participates in primary metabolite biosynthesis while the other is involved in the secondary metabolism. Recently importance of IDS gene in the regulation of terpenoids biosynthesis is unraveled. (Botella-Pavía *et al.*, 2004; Kim *et al.*, 2009).

G. biloba is a gymnosperm that has three copies of IDS gene. The isogenes are divided into two clades in phylogenetic analysis, and their functional study was reported by Kim et al (Kim *et al.*, 2008b). Their research supported regulation of metabolic flux between primary and secondary metabolisms; GbIDS1 was suggested to be involved in primary metabolism and GbIDS2 in secondary metabolism.

It was evident that overexpression of IDS gene in poplar resulted in accelerated growth and increased biomass. Diverse groups of terpenoids are involved in growth and development of plants, especially those derived from plastidal terpenoids precursors. For instance, terpenoids such as GA,

chlorophylls, and carotenoids are major group of plastid origin terpenoids. Phytol is a crucial component of chlorophyll structure and is formed from geranylgeranyl diphosphate which in turn originates from plastidal IPP and DMAPP (Joyard *et al.*, 2009). Carotenoids are C_{40} terpenoids whose precursors are derived from the MEP pathway (Walter *et al.*, 2002). Therefore, overexpression of IDS would result in increased level of isoprenoid precursors, IPP and DMAPP, and in turn affects contents of plastidal isoprenoid pigments and GA, which are essential components in plant growth. Therefore contents of chlorophyll and carotenoid were measured in transgenic poplars. The contents of chlorophyll and carotenoid were increased in transgenic poplar plants as expected (Figures 2.8 and 2.9). In addition, transcript levels of chlorophyll biosynthesis related genes were up regulated (Figure 2.10). The previous report presented that IDS is an important enzyme for regulation of carotenoid biosynthesis (Botella-Pavía *et al.*, 2004). This research also supports close relationship between IDS and biosynthesis of the plant pigments.

GAs promote plant growth and flowering (Hedden, 2012). Biosynthesis of GA was assessed in terms of transcript level of key GA-activating and inactivating enzyme gene. The transcript levels of GA biosynthetic genes, KS and GA20ox, were found to undergo dramatic increase, while little change in GA inacting enzyme, GA2ox, was observed (Figure 2.11). Therefore, one can conclude that increased IDS level induced transcription

of genes encoding enzymes in GA biosynthesis without much change of GA-degrading enzyme gene level.

In conclusion, the present research proved that overexpression of *GbIDS1* in poplar positively acted on key enzymes for plastidal isoprenoid biosynthesis. The experimental results, such as rapid growth, up-regulation of terpenoid related genes, and increase of biomass in *IDS1* overexpression transgenic poplars, proved that IDS is a valuable resource for genetic engineering of woody plant for biomass production.

2.6. ABSTRACT (IN KOREAN)

은행은 MEP 대사경로의 마지막 효소인 IDS를 세 벌 가지고 있다. 이들의 역할을 규명하기 위하여 은행 *IDS1*과 *IDS2* 유전자를 35S 프로모터가 삽입되어 있는 벡터에 삽입한 후 아그로박테리움을 이용하여 포플러에 형질전환을 실시하였다. *IDS1* 과발현 포플러 형질전환체는 10개 라인을 확립하였지만, *IDS2* 과발현 포플러 형질전환체는 한 라인도 얻을 수 없었다. 은행의 *IDS1* 유전자의 과발현은 식물의 생장을 촉진시키고 식물의 바이오매스를 증가 시킨다는 사실을 확인할 수 있었다. 실내환경에서 은행 *IDS1* 과발현 포플러는 야생형에 비해 식물체 신장이 25%까지 증가하였으며 2장의 잎을 더 가졌다. 야외 포장에서는 은행 *IDS1* 과발현 포플러는 야생형에 비하여 신장이 7% 증가하였고 동아형성이 지연되었다. *IDS1* 과발현체에서는 클로로필과 카로테노이드 함량이 대략 20%정도 증가함이 확인되었고, 클로로필 생합성 유전자인 *CHS*와 *CAO*의 전사수준이 각각 30%와 50%까지 증가하였다. *IDS1* 과발현 포플러와 야생형 포플러에서 지베렐린 조절 유전자인 *KS*, *GA20ox*, 그리고 *GA2ox*의 전사 수준을 측정하였다. 그 결과, *IDS1* 과발현 포플러에서는 *KS*와 *GA20ox*는 각각 170%와 85%까지 증가하였고, *GA2ox*는 40% 감소하였다. 이러한 결과들을 통하여 은행의 *IDS1* 유전자의 과발현이 IPP와 DMAPP생산을 증가시켜 하위 유전자들에 영향을 주고, 결과적으로 터펜 생산과 바이오 매스 증대에 기여했을 것이라는 판단을 내릴 수 있었다.

PART Ⅲ

Functional Analysis of Truncated *Ginkgo biloba* (*E*)-4-Hydroxy-3-methylbut-2-enyl Diphosphate Reductase/Isopentenyl Diphosphate Synthase (HDR/IDS) 2 Gene in Arabidopsis

3.1. ABSTRACT

(*E*)-4-Hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR) or isopentenyl diphosphate synthase (IDS) is the last step of MEP pathway enzyme, three copies of IDSs are present in *Ginkgo biloba*. In this research, the truncated form of GbIDS2 (tGbIDS2) devoid of signal peptide protein was studied. The *tGbIDS* fused with GFP was introduced into Arabidopsis protoplasts. In spite of signal peptide deletion, tGbIDS2 was targeted to the chloroplast. Although overexpression of *tGbIDS2* was known to lead rapid growth and early flowering in the heterozygote Arabidopsis plant, homozygous *tGbIDS2* overexpression transgenic Arabidopsis exhibited no significant phenotype changes compared to the Col-0 wild type (WT) Arabidopsis. Besides, little change was observed in chlorophyll and carotenoids contents in transgenic plant compared to WT. On the other hand, transcript levels of floral genes and *GA4* displayed differences between WT and transgenic Arabidopsis plants. *CO* levels were up-regulated by 60% but *FLC*, *SOC1*, and *LFY* were down-regulated by 26%, 32%, and 24%, respectively. Transcript level of *GA4* gene was increased by 56% in transgenic Arabidopsis, which infered decrease of GA content in transgenic Arabidopsis compared to WT.

Keywords

Ginkgo biloba, Isoprenoids, MEP pathway, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase/isopentenyl diphosphate synthase (HDR/IDS), flowering, chlorophyll, carotenoid, *CO*, *SOC1*, *FLC*, *LFY*

3.2. INTRODUCTION

Plants produce diverse group of natural products such as isoprenoids, alkaloids, and prenylpropanoids. In particular, isoprenoids comprise over 25,000 compounds and command a majority in natural products (Croteau *et al.*, 2000). Isoprenoids (also called terpenoids) participate in general living processes such as photosynthesis, respiration, and regulation of growth and development (Phillips *et al.*, 2008). Moreover, they provide secondary metabolites protecting plants against herbivores and pathogens, and have been commercially used as flavors, drugs, and so on (Rodriguez-Concepcion, 2004). Even though they are structurally and functionally complex, simple C5 building blocks, isopentenyl diphosphate (IPP) and dimethylally diphosphate (DMAPP), play their precursors. In plants, both of the isoprene units are synthesized by two distinct pathways, mevalonate (MVA) and 2-*C*-methyl-D-erythritol 4-diphosphate (MEP) pathways. The MVA pathway exists in plant cytosol and produces sequi- (C15), tri- (C30) terpenoids, while mono- (C10), di- (C20), tetra- (C40) terpenoids are synthesiszed by the MEP pathway in plant plastid (Croteau *et al.*, 2000).

Gikgo biloba L. has long fossil record since early Jurassic period so that it is called ‘living fossil’. The tree has been used for pharmaceutical purposes (Mahadevan and Park, 2008), and also its insecticidal activity was reported recently (Lee *et al.*, 2005). Ginkgolides, chemically classfied as diterpene lactones, are found in *G.biloba.* They are known to be derived from MEP

pathway (Singh *et al.*, 2008). The multi-copy nature of IDS, the terminal enzyme in MEP pathway, has been reported in gymnosperms such as *Pinus taeda*, *P. densiflora*, and *Cycas revoluta* (Kim *et al.*, 2008b; Kim *et al.*, 2009). In particular, *G. biloba* has three copies of IDSs, which are divided into two class (Kim *et al.*, 2008b). The previous report demonstrated tissue-specific expression pattern and response of GbIDSs toward to the light and MeJA treatment. The previous research supported that GbIDS1 is related with production of primary metabolites while GbIDS2 with secondary metabolites (Kim *et al.*, 2008b).

In tobacco plants, overexpression of *GbIDS2* resulted in rapid growth and increase of biomass, while in poplars no *GbIDS2* transformants were acquired (Kim, 2009). The heterozygous Arabidopsis *tGbIDS2* overexpression transformants also showed biomass increase (Sultana, 2010). This experiment was performed to confirm the previous work with heterozygous *tGbIDS2* overexpression Arabidopsis in homozygous lines.

3.3. MATERIALS AND METHODS

3.3.1. Construction of plasmid

The truncated form of *G. biloba IDS2* (*tGbIDS2*) region (Figure 3.1) was amplified with designed primers (forward: TCTAGAATGTGCCATGGAGTTTCCACT, reverse: GGATCCGCCGCTACTTGCAAGGTCTCTT) by PCR and inserted into the pBI121 vector (Figure 3.2). The construct was introduced into the *Agrobacterium tumefaciens* C58C1 strain for transformation using the freeze-thaw method (Weigel and Glazebrook, 2006).

Figure 3.1. Nucleotide sequences of *GbIDS2* and *tGbIDS2*.

3.3.2. Arabidopsis growth condition and transformation

Arabidopsis thaliana (Columbia ecotype) seeds were sterilized by soaking in 70% ethanol for 5 minutes and washed two times with absolute ethanol. And then the seeds were dried on the germ-free filter paper till the trace of alcohol was completely removed. After this process, the seeds were sown on 1/2 MS medium, and then kept on 4°C for 3 days for vernalization. Thereafter, the plates were transferred in a growth chamber at 23°C and plants were grown under 16/8 h light/dark photoperiod. To observe Arabidopsis phenotype, the 4-leaf-stage plants were transferred from plate onto soil, and grew under the same condition as plates until harvest.

The Arabidopsis transformation was performed using floral dip method (Clough and Bent, 1998), and the transformants were selected on the 1/2 MS medium containing kanamycin at 50 mg/L. For this study, T3 homozygous *A. thaliana* transgenic plants were used.

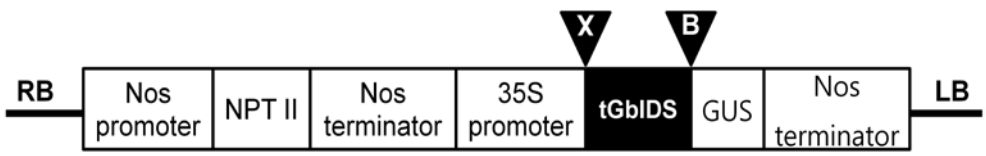


Figure 3.2. Construction of the T-DNA region of *tGbIDS2*.

X: *Xba*I, B: *BamH*I

3.3.3. Transient expression in Arabidopsis protoplast

The tGbIDS2 sequence was amplified with primers (forward: CACGGGGGACTCTAGAATGTGCCATGGAGTTTCCACT, reverse: TATATCTCCTGGATCCGCCGCTACTTGCAAGGTCTCTT) and fused to pSMGFP vector for the transient expression in Arabidopsis protoplast. The protoplasts were isolated using the Sheen's lab protocol with some modifications (Yoo *et al.*, 2007). Arabidopsis leaves from about 4-week-old plants were cut at intervals of about 1 mm and then transferred to lysis solution (1% cellulose R 10, 0.2% macerozyme R 10, 0.4 M mannitol, 20 mM KCl, 20 mM MES (pH 5.7), 10 mM $CaCl_2$, and 0.1% BAS). After overnight incubation with gentle shaking, the solution was filtered through a 100 μm nylon mesh. For precipitation of protoplasts, the filtered solution was provided with an equal volume of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 2 mM MES (pH 5.7) and centrifuged at 500 rpm for 5 min. The pelleted protoplasts were resuspended in 5 ml of cold W5 solution and incubated for 3 h at 4ºC. After centrifugation at 500 rpm for 5 min, the pelleted protoplasts were resuspended in MaMg solution (0.4 M mannitol, 15 mM $MgCl_2$, and 4 mM MES, pH 5.7). Protoplasts were transfected with the constructed plasmids using a modified polyethylene glycol method as described for *A. thaliana* (Abel and Theologis, 1994). Each plasmid of 20 μg DNA (1 μg/μl) was transferred into 300 μl protoplasts suspension (10^6/ml), and the transformed protoplasts were incubated at 22ºC overnight in the dark. Expression of the fusion protein

was observed with Image Restoration Microscopy (Deltavison RT, Applied Precision, USA)

3.3.4. Phenotypic assessment

The phenotype of the Col-0 and *tGbIDS2* overexpression transgenic Arabidopsis was observed regularly. The height and rosette leaf numbers of both Arabidopsis plants were measured 1 week after bolting. And also the height of both Arabidopsis plants were checked when the plant growth was completed.

3.3.5. Measurement of chlorophyll and carotenoid contents

Leaves of 14-day-old Arabidopsis seedlings were collected and weighed. The samples were ground in liquid nitrogen and added with 80% acetone, and then shaken in the dark for 15 min. The extracts were centrifuged at 4500 rpm for 15 min at 4ºC. The supernatants were collected into new containers then absorbance was measured at 470, 645, and 663 nm. The chlorophyll and carotenoid contents were calculated following the method of Ni et al. (Ni *et al.*, 2008).

Chl *a* (mg/g) = $(12.7 \times A_{663} - 2.69 \times A_{645}) \times V/(1000 \times W)$

Chl *b* (mg/g) = $(22.9 \times A_{645} - 4.86 \text{ x } A_{663}) \times V/(1000 \times W)$

Chl *a*+*b* (mg/g) = $(8.02 \times A_{663} + 20.20 \times A_{645}) \times V/(1000 \times W)$

Car (μg/ml) = $(1000 \times A_{470} - 1.82 \times \text{Chl a} - 85.02 \times \text{Chl b})/198$

V indicates volume of the extracts (ml) and W was weight of fresh leaves (g).

3.3.6. RNA preparation and reverse transcription

Total RNAs were isolated from young leaves of poplars growing in test tubes. The plants were ground in liquid nitrogen and then the fine powders were transferred into the 1.5 ml Eppendorf tubes. Same volume of RNA extraction buffer (200 mM Tris-HCl, pH 8.0; 20 mM EDTA; 300 mM NaCl; 1% SDS) and water saturated phenol was added to the sample and vortexed. Then the mixtures were centrifuged for 3 min at 10000 *g* at room temperature. The supernatants were extracted with 0.55 ml of chloroform, and then centrifuged again. Fifty five milliliters of sodium acetate solution (3 M, pH 5.3) and 1 ml absolute ethanol was added into the supernatant and chilled for 30 min at -20ºC. Then the samples were centrifuged for 15 min at 10000 *g* at 4ºC and the supernatants were removed. Three hundred microliters of 2 M LiCl was added to pellet and chilled on ice for 30 min. The samples were centrifuged again and the pellet was resuspended in 0.3 ml of ddH_2O. After 10 min of ice incubation, 3M sodium acetate (pH 5.3) and 0.3 ml of absolute ethanol were combined with the samples and chilled for 5 min at -20ºC. The samples were centrifuged at 10000 *g* at 4ºC for 10 min, supernatants were removed, and finally the pellets were dried. The total RNA was resuspended in 30 µl ddH_2O. The RNA concentration of each

sample was measured on a micro-volume spectrometer (ASP-3700, ACTgene, USA). One microgram of RNA was used to synthesize the first strand cDNA using the cDNA synthesis kit (Philekorea Technology, Korea) according to the manufacture's protocol.

3.3.7. qRT-PCR analysis

Transcript levels of various genes in poplars were determined by using quantitative real-time polymerase chain reaction (qRT-PCR) on Rotor-Gene 2000 (Corbett Research, Mortlake, NSW, Australia), using 50 ng of the cDNA with a QuantiMix SYBR Kit (Philekorea Technology). The PCR condition was according to the manufacturer's recommendation. The primers used in this study are listed in Table 2.3.

3.3.8. Statistical analysis

The data as obtained in this experiment was subject to statistical analysis. Student's t-test was performed by using SAS (version 9.1). Statistical significances were denoted as * ($P \leq 0.05$) and ** ($P \leq 0.01$).

Table 3.1. Primers used in qRT-PCR for Arabidopsis floral genes and *GA4*.

Primer	**Forward (5’ → 3’)**	**Reverse (5’ → 3’)**
CO	CGGGTCTGCGAGTCATGTGA	GTGAGTAGTGGTCATGGAGCTGAA
SOC1	GAGCTGCAACAGATTGAGCAACA	CGTCTCTACTTCAGAACTTGGGCTAC
FLC	GCATGGGTCGCTCTTCTCGT	CACAAGTTCAAGTAGCTCATAGTGTGAA
LFY	TCGTTGGGAGCTTCTTGTTG	ACCCTTCTTGGGAGAGAGCA
GA4	CGATTTCCGTAAACTTTGGC	ATCCATTGGATAGGATGTGG
APT1	TCCCAGAATCGCTAAGATTGCC	CCTTTCCCTTAAGCTCTG

3.4. RESULTS

3.4.1. RT-PCR analysis

To confirm the existence of tGbIDS2 transcripts, RT-PCR analysis was performed from Col-0 and homozygote transgenic Arabidopsis lines. Band of about 1,300 bp-long, as expected from the sequence of tGbIDS2, were amplified from each transgenic Arabidopsis lines, whereas no PCR bands were found in wild-type Col-0 Arabidopsis (Figure 3.3).

3.4.2. Targeting analysis of tGbIDS2 in Arabidopsis protoplasts

The N-terminal transit peptides of GbIDS2 localized the protein in chloroplast (Kim *et al.*, 2008b). In this experiment, predicted N-terminal transit peptide sequence was deleted and fused with pSMGFP vector for transient expression. Even the without the putative N-terminal transit peptide sequence, expression of tGbIDS2::GFP fusion protein was detected in chloroplast (Figure 3.4).

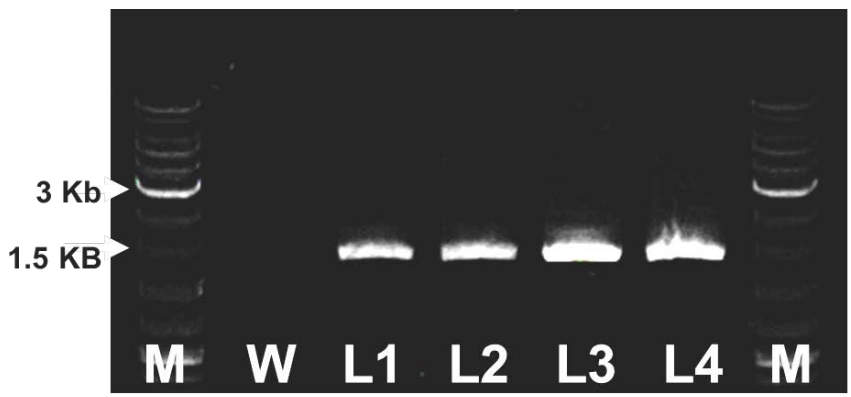


Figure 3.3. PCR bands of *tGbIDS2* coding region from Col-0 and *tGbIDS2* overexpression transgenic plants. M: Marker, W: Col-0, L1-L4: *tGbIDS2* overexpression transgenic Arabidopsis lines.

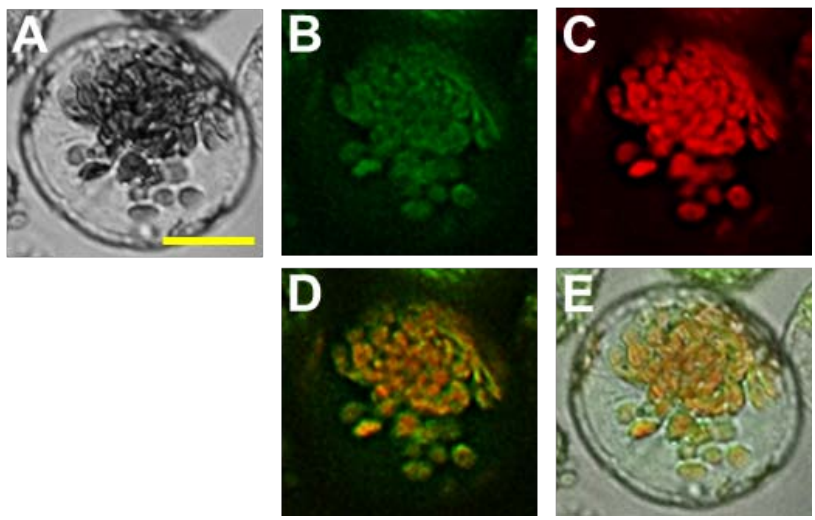


Figure 3.4. Subcellular localization of the fusion constructs of tGbIDS2 with GFP. Yellow line indicates 25 μm. A: bright image, B: GFP signal, C: chloroplast autofluorescence, D: B and C merged image, E: A, B, and C merged image.

3.4.3. Growth and flowering time of *tGbIDS2* overexpression transgenic Arabidopsis

The truncated form of *GbIDS2* (tGbIDS2) gene was overexpressed in Arabidopsis plants and their growth was observed. In homozygous *tGbIDS2* overexpression Arabidopsis plants, there were no significant phenotype changes compared to the Col-0 Arabidopsis plants (Figures 3.5 and 3.6). Rosette leaf numbers and days for bolting are considered as main characteristics deciding environmental factors and genotypes (Koornneef *et al.*, 1998). In this experiment, homozygous *tGbIDS2* overexpression Arabidopsis displayed normal rosette leaf numbers and flowering patterns in contrast to the heterozygote *tGbIDS2* overexpression transgenic Arabidopsis plants (Figure 3.5).

3.4.4. Chlorophyll and carotenoid contents

MEP pathway produces IPP and DMAPP, the precursors to construct the chlorophyll and carotenoids molecules. Chlorophyll and carotenoid contents could provide major information of plant growth and development status. In addition, the pigment contents provide evidence for role of IDS genes.

In heterozygote *tGbIDS2* overexpression Arabidopsis plants, contents of chlorophyll and carotenoid is reported to be higher than Col-0 Arabidopsis (Sultana, 2010). However, in the homozygote Arabidopsis, contents of both pigments were not significantly different from Col-0 plants (Figures 3.7 and 3.8).

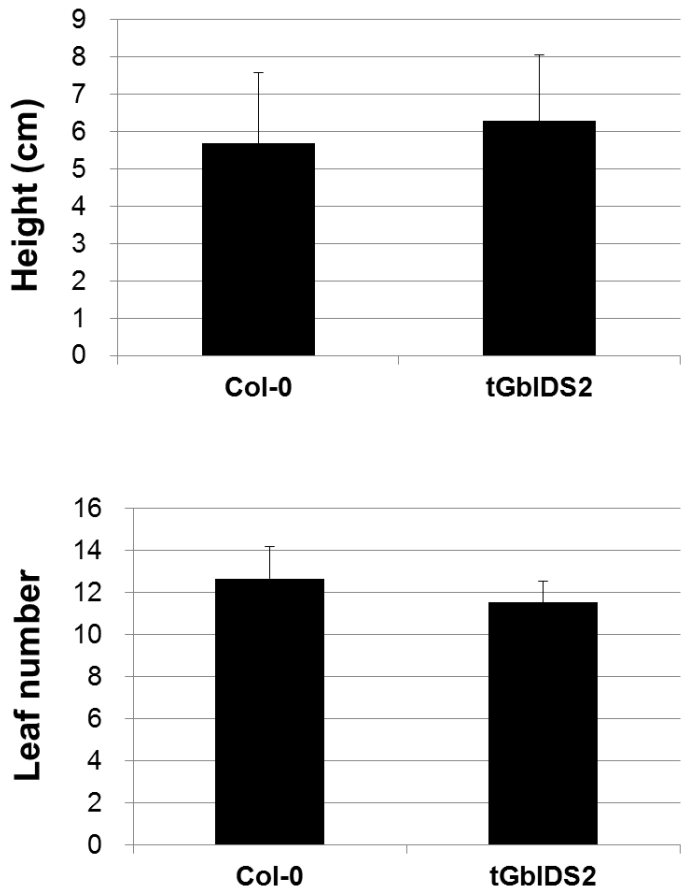


Figure 3.5. Inflorescence stem length and rosette leaf numbers of Col-0 and *tGbIDS2* overexpression Arabidopsis plants 7 days after bolting. (*n* = minimum 3, maximum 5).

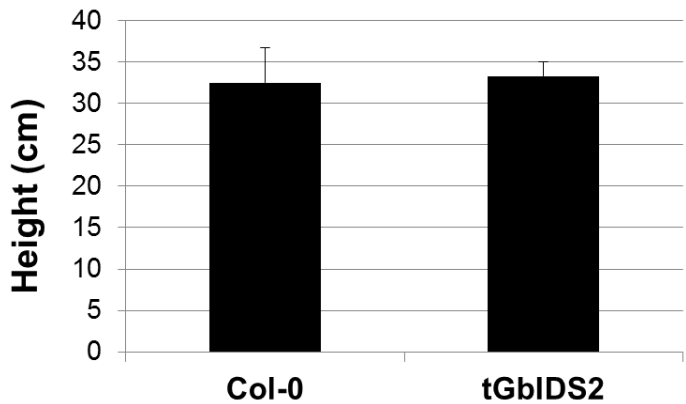


Figure 3.6. Inflorescence stem length of Col-0 and *tGbIDS2* overexpression Arabidopsis plants at harvest stage. (*n* = minimum 3, maximum 5).

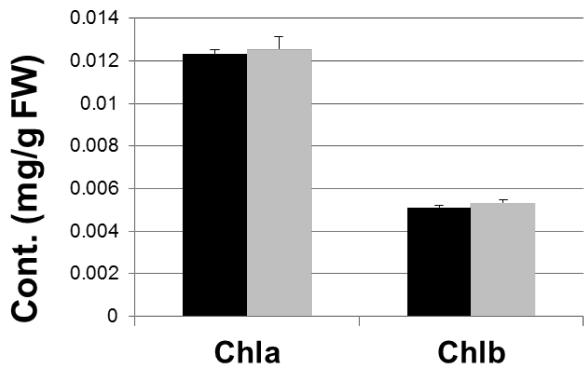


Figure 3.7. Chlorophyll contents of Col-0 and *tGbIDS2* overexpression transgenic Arabidopsis plants. Col-0 (black), tGbIDS2 (grey) (*n* = minimum 3, maximum 5).

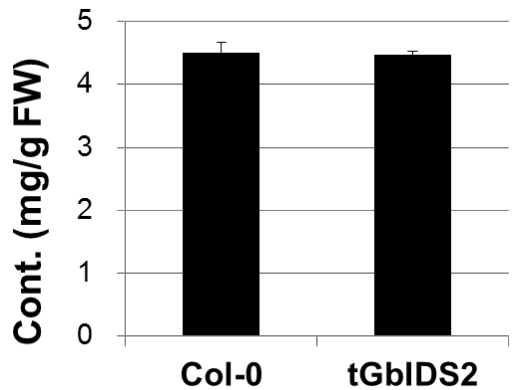


Figure 3.8. Carotenoid contents of Col-0 and *tGbIDS2* overexpression transgenic Arabidopsis plants. (*n* = minimum 3, maximum 5).

3.4.5. Transcript levels of floral genes

Some flowering regulatory genes, *CO*, *FLC*, *SOC1*, and *LFY* were selected to assess changes in their transcript levels upon homozygous overexpression of *tGbIDS2*. The *CO* transcript levels were up-regulated in homozygote *tGbIDS2* overexpression Arabidopsis by 60% (Figure 3.9A). In contrast to *CO*, transcript levels of *SOC1*, *FLC*, and *LFY* were decreased by 32%, 26%, and 24% respectively in transgenic plants (Figure 3.9B-D).

3.4.6. Transcript level of *GA4* gene

It is difficult to measure the GA levels because of the wide range of active and inactive GAs. In this reason, *GA4* gene was used as a marker for the determination of GA level (Cowling *et al.*, 1998). *GA4* gene is regulated by a negative feedback mechanism, therefore the relative level of the *GA4* gene show inverse relationship to the amount of active GA present in plants. In this analysis, *GA4* transcript level was higher by 56% in homozygote *tGbIDS2* overexpression transgenic plants than Col-0 Arabidopsis (Figure 3.10), signifying that homologous overexpression of *tGbIDS2* resulted in lowering the level of active GA level.

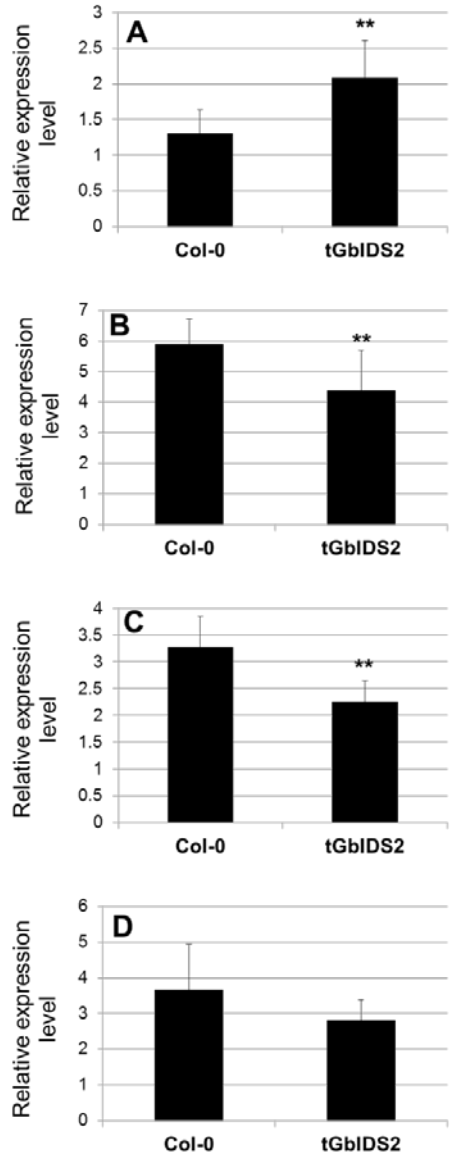


Figure 3.9. Transcript levels of floral genes in Col-0 and *tGbIDS2* overexpression Arabidopsis plants. A: *CO*, B: *SOC1*, C: *FLC*, D: *LFY*. (*n* = minimum 3, maximum 5).

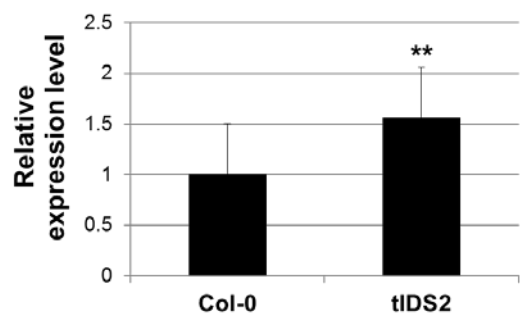


Figure 3.10. Transcript level of *GA4* gene in Col-0 and *tGbIDS2* overexpression Arabidopsis plants.
(n = minimum 3, maximum 5).

3.5. DISCUSSION

In the previous research, heterozygous overexpression of *tGbIDS2* in Arabidopsis has been shown to accelerate plant growth (Sultana, 2010). It is interesting that tGbIDS2 was targeted to chloroplast even without predicted targeting peptide sequence (Figure 3.4). In order to establish the stable tGbIDS2 transgenic Arabidopsis, homozygote lines were developed and phenotype of the lines were observed. However, the performance of homozygous Arabidopsis in terms of biomass was no better than WT. Particularly, no significant changes in growth rate, flowering time, rosette leaf numbers, and contents of chlorophylls and carotenoids were observed in transgenic Arabidopsis compared to Col-0. The phenotype of the homozygous transgenic plant was partially explained by floral gene expression and gibberellin level indirectly assessed by *GA4* gene in the homozygous plant. Flowering is regulated by the complex networks of genes such as *CO, SOC1*, *FLC,* and *LFY*. The up-regulation of *CO* and down-regulation of *FLC* in the homozygous transgenic plants were same as the behavior of the genes in the heterozygous transgenic plant. However, the down regulation of *SOC1*, as opposed to the previous finding with the heterozygous plant, was observed in the present work. The *CO* gene is activator of floral integrators *SOC1* and *LFY* (Samach *et al.*, 2000) and *FLC* a repressor. However, down-regulation of *SOC1* and *LFY* was observed in the present study. Therefore, factor(s) other than CO and FLC must operate

in the homozygous plant. *SPL*s, negatively regulated by miR156, are known to control age-related flowering pathway. It is highly possible that activation of miR156 pathway could be the reason for the reduced integrator gene expression.

In the part 2 of this thesis, it was noted that poplar lines overexpressing *GbIDS2* was not feasible to establish. However, in Arabidopsis, *GbIDS1* overexpression was difficult to obtain the tansfromatns had low germination and poor growth. It signifies that behavior of GbIDS1 and GbIDS2 in Arabidopsis might be different. Overproduction of isoprene precursors might be the reason for the negative effect. In conclusion, heterozygous *tGbIDS2* overexpression Arabidopsis showed accelerated growth and increased biomass, in while such positive effect was not observed in the homozygous plant. In fact, tGbIDS2 affected rather negatively in Arabidopsis growth was reported in homozygous *GbIDS2* overexpression tobacco. For this reason, it was impossible to apply tGbIDS2 and GbIDS2 in homozygous plant.

3.6. ABSTRACT (IN KOREAN)

은행에는 MEP pathway의 마지막 단계 효소인 IDS가 세 벌로 존재하며 본 연구에서는 GbIDS2의 시그널 펩타이드를 제거한 truncate 상태의 GbIDS2 (tGbIDS2)의 연구를 시행하였다.

tGbIDS2를 GFP 단백질과 결합시켜 애기장대의 원형질체에서 발현 시켜본 결과, 시그널 펩타이드를 제거했음에도 GFP 발현이 엽록체에서 나타났다. 애기 장대 식물에서 은행 *tIDS2* 유전자 과발현 homozygote 식물체에서는 heterozygote 식물체와 달리 빠른 식물 생장이나 개화 촉진과 같은 결과를 확인 할 수 없었다. 또한 식물의 클로로필과 카로테노이드의 함량 측정에서도 형질전환체 식물과 야생형에서 차이를 확인 할 수 없었다. 하지만 개화 유전자들과 *GA4* 유전자의 전사 수준을 측정한 결과, 형질전환체 식물과 야생형에서 차이가 나타났다. 개화 유전자들의 전사수준을 측정한 결과, *CO*는 형질전환체에서 60%까지 증대하였고, *FLC*, *SOC1*, 그리고 *LFY* 유전자들의 전사 수준은 각각 26%, 32% 그리고 24%까지 감소하였다. 또한 형질전환체에서 *GA4* 유전자의 전사 수준이 56%까지 증가한 것으로 보아 지베렐린 함량이 감소하였다는 것을 간접적으로 확인 할 수 있었다.

OVERALL ABSTRACT (IN KOREAN)

이소프레노이드 (isoprenoid)는 다섯 개의 탄소로 이루어진 기본 단위인 IPP와 이성질체인 DMAPP로부터 생합성되며, 터페노이드 (terpenoid) 라고도 알려져 있다. 이 화합물들은 모든 생명체에서 만들어지지만, 특히 식물에서는 좀 더 다양하고 많은 종류의 화합물들이 생성된다. 그리고 식물에는 이소프레노이드를 생산하는 MVA와 MEP pathway라는 두 가지 서로 다른 합성 경로가 존재한다. 본 연구에서는 이 두 생합성 경로 중 식물체 plastid내에 존재하는 MEP pathway의 마지막 효소인 IDS를 중점적으로 다루어보았다. '살아있는 화석'이라고도 불리는 은행 나무는 속씨 식물에 속하며, IDS가 세 벌로 존재하고 두 개의 그룹으로 나누어진다. GbIDS1은 첫 번째, GbIDS2와 GbIDS2-1은 두 번째 그룹에 속하며, 각각 일차와 이차 터펜 생합성 대사로 나누어 참여한다. 본 논문에서는 은행나무 IDS 유전자의 프로모터와 과발현 연구가 각각 애기장대와 포플러를 이용하여 수행되었다. 은행의 IDS1과 IDS2 프로모터들을 각각 GUS 단백질과 연결 시킨 후 애기 장대에 도입 하였다. 은행의 IDS1 프로모터가 도입된 애기 장대에서는 뿌리, 꽃잎 그리고 수술을 제외한 식물체 거의 모든 조직과 기관에서 GUS 발현이 확인되었다. 반면, 은행의 IDS2 프로모터를 도입한 애기장대에서는 식물의 어린 잎과 분화 조직 그리고 뿌리의 특정 부위(원뿌리와 하배축 사이의 접합 영역)에서만 GUS가 발현 되었다. 이러한 GUS 발현 양상은 RT-PCR과 qRT-PCR을 통해 확인 된 은행의 *IDS1*과 *IDS2*의

전사 양상과도 상관관계를 가진다. 은행 IDS 프로모터가 도입된 애기장대 식물에 스트레스 유도인자인 MeJA를 처리한 결과, 은행 IDS1 프로모터가 도입 된 식물체 잎에서는 GUS activity가 증가하였고 IDS2 프로모터가 도입 된 식물체의 뿌리에서는 activity가 감소하였다. 이와 같은 유전자 조절 양상은 IDS2 프로모터가 도입된 애기장대 뿌리에서 GA, ABA, 그리고 IBA를 처리하였을 때도 관찰되었다. 은행의 IDS 과발현 실험을 포플러에서 시행하기 위하여 *IDS1*과 *IDS2* 유전자를 35S 프로모터가 삽입되어 있는 벡터에 도입한 후 아그로박테리움을 이용하여 포플러에 형질전환을 실시하였다. 그 결과 10개 라인의 *IDS1* 과발현 포플러 형질전환체를 확립하게 되었다. 하지만 *IDS2* 과발현 포플러 형질전환체는 한 라인도 확립할 수 없었다. 은행 *IDS1* 과발현 형질전환 포플러는 실내 환경 하에서 25%의 신장 증대와 2장의 잎 수 증가를 보였으며, 실외 포장으로 옮겨진 후에는 야생형 포플러에 비하여 7% 정도의 신장이 증대 되었고 동아 형성이 지연되는 것이 관찰되었다. 또한 *IDS1* 과발현체에서는 클로로필과 카로테노이드 함량이 대략 20%정도 증가함이 확인되었고, 클로로필 생합성 유전자인 *CHS*와 *CAO*의 전사수준이 각각 30%와 50%까지 증가하였다. *IDS1* 과발현 포플러와 야생형 포플러에서 지베렐린 조절 유전자들의 전사 수준을 측정한 결과, *IDS1* 과발현 포플러에서는 *KS*와 *GA20ox*는 각각 170%와 85%까지 증가하였고, *GA2ox*는 40% 감소하였다. 이러한 결과들을 통하여 은행의 *IDS1* 유전자의 과발현이 IPP와 DMAPP생산을 증가시켜 하위 유전자들에 영향을 주고, 결과적으로 터펜 생산과 바이오

매스 증대에 기여했을 것이라는 판단을 내릴 수 있었다. 은행의 tGbIDS2(시그널 펩타이드를 제거한 GbIDS2)를 GFP 단백질과 결합시켜 애기장대의 원형질체에서 발현 시켜본 결과, 시그널 펩타이드를 제거했음에도 GFP 발현이 엽록체에서 나타났다. *tGbIDS2* 과발현 애기장대 homozygote 식물체에서는 heterozygote 식물체와 달리 빠른 생장속도나 개화 시기의 촉진을 확인 할 수 없었다. 또한 클로로필과 카로테노이드의 함량에서도 형질전환 애기장대와 야생형 애기장대에서 차이를 보여주지 못했다. 하지만 개화 관련 유전자와 *GA4*유전자의 전사 수준에서는 두 그룹간에 차이가 나타났다. 개화 유전자들의 전사수준을 측정한 결과, *CO*는 형질전환체에서 60%까지 증대하였고, *FLC*, *SOC1*, 그리고 *LFY* 유전자들의 전사 수준은 각각 26%, 32% 그리고 24%까지 감소하였다. 또한 형질전환체에서 GA4 유전자의 전사 수준이 56%까지 증가한 것으로 보아 지베렐린 함량이 감소하였다는 것을 간접적으로 확인 할 수 있었다. 이러한 실험들을 통하여 은행 IDS들의 생리학적 차이점들을 규명할 수 있었으며, 은행 IDS 유전자들의 유전공학적 응용 가능성을 평가해 볼 수 있었다.

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