5-2007

Genetic manipulation of auxin and ethylene production to alter the growth and development of *Populus*

Joo Young Kim
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Joo Young Kim entitled "Genetic manipulation of auxin and ethylene production to alter the growth and development of *Populus*." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant Sciences.

Zong-Ming Cheng, Major Professor

We have read this thesis and recommend its acceptance:

Robert N. Trigiano, Gerald A. Tuscan, Timothy J. Tschaplinski, Timothy G. Rials

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting herewith a thesis written by Joo Young Kim entitled "Genetic manipulation of auxin and ethylene production to alter the growth and development of *Populus*." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science, with a major in Plant Sciences.

_____

Zong-Ming (Max) Cheng, Major Professor

We have read this thesis
and recommend its acceptance:

Robert N. Trigiano
_____

Gerald A. Tuscan
_____

Timothy J. Tschaplinski
_____

Timothy G. Rials
_____

Accepted for the Council:

Linda R. Painter
_____

Vice Provost and Dean of the
Graduate Studies

(Original signatures are on file with official student records.)

GENETIC MANIPULATION OF AUXIN AND ETHYLENE PRODUCTION TO ALTER THE GROWTH AND DEVELOPMENT OF *POPULUS*

A thesis
Presented for the
Master of Sciences Degree
The University of Tennessee, Knoxville

Joo Young Kim
May 2007

DEDICATION

This thesis is dedicated to my husband, daughter and son,

Keun Ho, Grace and Joseph.

Because of their support, patience, trust, and encouragement,

I could achieve my goal.

I love and thank them from the bottom of my heart.

ACKNOWLEDGEMENTS

I would like to thank all those who helped me finish my Master of Sciences degree in the Department of Plant Sciences at the University of Tennessee. First, I thank my major professor, Dr. Zong-Ming (Max) Cheng who led me with his guidance and effort. Without his trust, I might not have completed this work. I also thank Dr. Timothy G. Rials, Dr. Robert N. Trigiano, Dr. Gerald A. Tuscan, and Dr. Timothy J. Tschaplinski for serving on my committee. They helped me get familiar with my study and gave me challenges and advice. I specially appreciated Dr. Timothy Tchaplinski for measuring auxin contents for this work and Dr. Carl Sams for his advice and help in measuring ethylene contents.

I would also like to thank my lab colleagues, Lori Osburn, Kim Pickens, Byung Guk Kang, and Jianhua (Jim) Wei. They helped in so many ways whenever I faced hardship. They always listened to me, and are real friends who shared my feelings. Especially, Lori has always helped me whenever I needed, and I thank her for her kindness.

Most of all, I'd like to thank Keun Ho, Grace and Joseph, my husband and young children for their trust, encouragement, and patience, although I have not paid scrupulous care on them. They always love me and understand me. They are priceless to me, and I love them from the bottom of my heart. I would also like to thank my mother and mother-in-law for their love and concern for me.

Lastly, I am thankful to God for his unchangeable love and care. I have been able to continue the challenging research and complete my degree because of his encouragement whenever I was in stress and fell down. He always makes me stand up again and again, and gives me much stronger will to move on. Without him, I never would have had confidence in myself and finished my degree. The accomplishment of my degree makes me realizes me that I will always face challenges, and the willingness and commitment to complete my work will make me successful in my future. I really appreciate all who helped me to get this precious experience.

ABSTRACT

Populus is one of the most important tree species for pulp, paper, wood products, and more recently for biomass energy. The increasing need for wood and decreasing land area for forest trees demand the development of fast-growing trees with desirable quality. This experiment was conducted to alter poplar growth and development by manipulating endogenous auxin and ethylene levels through genetic transformation.

Since auxin stimulates vascular differentiation and wood formation, indole-3-acetic acid (IAA) biosynthetic gene, *iaaM*, driven by a vascular specific promoter, glycine-rich protein promoter (GRP), was inserted into a hybrid aspen (*P. canescens* x *P. grandidentata*) to increase the endogenous auxin level. However, because elevated auxin can trigger overproduction of ethylene, which can inhibit plant growth, an ethylene inhibition gene, *1-acetocyclopropane-1-carboxylic acid deaminase* (*ACC deaminase*) under the control of cauliflower mosaic virus 35S (CaMV35S) promoter conjugated with GRP-*iaaM* gene was also inserted into the aspen via the *Agrobacterium tumefaciens* Ti plasmid vectors. Fourteen transgenic plants having GRP-*iaaM* and thirteen transgenic plants having GRP-*iaaM*-35S-*ACC deaminase* genes were confirmed by polymerase chain reaction (PCR) and Southern blot. Transgenic plants were propagated by cuttings and their heights and diameters were measured to determine the effect of the inserted genes on aspen. The amounts of IAA and ethylene were also measured to investigate the expression of the inserted genes.

Two lines in 2003 and four lines in 2004 of the GRP-*iaaM*-35S-*ACC deaminase* transgenic plants were significantly taller than non-transgenic plants while the GRP-*iaaM* transgenic plants were shorter or similar height with non-transgenic plants. No significant differences in the growth of height and diameter of the GRP-*iaaM* transgenic plants may be due to the use of a weak promoter because the levels of IAA showed slightly higher than non-transgenic plants, but the differences were not statistically significant. The GRP-*iaaM*-35S-*ACC deaminase* transgenic plants showed no significant differences in IAA levels, but had less or similar levels of ethylene compared to non-transgenic control while the GRP-*iaaM* transgenic plants had elevated ethylene amount. This indicates that the *iaaM* gene expressed at a low level increased the level of ethylene, but the *ACC deaminase* gene appeared to reduce the elevated ethylene in the GRP-*iaaM*-35S-*ACC deaminase* transgenic plants.

The GRP-*iaaM*-35S-*ACC deaminase* transgenic plants having modified IAA and ethylene levels showed more growth in height and volume than the GRP-*iaaM* transgenic in the linear contrast, and a negative correlation between ethylene amounts and heights was shown. This result indicates that an increased auxin level might have a negative effect in the growth of trees resulted from elevated ethylene level triggered by elevated auxin, but a decreased ethylene level in addition to an increased auxin level might have a positive effect on the growth of trees. Although further detailed analyses are needed, this research suggests that it is possible to manipulate plant hormones, especially ethylene, to change plant growth.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ACC	1-acetocyclopropane-1-carboxylic acid
BA	benzyl aminopurine
CAD	cinnamoyl alcohol dehydrogenase
CaMV	cauliflower mosaic virus
COMT	caffeic acid O-methyl transferase
Ethrel	2-chloroethylphosphonic acid
GRP	glycine rich protein
GUS	β-glucuronidase
IAA	indole-3-acetic acid
IAM	indole-3-acetamide
35S	cauliflower mosaic virus 35S
nptII	neomycin phosphotransferase II gene
PCR	polymerase chain reaction
WPM	woody plant medium

CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1. Introduction

Populus is one of the most important genera of forest trees utilized for pulp and paper, many different wood products and biomass energy (Tzfira et al., 1998b). The commercial poplar plantation area is growing rapidly in the United States (Han et al., 1997; Tuskan, 1998). In addition, *Populus* trees play important roles in soil conservation, oxygen production and carbon sequestration, and potentially in the phytoremediation of contaminated soils and groundwater (Tuskan, 1998; Rishi et al., 2001). *Populus* also contribute to the aesthetic and recreational aspects of urban and rural areas.

Long generation time, large size, and the lack of genetic information of forest trees have been main obstacles to efficient traditional forest breeding (Tzfira et al., 1998b). The progress of breeding of many tree species is limited because of the long time from seed germination to flowering, and because most forest tree traits can be assessed only when the tree reaches maturity (Altman, 2003). As in agronomic crop breeding, tree breeders choose parent trees that have particularly desirable characteristics, make crosses, evaluate seedling, and select new hybrids in field trials (Cheliak and Rogers, 1990). These traditional techniques have been costly and time consuming as well as requiring a large space in the field. Biotechnological approaches, especially genetic transformation, can complement conventional breeding by the insertion of genes which confer desired

traits are not readily available in sexually accessible gene pools (Han et al., 1997; Dinus et al., 2001). Genetic engineering has made considerable contributions to plant genetic improvement (Brunner et al., 1998).

Wood formed by the vascular cambium during secondary growth is one of the most important resources, and demand for traditional wood products and the use of wood for biomass energy is growing (Tzfira et al., 1998b). As forest trees are threatened by pests, acid rain, and desertification and deforestation, more effort has been placed on the production of fast growing and more pest- and stress-tolerant high quality trees (Tzfira et al., 1996). One of the important goals in forest tree breeding is to alter plant growth rate and wood properties. This may be achieved by genetic transformation to alter endogenous hormone levels. Auxin is a crucial factor for stimulating vascular differentiation and wood development (Eklund and Little, 1994; Avsian-Kretchmer et al., 2002). The increase of endogenous auxin level can be accomplished by the expression of IAA biosynthetic *iaaM* and *iaaH* genes of *Agrobacterium tumefaciens* T-DNA (Klee and Lanahan, 1995). The *iaaM* gene encodes for tryptophan monooxygenase which catalyzes the conversion of tryptophan into indole-3-acetamide, and the *iaaH* gene encodes for indole acetamide hydrolase which converts indole-3-acetamide into indole-3-acetic acid (IAA) (Follin et al., 1985). It has been demonstrated that increased wood production can be achieved by the over-production of endogenous auxin in transgenic plants (Tuominen et al., 1995; Grünward et al., 2000). However, auxin stimulates the production of ethylene (Mckeon and Yang, 1987). The over-production of ethylene mediated by elevated auxin was reported in the transgenic plants expressing the *iaaM* gene (Romano

et al., 1993). The down-regulation of ethylene can be achieved by the insertion of *ACC deaminase* gene. ACC deaminase is an enzyme that degrades 1-acetocyclopropane-1-carbolylic acid (ACC) to α-ketobutyric acid, which prevents ethylene synthesis and reduces ethylene levels. The elevated level of ethylene triggered by the expression of the *iaaM* gene was efficiently reduced by a transformation with the *ACC deaminase* gene as demonstrated in tomato plants (Klee et al., 1991; Romano et al., 1993).

1.2. Rationale and objectives

Wood and fibers are leading industrial raw materials and have an important impact on the economy. *Populus*, being one of the most important tree genera, has great potential as a biomass and energy crop (Tuskan, 1998). Since auxin stimulates xylogenesis and wood formation, the over-production of IAA by the expression of the auxin biosynthetic gene, *iaaM* of *Agrobacterium tumefaciens*, may induce the increased growth and modification of wood. However, the over-expression of the auxin biosynthesis gene may also trigger the over-production of ethylene which generally inhibits plant growth. The major goal of this research is to genetically transform aspen with the *iaaM* and *ACC deaminase* genes to improve productivity.

The specific objectives of this research were:

1) To transform hybrid aspen, *P. canescens* x *P. grandidentata* with the *iaaM* gene

driven by a vascular specific promoter, glycine rich protein (GRP) (Keller et al., 1989) (GRP-*iaaM*), and *ACC deaminase* gene under the control of cauliflower mosaic virus 35S promoter (CaMV 35S) along with the GRP-*iaaM* (GRP-*iaaM*-35S-*ACC deaminase*) gene,

2) To confirm genetic transformation by polymerase chain reaction (PCR) and Southern blot analysis,
3) To characterize the growth of the transgenic plants,
4) To quantify the auxin and ethylene in the transgenic aspen, and
5) To correlate the auxin and ethylene data with the plant growth data to determine the effect of GRP-*iaaM* and GRP-*iaaM*-35S-*ACC deaminase* genes on aspen growth.

CHAPTER II

LITERATURE REVIEW

2.1. General introduction to *Populus* species and their significance

The genus *Populus* is a member of the willow family (Salicaceae) and is composed of five sections (Turanga, Leucoides, Leuce, Tacamahaca and Aigeiros). It consists of nearly 35 species of poplar, cottonwoods, and aspens with a wide natural distribution in the Northern hemisphere. Among those sections, Leuce section is a complex group containing aspens and white poplars. White popular includes *P. alba* and aspen includes *P. tremula*, *P. tremuloides*, and *P. grandidentata* (Dickmann and Stuart, 1983). *Populus* also has genetic variations due to natural and artificial hybridization that usually show a hybrid vigor, such as *P. canescens* x *P. grandidentata* used in this research (Li et al., 1993). *Populus* species grow very fast, are easy to propagate vegetatively, and can grow in various soil and environmental conditions (Rishi et al., 2001).

Wood is a source of raw materials important for pulp, paper, and biomass energy. Wood is both a supporting and a conductive structure for trees to grow to big size. Wood has remarkable physical and structural properties which have made it valuable to humanity. The Food and Agriculture Organization (FAO) has estimated that a total of 3.4 billon m^3 of wood was consumed in 2000; 1.8 billion m^3 of wood was burned as fuel in 2000 and 1.6 billon m^3 of wood was harvested for industrial use. The global demand

for wood is growing at 1.7 % annually (Fenning and Gershenzon, 2002). The world's forest area is estimated to cover 3.2 to 3.9 billion hectares or about 30% of the earth's land area. However, this has decreased dramatically due to forest degradation and deforestation for crop production. In addition, 50% of the forest area is either nominally protected or too remote to harvest.

2.2. Genetic improvement of *Populus*

The increasing demand for wood and the reduction of available forest land fuel an urgent demand for the introduction of new and more efficient breeding tools for tree improvement. Breeders have been using conventional hybridization methods for many years. The seeds generated through the crosses are used for screening and evaluation. In contrast to other agronomic and horticultural species, tree breeding has been hampered by a long generation of life cycle and lack of genetic information although much progress has been made in *Populus* breeding (Dinus et al., 2001; Rishi et al., 2001; Giri et al., 2004).

In contrast to sexual breeding, genetic engineering allows new genes to be added while the desirable genotype is preserved, therefore, reducing the time required for producing an elite line (Han et al., 1997). Thus, genetic engineering has potential to become an important means of improving forest tree species especially by expanding the gene pool (Tzfira et al., 1997a). Although it is routine in several major agronomic crops,

genetic engineering in most forest tree species has been far behind. Most of the reports on the genetic transformation of forest trees focused on determining different factors affecting *Agrobacterium* infection and establishing protocols for obtaining successful and stable transformation leading to regeneration of the species. One exception is *Populus*. Hybrid aspen and other *Populus* species have become model species because of small genomes which are about four times larger than that of *Arabidopsis* (Bennett and Leitch, 1997; Tuskan et al., 2004). Since Fillatti et al (1987) first reported successful genetic transformation of *Populus*, many transgenic *Populus* have been reported by several laboratories and efficient transformation protocols have been developed for various *Populus* genotypes (Han, 2001; Dai et al., 2004). Several economically significant genes have also been transferred to various poplar species, including herbicide resistance (Donahue et al., 1994; Confalonieri et al., 2000), insect tolerance (Kleiner et al., 1995; Delledonne et al., 2001), early flowering ability (Weigle and Nilsson, 1995), and improved rooting ability (Tzfira et al., 1997b, 1998a; Dai et al., 2003).

Since wood is the major harvested part of forest trees, many tree breeding and biotechnology programs have been focusing on the modification of wood characteristics and trunk structure to increase growth rate and to alter tree architecture (Altman, 2003). The expression of auxin-biosynthetic genes, *iaaM* and *iaaH* genes from *A. tumefaciens*, as well as the *rolB* gene from *A. rihizogenes*, has been shown to alter the growth patterns and development of transgenic *Populus* (Fladung et al., 1997; Tuominen et al., 1995, 2000; Tzfira et al., 1997b, 1999). Interestingly, Tzfira et al. (1999) reported that the expression of *rol* genes in transgenic aspen plants resulted in enhanced growth rate and

higher cumulative stem length with breaking of stem apical dominance. In contrast, Grünward et al. (2000) reported that *rolC* transformed aspen trees showed stunted growth with light green leaves of reduced size, thinner fiber cell walls, and delayed xylem cell formation.

Lignin is a complex phenolic compound which is located in the cell wall of higher plants and has important functions for mechanical support, solute transport, and disease resistance. However, lignin must be removed from wood during paper manufacture, which consumes energy and chemicals and causes environmental pollution (Herschbach and Kopriva, 2002). Therefore, the reduction of lignin content and manipulation are one of the most important targets of tree biotechnology. The effort to reduce lignin content via biotechnology was shown by reducing the activity of enzymes which are important for lignin production including caffeic acid O-methyl transferase (COMP), cinnamoyl alcohol dehydrogenase (CAD) and 4-coumarate coA ligase. Transgenic poplar showed reduced activity of COMT by the insertion of an antisense gene of COMT, but the lignin content did not vary from that of control plants (Dumas et al., 1992). Transgenic aspen containing complete COMT cDNA sequence showed modified lignin structure and reduced COMT activity, but the lignin content did not differ from that of control plants (Tsai et al., 1998). Recently, the transgenic plants expressing an antisense gene which down-regulates CAD showed a slight reduction of lignin content (Lapierre et al., 1999). Hu et al. (1999) reported that the down-regulation of 4-coumarate coA ligase gene showed reduced lignin, increased cellulose, and enhanced growth without altered lignin composition in transgenic aspen.

2.3. Vascular tissue and wood development

Vascular tissue is composed of xylem and phloem and is formed from the division of vascular cambium and procambium. During the primary growth of stems and roots, procambium initials derived from an apical meristem produce primary xylem and primary phloem (Ye, 2002). In woody plants, the primary vascular tissues of shoots and roots are replaced in later development by secondary vascular tissues, which are produced by a secondary meristem. The secondary vascular system is comprised of secondary phloem (bark), vascular cambium (cambium), and secondary xylem (wood). Wood is formed by the successive addition of secondary xylem, and it occurs as a continuous ring of cells between the xylem and the phloem which forms a cylindrical sheet in a tree stem (Chaffey, 1999).

In vascular cambium, fusiform initials give rise to axial wood elements such as vessels, fibers, and tracheids, and ray initials give rise to horizontally oriented ray cells. In angiosperm species such as poplar, wood has few small vessels and fibers with an inner gelatinous cell wall layer that consists of almost pure cellulose with microfibrils (Hellgren et al., 2004). The cell wall provides rigidity and protection to the plant cell without preventing the diffusion of water and ions from the environment. The typical primary cell wall of a woody plant consists of 25-30% cellulose, 15-25% hemicellulose, 35% pectin, and 5-10% protein on a dry weight basis. Forty to seventy linear chains of glucose are held together by hydrogen bonds to form a crystalline structure, a microfibril. Microfibrils are arranged in a transverse axis, which are interconnected to cross-linking

glycans, putative networks involving structural proteins or phenylpropanoid compounds and elements of a pectin network. Secondary cell wall contains a higher proportion of cellulose than the primary cell wall. A critical component of secondary cell wall is lignin, which constitutes 15-35% of dry weight of woody tissues. Lignin is located in the wall matrix and plays an important role in the attainment of wall rigidity (Taiz and Zeiger, 1991).

Cell expansion prior to secondary cell wall thickening involves extensive changes in the mass and composition of the cell wall. During elongation or expansion, the incorporation of new materials increases the surface area of cells and changes the existing cell wall architecture. The cell wall is extensible, allowing discrete biochemical loosening of the cell wall matrix and permitting microfibril separation and insertion of newly synthesized polymers (Buchanan et al., 2000). The application of plant growth regulators such as auxin and gibberellins changes the orientation of cortical microtubules and cellulose microfibrils. As the rate of growth changes, the molecules from microfibrils dissociate or break down. Altering the interaction between the cross-linking glycans and cellulose is the principal determination of a rate of cell expansion. Tracheid and ray cells also expand in a radial direction, and vessels expand both radially and tangentially by the expansion of the radial wall. Once expansion is complete, xylem elements start to form a secondary cell wall, and lignification is initiated in the middle lamella and continues throughout the cell wall layer toward the cell lumen (Hellgren et al., 2004).

Wood formed by the vascular cambium during secondary growth can be increased by genetic engineering (Hu et al., 1999). In forest trees, the modification of endogenous hormone levels by genetic engineering has been used to modify wood properties, and this can be obtained by the insertion of *iaaM* or *rolC* gene into plants. Tuominen et al. (1995) reported that transgenic hybrid aspen expressing the *iaaM* gene caused a change in the xylem structure. The transgenic aspen showed lower rates of cambial cell division, longer duration of xylem cell expansion, decreased xylem production, and larger fiber lumen area. These trees displayed an alteration in cambial growth which was related to changes in concentration and a radial distribution pattern of IAA across the cambial meristem (Tuominen et al, 1997). Transgenic *Populus* expressing the *rolC* gene showed not only altered growth and development but also accelerated stem growth (Nilsson et al., 1996), delayed formation of xylem cells, and lower deposition of lignin in less differentiated xylem cells as compared to non-transgenic aspen (Grünward et al., 2000). Transgenic hybrid aspen expressing 35S-*rolC* also showed thinner fiber walls as compared to the control plant (Grünward et al., 2001).

2.4. The effect of auxin on plant growth and wood formation

The plant hormone auxin is involved in diverse developmental processes including cell enlargement, vascular tissue differentiation, root initiation, gravitropic and phototropic responses, and apical dominance (Davies, 1987; Kende and Zeevaart, 1997). IAA is the major form of auxin in plants and plays an important role in the control of

many processes. A complex balance among biosynthesis, conjugation, catabolism, and efflux regulates the level of free IAA. The IAA pool is fed by *de novo* synthesis from tryptophan and non-tryptophan precursors and by the hydrolysis of IAA conjugates (Bandurski et al., 1995). IAA contents in individual tissues also are influenced by a basipetal polar transport system that results in the downward movement of IAA from apical meristems and young leaves towards root systems. Plants can synthesize IAA from L-tryptophan by three different routes: indole-3-pyruvic acid, indole-3-acetyldoxime, and tryptamine pathways. The major pathway appears to proceed via indole-3-pyruvic acid and indole-3-acetaldehyde (Normanly et al., 1995). Most of the IAA in plant tissues is in a conjugated form. The structures of numerous IAA conjugates have been elucidated, and these include conjugates of the carboxyl group of IAA to sugars, high molecular weight glycans, amino acids, and peptides (Bartel, 1997). Auxin activity is lost through IAA catabolism by IAA oxidases, which irreversibly decreases of the size of the IAA pool (Buchanan et al., 2000).

Auxin has been shown to play an important role in cell elongation. When auxin was added to isolated stem segments and coleoptiles, auxin induced elongation (Davies, 1987). The response begins within 10 minutes and can result in a 5- to 10-fold increase in growth rate that can persist for days (Evans, 1985). The transgenic plants expressing 35S-*iaaL* gene, which reduces free auxin level, showed reduced auxin levels consequently causing reduced plant size, leaf size, apical dominance, fertility, and hypocotyl length in the light (Romano et al., 1991). In another report, the hypocotyl growth was inhibited when wild type *Arabidopsis* seedlings were grown on media

containing a range of auxin concentrations, and transgenic plants expressing 35S-*iaaL* showed promoted hypocotyl growth in the conditions on which wild type hypocotyl growth was inhibited (Collet et al., 2000). This suggests that auxin level in the wild type hypocotyls is optimal for elongation and additional auxin is inhibitory. Recently, van der Graff et al. (2003) also reported that increased endogenous auxin by inserting *iaaM* and *iaaH* genes in *Arabidopsis* caused increased apical dominance, hypocotyl length, and petiole length with epinastic leaves and cotyledons.

Vascular differentiation is also influenced by auxin flux. Auxin is transported from a shoot apical meristem and young leaves and induces formation of procambial cells (Aloni, 1987; Ye, 2002). Avsian-Kretchmer et al. (2002) showed that IAA distribution coincides with the vascular differentiation of *Arabidopsis* during leaf expansion. In forest trees, the polar flow of IAA is crucial for maintaining the structure and activity of the vascular cambium, which gives rise to secondary growth of the stem (Sundberg et al., 1994; Uggla et al., 1998). It is well established that exogenous IAA affects several aspects of secondary growth of the stem such as cambial cell division and the radial enlargement of xylem elements. The basipetal polar transport of IAA in the cambial region was shown by stem girdling and debudding. Stem girdling resulted in increased tracheid production and IAA concentration above the girdle, and decapitation led to a decrease of the production of tracheids and the concentration of IAA. The application of IAA also increased the production of tracheids and IAA levels (Little and Savidge, 1987).

The development of techniques for measuring IAA in small samples of plant tissues has greatly facilitated the ability to determine the physiological effects of IAA in regulating cambial growth. The internal concentrations of IAA have been shown to correlate with the application of exogenous IAA or IAA transport inhibitors. The application of IAA transport inhibitors resulted in both a depletion of IAA and an inhibition of cambial growth below the point of application. This manipulation of the levels of IAA in the vascular cambium region affected cambial growth including xylem production, size and wall thickness of xylem cells, wood and vessel density (Sundberg et al., 1994). This observation demonstrates that cambial growth is stimulated by IAA through a polar transport system. Numerous experiments have demonstrated the potential of IAA to affect most aspects of cambial growth (Little and Pharis, 1995). However, it has failed to establish a consistent relationship between internal IAA concentration and variations of cambial growth rate until mass spectrometry (MS) was introduced. By using MS technique with increased sensitivity, it has become possible to demonstrate that endogenous IAA is distributed as a steep concentration gradient across cambial meristem and differentiating derivatives in Scots pine (Uggla et al., 1996) and hybrid aspen (Tuominen et al., 1994, 1997). Uggla et al. (1998) suggested that IAA functions as a morphogen and acts as a positional signal that controls cambial growth rate by regulating the number of dividing cells, and showed that the radial IAA gradient had different cambial growth rates.

2.5. Genetic engineering to modify IAA level

Although the exact molecular mechanisms by which IAA acts on wood formation are largely unknown, it can be concluded that IAA is critical for growth and development, especially for the amount and quality of wood produced (Tuominen et al., 1995). Many researchers are interested in modifying the endogenous level of IAA in stem tissues. With the development of gene transfer technology, the modification of endogenous hormone balance became possible. Increase of endogenous auxin levels can be accomplished by the expression of the IAA-biosynthetic *iaaM* and *iaaH* genes of *A. tumefaciens* T-DNA (Klee and Lanahan, 1995). The *iaaM* gene encodes for tryptophan monooxygenase which catalyses the conversion of tryptophan into indole-3-acetamide, and the *iaaH* gene encodes for indole acetamide hydrolase which converts indole-3-acetamide into IAA (Follin et al., 1985).

In the early phases of genetic engineering the transgenes were constitutively expressed mainly using the 35S-promoter of cauliflower mosaic virus (CaMV) or nopaline synthase gene promoter, which drives constitutive gene expression in a whole plant. Since desirable and undesirable aspects of an interesting trait are often expressed under constitutive promoters, many studies about tissue-specific promoters have been reported to reduce the undesirable gene expression (Rotino et al., 1997). If a foreign gene can be expressed only when or where its gene product is needed, this should avoid non-target effects of a transgene. Many genes found in living organisms are regulated by tissue- or development-, or environmentally-induced promoters. These promoters

include a vascular-specific promoter (Keller and Baumgartner, 1991), ovule-specific promoter (Ficcadenti et al., 1991), mesophyll-specific promoter (Stockhaus et al., 1997), and cambium-specific promoter (Tuominen et al., 2000). Keller et al. (1989) reported that GRP associated with cell wall protein is specially synthesized in protoxylem tracheary elements of a vascular system in bean. A promoter fragment of 494 bp including 427 bp upstream from transcription start site was used for driving β-glucuronidase (GUS) reporter gene, and transgenic tobacco plants showed vascular-specific expression in all tissues including roots, stems, leaves, and flowers. The gene was developmentally expressed during the differentiation of both primary and secondary vascular tissue and was also rapidly induced after excision-wounding of young stems (Ringli et al., 2001a, b).

The increase of IAA levels in plants can be achieved by expressing the *iaaM* and *iaaH* genes, and this has been used successfully to study physiology and the metabolism of IAA. Klee et al. (1987) transformed petunia and Sitbon et al. (1992) transformed tobacco plants with the *iaaM* and *iaaH* under the control of a strong CaMV 19S or 35S promoter. These transgenic plants resulted in a several-fold increase in the concentration of IAA. Consequently, abnormal morphology including smaller and narrower leaves with curling, apical dominance, elongated stem and woody tissues, and alteration of xylem formation also occurred. Klee et al. (1987) also used soybean 7S storage protein gene promoter, which showed embryo-specific expression, to drive the *iaaM* gene. The transformed shoots initially exhibited the same phenotypes as the transgenic petunia with CaMV 19S promoter, but they soon outgrew the abnormal phenotype. Klee et al.

suggested that this result was most likely due to the expression of the gene at a significant level in transformed callus but proper regulation of the gene following organogenesis. In transgenic tobacco with a heat shock promoter-*iaaM* (Kares et al., 1992), elevated indole-3-acetamide (IAM) resulted in plants with narrow leaves, elongated stems, and a tendency to form roots along the stems. Romano et al. (1993) also showed that the expression of the *iaaM* driven by the CaMV 19S promoter resulted in alteration in development such as epinastic leaves, elongated hypocotyl length, and increased apical dominance. Ficcadenti et al. (1999) inserted the *iaaM* gene with a placental- and ovule-specific promoter DefH9 from *Anthirrhium majus* into tomato. The resulting transgenic tomato plants showed no visible alterations in their vegetative growth except in fruit development. Recently, transgenic *Arabidopsis* expressing the *iaaM* or *iaaH* gene with natural promoters exhibited increased hypocotyl length and increased apical dominance with increased IAA level (van der Graaff et al., 2003). The longitudinal section of the transgenic plants not only showed increased cell length and a smaller cell diameter of *Arabidopsis* hypocotyl and stem but also showed epinastic leaves and cotyledons, necrotic spots on leaves and bracts, and reduced amounts of vascular tissue. The relationship between auxin level and phenotype was shown in transgenic tobacco expressing the *iaaM* gene driven by the double CaMV 35S promoter. The transgenic plants showing abnormal phenotypes had high levels of auxin and the transgenic plants showing normal phenotypes had lower levels of auxin (Alekseeva et al., 2004).

Insertion of the *iaaM* into plants has also been used for studies of wood formation in forest trees. The transformation of trees with the the *iaaM* resulted in a change in the

xylem structure of those transgenic trees. The *iaaM* gene was expressed in a hybrid aspen, *P*. *tremula* x *P*. *tremuloides*, driven by a weaker mannopine synthase promoter which has a relatively low level of expression and vascular-specificity. The transgenic aspen had decreased height and stem diameter, reduced leaf area, lower peak levels, and a wider radial gradient of IAA than the wild type plants (Tuominen et al., 1995). They also showed lower activity of cambial cell division, longer duration of xylem cell expansion, decreased xylem production, and larger fiber lumen area. A detailed analysis of these trees revealed that the alteration in cambial growth was related to alterations in concentration and radial distribution pattern of IAA across the cambial meristem (Tuominen et al, 1997). More recently, Tuominen et al. (2000) investigated the effect of localized expression of the IAA-biosynthetic genes on cambial growth of hybrid aspen. The *iaaM* or *iaaH* gene and the GUS reporter gene driven by the *rolC* promoter resulted in localized expression in the cambial meristem, expanding cambial derivatives and mature phloem. The transgenic plants showed an increased IAA concentration in the cambial region tissue but no changes in the radial distribution pattern of IAA compared with wild-type plants. Interestingly, the plants expressing the *iaaM* gene were not significantly different from control plants in the developmental patterns of xylem such as the rate of xylem cell production and xylem morphology (Tuominen et al., 2000). The research showed that the increase of IAA did not result in any change in the developmental pattern of cambial derivatives and cambial growth rate, suggesting that a moderate increase in IAA concentration does not necessarily stimulate growth.

2.6. The effect of ethylene on plant growth and wood formation

The plant hormone ethylene triggers a wide range of physiological phenomena, such as fruit ripening, senescence, and leaf abscission in response to external stresses (McKeon and Yang, 1987). Ethylene also plays roles in pollination-regulated flower development (O'Neill, 1997; Trebitsh et al., 1997), regulation of plant development, and defense response in many aspects (Shinshi et al., 1995). The biosynthetic pathway of ethylene in higher plants is well characterized and involves a substrate, ACC. ACC is produced from S-adenosylmethionine by ACC synthase and converted into ethylene by ACC oxidase (Yang and Hoffman, 1984; Sheehy et al., 1991; Kieber, 1997). The expression of ACC synthase is controlled by different physiological signals (Olson et al., 1991), and ACC oxidase regulates the production of ethylene in a variety of plant organs including flowers and climacteric fruits (Oeller et al., 1991). Auxin stimulates ethylene biosynthesis (McKeon and Yang, 1987) and has been shown to induce *de novo* synthesis of ACC synthase through the increased expression of specific ACC synthase genes or post-transcriptional regulation (Taiz and Zeiger, 1998).

It is reported that the application of ethylene inhibits shoot growth. The dramatic effect of ethylene on the growth and development of seedlings is the triple response involving reduction in elongation, swelling of the hypocotyls, and a change in the direction of growth (Kieber, 1997). These changes presumably result from a redistribution of auxin in response to the stress-induced ethylene. Ethylene may also modify shoot growth. Reduced growth and improved stem diameter of unstaked shade

trees is suggested to be due to ethylene which is produced in response to flexing stress (Davies, 1987). The transgenic tobacco showing the highest ethylene production displayed significant dwarf morphology, and the change resembled the phenotype of a wild-type plant exposed to excess ethylene (Araki et al., 2000). Hansen and Grossmann (2000) reported that auxin-induced ethylene showed growth inhibition in tomato.

It is well established that the application of the ethylene-releasing compound, 2-chloroethylphosphonic acid (ethrel) promotes xylem differentiation in *Lactuca* pith explants (Miller et al., 1984) and increased tracheid production and xylem increment in *Pinus* species (Yamamoto and Kozlowski, 1987). Savidge (1988) reported that the application of ethrel increased ray size and ray cell number and accelerated cortex growth of hardwood and conifer seedlings. Ethrel was also shown to alter the cell wall composition of *Picea abies* hypocotyls resulting in an increase the content of cellulose and a decrease non-cellulosic polysaccharides (Eklund, 1991). Ethylene evolution from the stem was higher during the period of xylem production than during the period of cambium dormancy (Ingemarsson et al., 1991). However, Eklund and Little (1994) reported that such applications resulted in stimulation in some species and inhibition of cambial cell division and diameter growth in other species. They mentioned that ethylene can promote tracheid production, but only when applied in a high concentration in the localized cambial region. It is not known whether ethylene is an essential factor for any of the cellular processes of wood formation and not much has been concluded about the exact role of ethylene in cambial growth and wood development (Andersson-Gunnerås et al., 2003).

2.7. Genetic engineering to modify ethylene level

The ability to inhibit the synthesis of ethylene in a plant without exogenous chemical application can be used to elucidate the precise role of ethylene in a variety of developmental- and stress-related phenomena (Klee et al., 1991; Kende and Zeevaart, 1997). A genetic approach for preventing ethylene synthesis can be obtained by genetic engineering of enzymes that degrade ACC. Irreversible degradation of the precursor of ethylene, ACC, is likely the only biochemical inhibition of ethylene synthesis because ACC is the only known precursor that participates in ethylene synthesis. The antisense of ACC oxidase (Hamilton et al., 1990) or ACC synthase (Oeller et al., 1991; Olson et al., 1991) has greatly reduced ethylene synthesis in transgenic tomato plants by blocking the conversion ACC into ethylene. ACC degradation could be catalyzed by two enzymes. ACC deaminase degrades ACC to α-ketobutyric acids and ammonia, and the gene has been cloned from a soil bacterium, *Pseudomonas* (Honma and Shimomura, 1978). Another enzyme, ACC N-malonyl transferase malonylates ACC into malonyl-ACC (McKeon and Yang, 1987). Klee et al. (1991) reported that transgenic tomatoes expressing *ACC deaminase* resulted in a high level of ACC deaminase, up to 0.5% of the total protein and the reduction of ethylene up to 97%. The transgenic tomato also showed significant delays in ripening.

In transgenic plants expressing the *iaaM* gene, auxin levels are typically elevated and associated with increased apical dominance, decreased internode elongation, and excessive leaf curling or epinasty (Klee et al., 1987; Sitbon et al., 1992). However,

because auxin stimulates the production of ethylene (McKeon and Yang, 1987), it is often unclear if the effects of the *iaaM* gene are due to auxin alone, ethylene alone, or the interaction of both. To effectively uncouple these effects, Romano et al. (1993) hybridized plants which showed over-production of IAA by the *iaaM* gene with plants showing decreased ethylene by the *ACC deaminase* gene. They found that most of the effects were primarily controlled by auxin rather than ethylene, but ethylene is partially responsible for the inhibition of stem elongation. This strategy allowed the production of plants that over-produce auxin, but synthesize normal levels of ethylene.

CHAPTER III

MATERIALS AND METHODS

3.1. Plant material

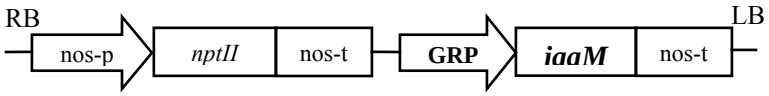
Hybrid aspen (*P. canescens* x *P. grandidentata*, Clone 6) was used in this research. *In vitro* shoots were maintained on woody plant medium (WPM; Lloyd and McCown, 1980) supplemented with 5.0 μM benzylaminopurine (BA) and 2% (w/v) sucrose. The medium was solidified with 0.7% (w/v) agar after adjusting to pH 5.7-5.8. They were cultured in a growth room at 22-25°C, with a 16h photoperiod provided with wide spectrum fluorescent lights at a light density of approximately 35 μmol m^{-2} s^{-1}. The cultures were sub-cultured every four weeks. The details were described by Dai et al. (2003).

3.2. Bacterial strain and vectors

The disarmed binary Ti plasmid pBI101 (Clontech, CA) was used for constructing transformation vectors. The plasmid pBI101 contained a promoter-less *β*-glucuronidase gene (GUS) cassette in a binary vector pBIN19. This plasmid also contained neomycin phosphotransferase gene (*nptII)* which confers kanamycin resistance, thus kanamycin was used for the selection of putative transgenic plants. The GRP-*iaaM* gene or GRP-*iaaM*-35S-*ACC deaminase* gene was inserted in *sal*I and *EcoR*I cutting sites in pBI101

after the GUS gene was removed. The *iaaM* gene was from *A. tumefaciens* and was driven by the vascular tissue specific promoter, GRP and nopaline synthase gene terminator. The GRP promoter was isolated from a bean glycine rich protein (Keller and Baumgartner, 1991). The resulting plasmid was designated as pGI. The other plasmid contained the *iaaM* gene along with the *ACC deaminase* gene under the control of the CaMV 35S promoter and nopaline synthase terminator. The *ACC deaminase* gene was PCR-cloned from *Pseudomonas* sp Strain 6G5 (Klee et al., 1991). The resulting plasmid was designated as pGIA. The plasmids pGI and PGIA were transferred separately into *A. tumefaciens* strain EHA105 (Hood et al., 1993) by electroporation. The resulting *Agrobacterium* was used for aspen transformation. The T-DNA portions of the plasmids pGI and pGIA are shown in Fig.1. The plasmids were provided by Dr. Y. Li at University of Connecticut.

(A) pGI



(B) pGIA

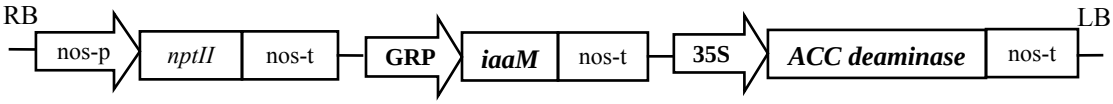


Figure 1. Schematic representation of the T-DNA portions of pGI (A) and pGIA (B). RB: right border; nos-p: nopaline synthase gene promoter; *nptII*: neomycin phosphotransferase gene; nos-ter: nopaline synthase gene terminator; GRP: glycine-rich protein gene promoter; *iaaM*: IAA biosynthetic gene; 35S: cauliflower mosaic virus 35S; *ACC deaminase*: ACC deaminase gene; LB: left border.

3.3. Transformation

Agrobacterium cells were grown in 10 ml Luria Bertani (LB) liquid medium containing 50 mgL^{-1} kanamycin at 28°C for 20 hrs on a shaker at 200 rpm. The cells were collected by centrifugation at 3000 rpm for 15 min, re-suspended in 50 ml LB medium with 20 μM acetosyringone, and cultured at 28°C for 6 hrs to a density of 1.0-1.5 (OD_{600}).

Leaves were infected using the technique based on that of Dai et al. (2003). Leaves were cut into 0.5 cm^2 segments from micropropagated shoots *in vitro* and incubated in an *Agrobacteria* suspension for 30 min on a shaker. They were blotted dry with sterile paper towels to remove the excess bacteria and then placed onto WPM medium containing 9.12 μM zeatin, 5.37 μM NAA, and 200 μM acetosyringone at 25°C in the dark. After three days of co-cultivation, leaf explants were washed with sterile water containing 500 mgL^{-1} carbenicillin, 250 mgL^{-1} cefotaxime, and 80 mgL^{-1} kanamycin (CCK antibiotics). They were blotted dry with sterile paper towels, transferred to callus induction medium (WPM medium containing 9.12 μM zeatin, 5.37 μM NAA, and CCK antibiotics), and cultured at 25°C in the dark. Ten leaf segments were put on each Petri plate with 4 replicate plates. After 4 weeks, induced callus tissues were transferred to shoot induction medium (WPM medium containing 9.12 μM zeatin and CCK antibiotics) in the light. Induced shoots were sub-cultured twice on the same media to multiply shoots at the same conditions described above. Transformation frequency was defined as

the percentage of inoculated leaf segments that produced at least one kanamycin-resistant shoot based on that of Confalonieri et al. (2000).

3.4. Rooting and transplanting

Induced shoots longer than 0.5 cm were excised and transferred to root induction medium (WPM medium without plant growth regulators) for rooting. After four weeks, the rooted plants longer than 5 cm were transferred to 1-inch rose pots with sterile potting mixture (Pro-Mix®) in a growth room. Trays holding the plants were covered with clear plastic lids to maintain a high level of humidity for two weeks. The plants were acclimated to ambient conditions by removing the lids gradually for two weeks. The plants were transferred to ½ gallon pots and grown in a greenhouse. The greenhouse was maintained at 70 °F/ 68 °F (day/night) by a Wadsworth STEP 50A controller. The plants were fertilized using Peters® Professional® 20-10-20 (N-P-K) at 100 ppm CLF (Constant Liquid Feed) with watering. Osmocote® was added to the pots every three months. After being grown in a greenhouse for one year, the plants were moved and grown outside.

3.5. Molecular analyses

Transformation was verified by polymerase chain reaction (PCR) and Southern blot analysis. Genomic DNA was isolated from young leaves of selected plants from both

kanamycin-resistant and non-transformed control plants. One gram of young leaf tissue per sample was ground to a fine powder with a sterile mortar and pestle in liquid nitrogen. DNA was extracted using a DNA isolation kit (Puregene®) and PCR was performed with 1 unit of *Taq* DNA polymerase (Promega, Madison, WI) and 100 ng of DNA. The amplification was subjected to 5 min at 94 °C for denaturation and 30 cycles of 1 min at 94 °C, 1 min at 56 °C, 1 min at 72 °C, and 10 min at 72 °C for extension. Two specific primers were used in the PCR amplification: forward primer, 5′-AGA TGT GCA CCG ATT GAT CTT-3′ and reverse primer, 5′-CAA GTA AGT GTC GAC TGT GC-3′ for the *iaaM* genes. For the *ACC deaminase* gene, forward primer, 5′-CCT TCG GTC CTT CTC CCA TC-3′ and reverse primer, 5′-TGC CGT GCA TCG ATT TAC C-3′ were designed specifically. The amplification was subjected to 5 min at 94 °C for denaturation, 5 cycles of 1min at 94 °C, 1 min at 45 °C, 1 min at 72 °C, 25 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, and 10 min at 72 °C for extension. The amplified fragments were electrophoresed on an 0.8% (w/v) agarose gel containing ethidium bromide and visualized under UV light.

For the Southern blot procedure, total genomic DNA was isolated using the method of Doyle and Doyle (1987) from young leaves of confirmed transgenic plants by PCR. Genomic DNA (20 μg) from each plant transformed with pGI or pGIA was digested with *Sac*I at 37°C overnight. The genomic DNA from the plants transformed with pGIA were also cut with another restriction enzyme, *Sal*I to confirm the inserted gene. The *Sal*I enzyme cut the 5′ end of the GRP promoter and the 3′ end of the *iaaM* gene, resulting in

a 1.35 kb fragment. Therefore, multiple copies of the inserted gene were expected to be shown as a strong signal of hybridization. The restriction fragments were separated on an 0.8% (w/v) agarose gel at 30-35 V for 16 h and transferred onto a charged nylon membrane (Amersham Pharmacia Biotech, NJ) according to the manufacturer's instructions using a vacuum transfer apparatus. After pre-hybridization at 42°C with a DIG Easy Hybridization solution (Roche, Germany) for 2 hrs, the membrane was hybridized overnight at 42°C with a digoxigenin-labeled probe according to the manufacturer's instruction. The probe was prepared by PCR DIG probe synthesis kit (Roche, Germany) for the *iaaM* gene and added directly into the hybridization solution after purification with a High Pure PCR product Purification kit (Roche, Germany). After hybridization, the membrane was washed and soaked in blocking buffer following the manufacture's instruction (Roche, Germany) at room temperature for 45 min, and then treated with the anti-digoxigenin-AP buffer (100 ml blocking buffer, 5 µl anti-digoxigenin-Alkaline Phosphate) for 30 min to the allow anti-digoxigenin-alkaline phosphate to combine with digoxigenin. After washing, the membrane was treated with a detection buffer (100 mM tris- pH9.5, 100mM NaCl) for 5-10 min, then CDP-Star (Roche, Germany) was added to the membrane. The membrane was exposed on a film (Fuji) to detect fluorescence, and the film was developed. The number of inserted gene copies were counted to examine the effect of transgene cosuppression.

3.6. Analyses of growth pattern

Since transgenic plants were produced through two transformation experiments, they were at two different developmental stages. Thus, 13 cm long shoot tips were cut from transgenic and non-transgenic plants and rooted to place all plants at the same physiological condition and growing stage in 2003. The basal end of the cuttings were dipped in 300 ppm IBA for 2 seconds and inserted into a soilless potting medium (Pro-Mix®) for rooting. Cutting trays were sterilized with a 10 % Clorox solution (6 % sodium hypochlorite), and a fungicide (Subdue MAXX™, Syngenta, Wilmington, DE) was applied to the cuttings and the potting medium to prevent infection by fungi following the manufacturer's instruction. The cuttings were placed under overhead mist with a 47% shade cloth for three weeks. The mist duration was 8 seconds at an interval of 10 seconds the first week, then the interval of mist was increased gradually during five weeks. After six weeks post-cutting, the rooted plants were transferred to ½-gallon pots, and the fertilizer was applied as described above. The heights and diameters of the plants were measured every five weeks from May to October 2003. The height was measured from the base to the tallest shoot, and the diameter was measured at about 5 cm from the base. After finishing the measurements, stems of the plants were cut and stored at 4°C for three months to meet chilling requirements. 13 cm long cuttings from the stored stems were inserted into potting medium (Pro-Mix®) for rooting in spring 2004. The cutting propagation and growth measurements were performed as described above. The heights and diameters of the plants were measured every five weeks from April to

September 2004. Relative height (RH), relative diameter (RD), and volume (Vol) were calculated using following formulas:

$RH = [(H2 - H1) / H1] * 100$, H1 and H2: height

$RD = [(D2 - D1) / D1] * 100$, D1 and D2: diameter

$Vol = [(D/2) * (D/2) * \pi] * H$, H: height, D: diameter

3.7. Analysis of IAA content

For IAA analysis, actively growing apical shoots with three unexpanded leaves were collected. The procedure for measuring IAA was according to that of Sundberg (1990). One gram of fresh tissue was ground with a chilled mortar and pestle in liquid nitrogen, put into a pre-chilled 15 ml centrifuge tube with 5 ml extraction buffer (0.05M NaH_2PO_4, 0.02% (w/v) sodium diethyldithiocarbamate, ~1 mg $^{13}C_6$-IAA, pH to 7.5 with 5N NaOH), and then incubated at 4°C for 5-8 hrs in the dark. After centrifugation at 600 xg for 15 min, the supernatant was collected in a new centrifuge tube and partitioned three times with ethyl acetate. The aqueous phase was adjusted with HCl to pH 2.6-2.7 and then partitioned with ethyl acetate three times. After that, the ethyl acetate phase was collected and dried under a stream of nitrogen gas. The samples were sent to Dr. T. Tschaplinski at Oak Ridge National Laboratory for quantification of IAA content by gas chromatography-mass spectrometry (GC-MS). An HP 5890 Series II GC and 5972 MS (Hewlett-Packard Co. PA, USA) equipped with an capillary column of 30 m x 0.25 mm x

0.25 mm (HP-5MS, crosslinked 5% PH ME Silotane) was used for the analysis of IAA content.

3.8. Analysis of ethylene content

For ethylene measurement in plant tissues, 5 g of fully expanded fresh leaves on lower branches were cut from transgenic and non-transgenic plants, placed into a 500 ml jar (Mason), and sealed with a lid having a syringe port. After 2 days of incubation, 5 ml of gas was withdrawn with a syringe and the amount of ethylene gas was measured by gas chromatography (Agilent technologies 6850 network GC system). The system was equipped with a capillary column of 50.0 m x 530.0 µm x 15.0 µm (Agilent 19095P-S25E), flame ionization detector (FID), and integrator. Hydrogen gas was used as a carrier gas at 10 ml min^{-1}, and the temperatures of the injector, column, and detector were 60°, 150°, and 265 °C, respectively. The retention time of ethylene was 1.5 min. The concentration of the sample on a fresh weight basis was calculated based on 1 ng/g standard of ethylene (Scott Specialty Gases, PA).

3.9. Statistical analyses

Analysis of variance (ANOVA) from GLM procedures in SAS (Statistical Institute Inc, 1990) was performed on each data set to determine the significance of the effects of

the *iaaM* and *ACC deaminase* genes on tree growth, auxin and ethylene quantity. The significance ($P < 0.05$) of the difference in effect induced by individual transgenic plants was determined by least significant difference test (LSD) and t-test. To investigate a difference between groups including GRP-*iaaM* transgenic plants, GRP-*iaaM*-35S-*ACC deaminase* transgenic plants, and non-transgenic plants, linear contrast was performed. Linear regression method was used to determine the relationships between auxin or ethylene and growth.

CHAPTER IV

RESULTS

4.1. Transformation

One hundred percent of leaf explants co-cultivated with *Agrobacterium* formed callus (Fig. 2A). Shoot initiation was observed two weeks after inoculation (Fig. 2B) and shoots appeared four weeks after inoculation (Fig. 2C). Six and seven kanamycin-resistant shoots from forty leaf explants transformed each with pGI and pGIA, respectively, were produced in the first transformation experiment. Nine and eleven kanamycin-resistant shoots from eighty leaf explants transformed with pGI and pGIA, respectively, were produced in second transformation experiment. Thus, a total of thirteen shoots transformed with pGI and eighteen shoots transformed with pGIA were selected with kanamycin from one hundred twenty co-cultivated leaf explants. The transformation frequency with pGI and pGIA was 15.0% and 10.83%, respectively. The number of shoots produced per leaf explant varied from 2.21 to 3.18. The induced shoots were transferred to shoot induction medium and multiplied *in vitro* for twelve weeks (Fig. 2D). Putative transgenic plants did not display abnormal morphology or development pattern during *in vitro* culture. The selected plantlets were transferred to 1-inch rose pots and acclimated in a growth room for four to six weeks. The plants were transferred to ½-gallon pots (Fig. 2E), grown in a greenhouse for one year (Fig. 2F), and then transferred outside (Fig. 2G). These transgenic plants grew very rapidly, reaching up to 180 cm after two years in 1-gallon pots.

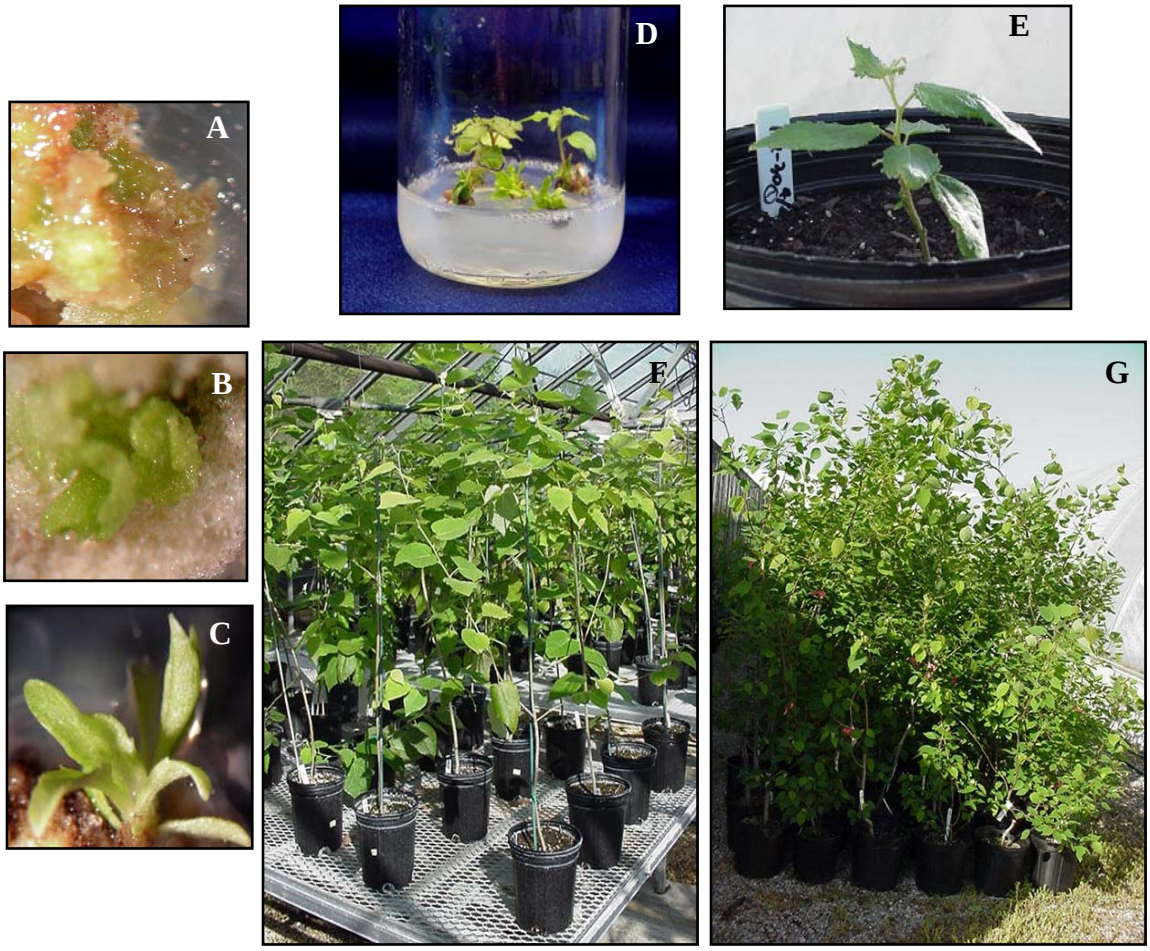


Figure 2. The transformation process of *Populus canescens* x *P. grandidentata* and acclimation of transgenic plants. A: Callus formation and shoot initiation at two weeks after infection; B: Shoot initiation at four weeks after infection; C: Elongation of shoot at six weeks after infection; D: Shoots were transferred to a jar at eight weeks after infection; E: Transplanting the plant in ½-gallon pot in a greenhouse after acclimation; F: One-year-old transgenic plants; G: Two-year-old transgenic plants.

Fourteen transgenic lines transformed with pGI were confirmed by PCR with the primers specific to the *iaaM* gene, amplifying the 0.58 kb *iaaM* gene sequence (see examples in Fig. 3A). Thirteen regenerated lines transformed with pGIA were also confirmed by PCR with the primers specific to the *iaaM* gene and *ACC deaminase* gene. The 0.58 kb *iaaM* gene (see examples in Fig. 3A) and 0.84 kb *ACC deaminase* gene (see examples in Fig. 3B) were amplified, respectively. Five of the 31 kanamycin-resistant plants (16.13%, data not shown) did not show amplification of the *iaaM* gene or *ACC deaminase* gene.

Southern blot hybridization analyses were performed on non-transformed plants and all transgenic lines which were confirmed by PCR. The genomic DNA was digested with *Sac*I and then hybridized with the probe specific to the *iaaM* gene. Figures 4 and 5A show the hybridization with the *iaaM* gene for the transgenic lines expressing the GRP-*iaaM* gene and the transgenic lines expressing the GRP-*iaaM*-35S-*ACC deaminase* gene, respectively. Positive hybridizations were detected with the digested DNA of all transgenic plants analyzed but not with the non-transformed plants. The genomic DNA from the transgenic lines with the GRP-*iaaM*-35S-*ACC deaminase* genes was also cut with another restriction enzyme, *Sal*I, to confirm the inserted gene. The *Sal*I enzyme cuts the 5′ end of the GRP promoter and the 3′ end of the *iaaM* gene resulting in a fragment of 1.35 kb, which is the same size as that in the vector. Therefore, multiple copies of inserted gene were shown as a strong signal of hybridization (Fig. 5B). Most transgenic lines contained either one or two copies of the inserted gene, but multiple copies of the *iaaM* gene were inserted into poplar genome of two GRP-*iaaM* transgenic lines, B21,

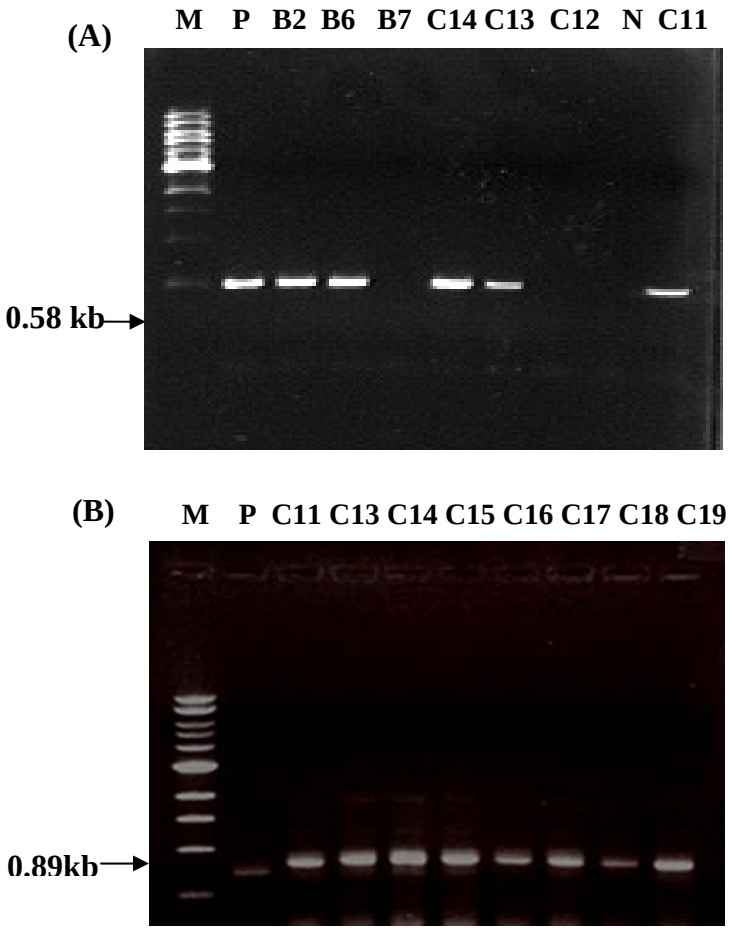


Figure 3. Confirmation of transformation by polymerase chain reaction (PCR). **(A)** Amplification of the *iaaM* gene by using the primer specific to the *iaaM* gene (0.58 kb). M: 1kb ladder; P: pGI as a positive control; B2, B6, B7: plants transformed with the GRP-*iaaM*; C14, C13, C12, C11: plants transformed with the GRP-*iaaM*-35S-*ACC deaminase*; N: non-transgenic plant. **(B)** Amplification of the *ACC deaminase* gene by using the primers specific to the *ACC deaminase* gene (0.84 kb). M: 1kb ladder; P: pGIA as a positive control; C11, C13, C14, C15, C16, C17, C18, C19: plants were transformed with the GRP-*iaaM*-35S-*ACC deaminase* gene.

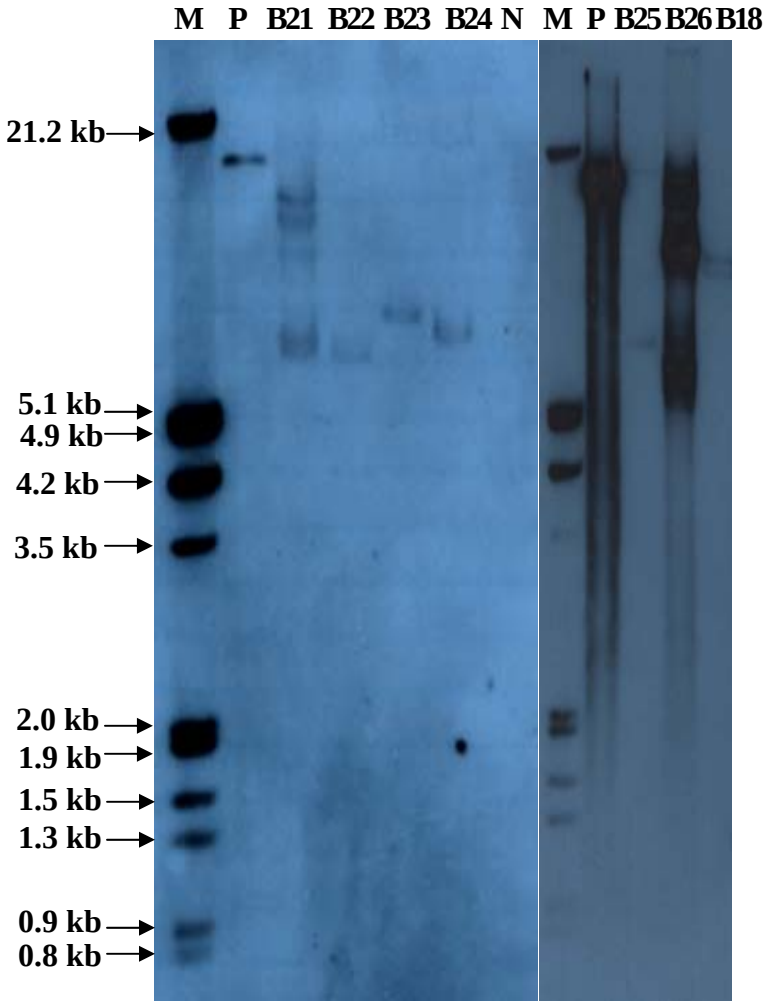


Figure 4. Southern blot analysis of putative transgenic hybrid aspens with the *iaaM* gene probe. Genomic DNA extracted from different transgenic hybrid aspen lines and a non-transformed control plant was digested with *Sac*I and hybridized with the probe specific to the *iaaM* gene. M: Dig-labeled 1kb ladder; P: pGI as a positive control; B18, B21, B22, B23, B24, B25, B26: the GRP-*iaaM* transgenic lines; N: non-transgenic plant.

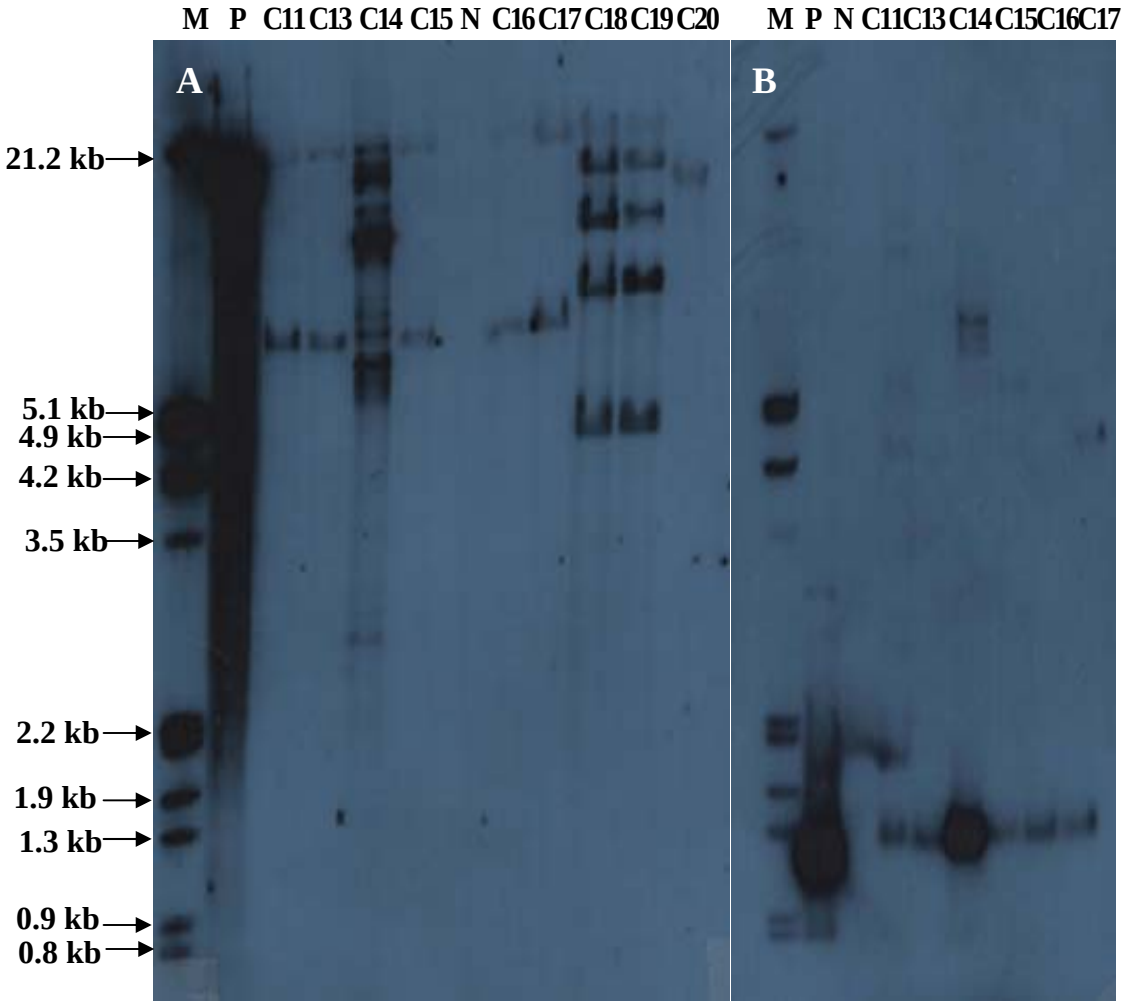


Figure 5. Southern blot analysis of putative transgenic hybrid aspens with the GRP-*iaaM*-35S-ACC *deaminase* gene probe. Genomic DNA extracted from different transgenic hybrid aspen lines and a non-transformed plant was digested with *Sac*I (A) and *Sal*I (B), and hybridized with the probe specific to the *iaaM* gene. *Sal*I resulted in a fragment including the GRP promoter and the *iaaM* gene, which shows the same size (1.35 kb) band as the *iaaM* gene in a positive vector. As a result, the multiple copies of the *iaaM* gene of C14 line in panel A were shown as a strong hybridization in panel B. M: Dig-labeled 1kb ladder; P: pGIA as a positive control; N: non-transgenic plant; C11, C13-C20: transgenic lines with the GRP-*iaaM*-35S-ACC *deaminase* gene.

B26, and three GRP-*iaaM*-35S-*ACC deaminase* transgenic lines, C14, C18, C19 (Table 1).

4.2. The growth patterns of the transgenic plants

The apical cuttings were performed in order to evaluate the growth of transgenic plants because all transgenic aspens were produced through two transformations during the experimental periods, which resulted in the different developmental stages of the transgenic plants. Therefore, cuttings were collected on March 22, April 4, April 18, 2003, and March 31, 2004. The cuttings of each transgenic plant were rooted and grown under the same environmental conditions to allow all of the plants to be at the same growing stage. However, due to infection by fungi, the cuttings propagated on March 22 and April 4 in 2003 did not survive. After cuttings and potting medium were treated with a fungicide (Banrot®, Scotts, Marysville, OH) on the date of April 18, 2003 and March 31, 2004, all shoots rooted and grew successfully. As a result of the rooting experiment in 2003, the cuttings in eleven of the fourteen GRP-*iaaM* transgenic lines and twelve of the thirteen GRP-*iaaM*-35S-*ACC deaminase* transgenic lines rooted successfully with each having at least three replicates. In 2004, the cuttings in twelve of the fourteen GRP-*iaaM* transgenic lines and nine of the thirteen GRP-*iaaM*-35S-*ACC deaminase* transgenic lines rooted successfully. The cuttings that resulted in two or less replicates were not included in the growth measurements. The plants were infected by spider mites in a greenhouse, thus they were treated with Softer®soap.

Table 1. Summary of confirmation of the inserted genes by PCR and Southern blot analysis[z].

Plasmid/ Inserted gene	Transgenic line	PCR confirmation		Southern blot (# of copy)
		iaaM	*ACC deaminase*	
Non-transgenic		-	NP[y]	-
pGI/ *iaaM*	B2	+	NP	+ (1)
	B6	+	NP	+ (1)
	B8	+	NP	+ (2)
	B11	+	NP	+ (1)
	B13	+	NP	+ (1)
	B14	+	NP	+ (1)
	B17	+	NP	+ (2)
	B18	+	NP	+ (2)
	B21	+	NP	+ (4)
	B22	+	NP	+ (1)
	B23	+	NP	+ (1)
	B24	+	NP	+ (1)
	B25	+	NP	+ (2)
	B26	+	NP	+ (>5)
pGIA/ *iaaM-ACC deaminase*	C1	+	+	+ (2)
	C4	+	+	+ (1)
	C5	+	+	+ (1)
	C7	+	+	+ (1)
	C11	+	+	+ (2)
	C13	+	+	+ (2)
	C14	+	+	+ (>5)
	C15	+	+	+ (2)
	C16	+	+	+ (2)
	C17	+	+	+ (2)
	C18	+	+	+ (5)
	C19	+	+	+ (5)
	C20	+	+	+ (1)

[z]: PCR was performed with two specific primers to the *iaaM* and the *ACC deaminase* gene, respectively, and Southern blot was hybridized with the *iaaM* probe.
[y]: Not performed because the GRP-*iaaM* transgenic plants were not transformed with the *ACC deaminase* gene.

After transplanting the rooted cuttings to pots in the greenhouse, the height and diameter of the aspen plants were measured every five weeks for five months (May 30, July 4, August 8, September 12, October 17) in 2003 and every five weeks (May 12, June 16, July 21, August 25, and September 29) in 2004. To compare their growth effectively, relative height or diameter and volume between five and fifteen weeks after transplanting were calculated. Due to the limitation of greenhouse space and the structure configuration after end of September, the greenhouse environment was not uniform. That is, some of the transgenic lines placed near the window of greenhouse were still growing and some of the transgenic lines located in the center of the greenhouse stopped the growth Thus, the height and diameter of the plants at 20 weeks were not included in the statistical analyses.

In 2003, 15 weeks after transplanting, the GRP-*iaaM* transgenic lines B2, B14, B17, B24, B26, and the GRP-*iaaM*-35S-*ACC deaminase* transgenic line C15 were significantly shorter, but the GRP-*iaaM*-35S-*ACC deaminase* transgenic lines C5 and C18 were significantly higher than non-transgenic control (Fig. 6 and Table 2). However, no significant differences in relative height, diameter, and relative diameter were found between both transgenic plants and the non-transgenic plants (Fig.7-9 and Table 3-5). The volume showed the same pattern with the results of height. Seven of eleven GRP-*iaaM* transgenic lines (B2, B8, B14, B17, B23, B24, B26) and two of twelve GRP-*iaaM*-35S-*ACC deaminase* transgenic lines (C15, C17) had significantly smaller volume (Fig. 10 and Table 6). Most of the GRP-*iaaM*-35S-*ACC deaminase* transgenic lines exhibited larger volumes than non-transgenic control, but the differences were not significant (Fig

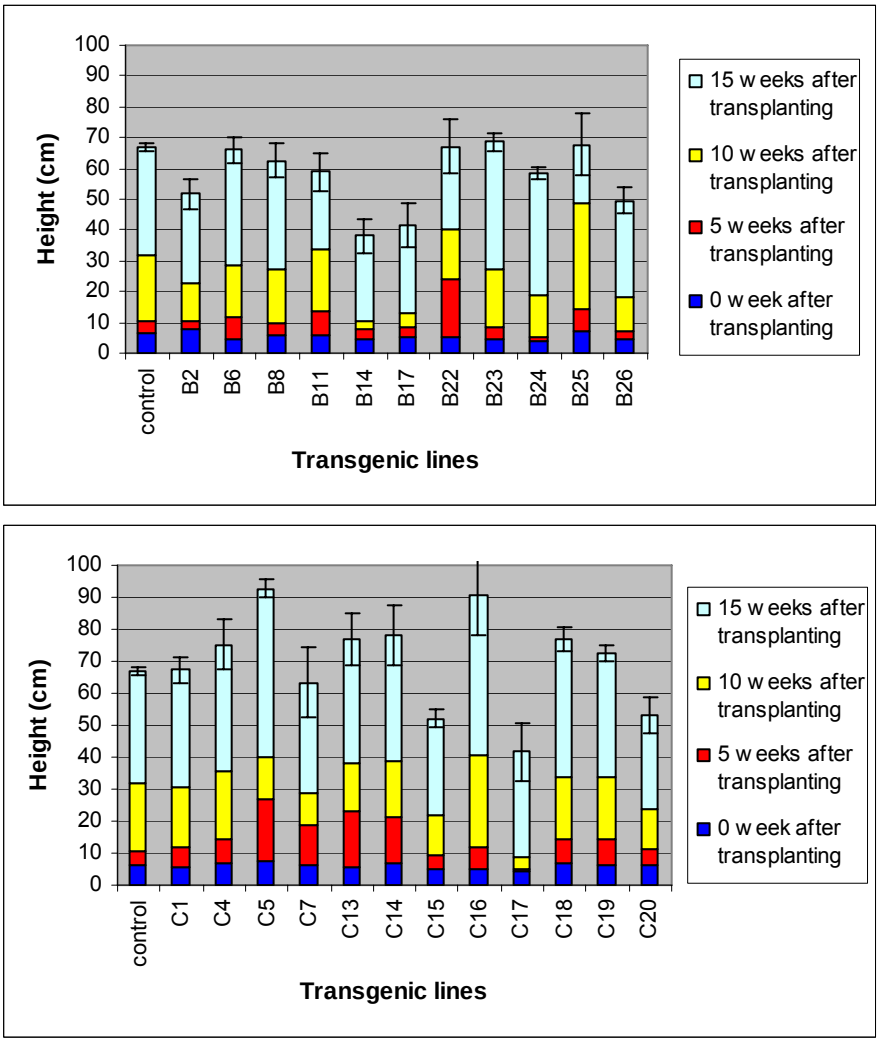


Figure 6. The means of heights of transgenic and non-transgenic plants at 0, 5, 10, and 15 weeks after transplanting in 2003. Control: non-transgenic plant; B2, B6, B8, B11, B14, B17, B22, B23, B24, B25, B26: transgenic plants expressing the GRP-*iaaM* gene; C1, C4, C5, C7, C13, C14, C15, C16, C17, C18, C19, C20: transgenic plants expressing the GRP-*iaaM*-35S-*ACC deaminase* genes. The bar means standard error.

Table 2 . Results of T-test of heights between transgenic and non-transgenic plants 15 weeks after transplanting in 2003.

Height of control (cm)	***iaaM*** [z]	Height (cm)	T-value (P-value)	***iaaM-ACC deaminase*** [y]	Height (cm)	T-value (P-value)
66.7	**B2**	51.8	3.177 (0.05)*	**C1**	67.2	-0.126 (0.905)ns
	B6	66.0	0.629 (0.553)ns	**C4**	70.6	-0.183 (0.862)ns
	B8	62.6	1.030 (0.343)ns	**C5**	92.7	-8.309 (<0.001)***
	B11	59.0	1.201 (0.353)ns	**C7**	79.7	-0.955 (0.440)ns
	B14	38.0	4.905 (0.016)*	**C13**	77.0	-1.271 (0.332)ns
	B17	41.8	3.575 (0.037)*	**C14**	78.3	-1.227 (0.345)ns
	B22	67.0	0.234 (0.836)ns	**C15**	52.1	4.972 (0.016)*
	B23	68.6	0.129 (0.901)ns	**C16**	90.8	-1.874 (0.202)ns
	B24	58.3	3.247 (0.023)*	**C17**	41.7	2.715 (0.113)ns
	B25	67.8	0.126 (0.911)ns	**C18**	76.8	-2.466 (0.0345)*
	B26	49.4	3.718 (0.007)**	**C19**	72.7	-2.165 (0.074)ns
				C20	52.8	2.391 (0.139)ns

[z]*iaaM*: GRP-*iaaM* transgenic plants.
[y]*iaaM-ACC deaminase*: GRP-*iaaM*-35S-*ACC deaminase* transgenic plants.
***: significant at 0.1% (P < 0.001); **: significant at 1% (0.001<P< 0.01); *: significant at 5% (0.01<P<0.05); ns: not significant.

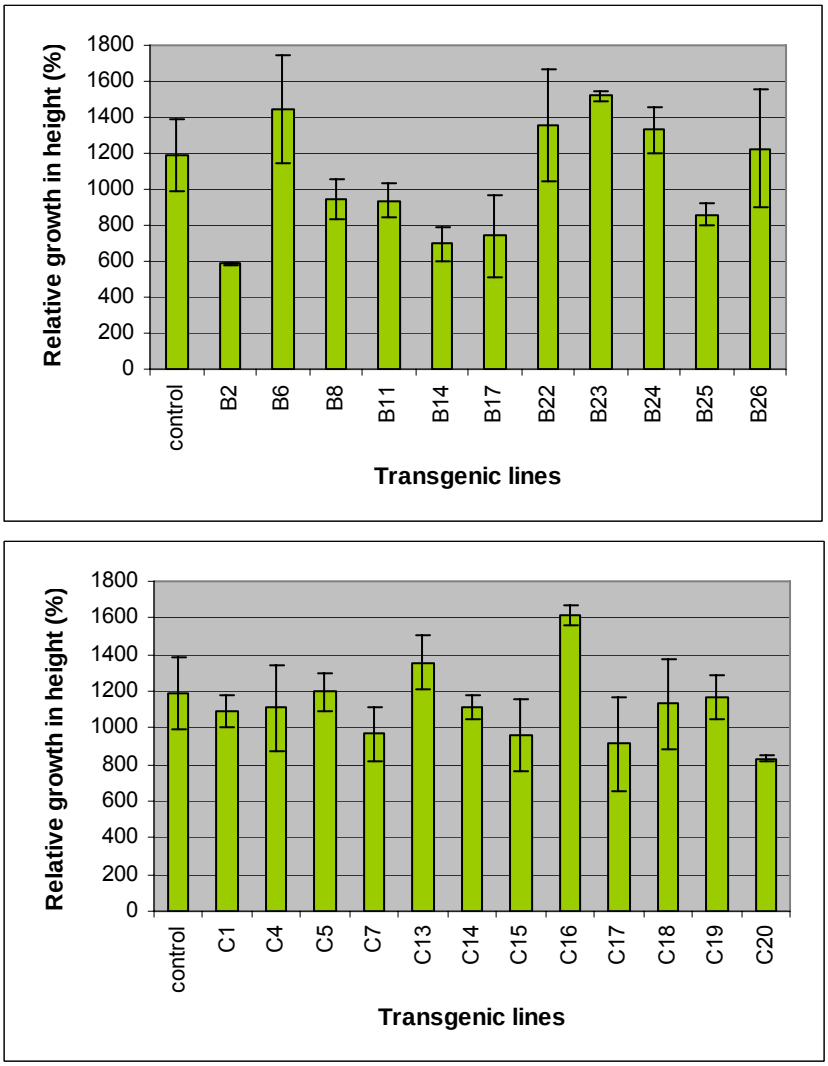


Figure 7. The means of relative growth in height of transgenic and non-transgenic plants in 2003. Control: non-transgenic plant; B2, B6, B8, B11, B14, B17, B22, B23, B24, B25, B26: transgenic plants expressing the GRP-*iaaM* gene; C1, C4, C5, C7, C13, C14, C15, C16, C17, C18, C19, C20: transgenic plants expressing the GRP-*iaaM*-35S-*ACC deaminase* genes. The bar means standard error.

Table 3. Results of T-test of relative growth in height between transgenic and non-transgenic plants in 2003.

Relative height of control (%)	***iaaM*** [z]	Relative height (%)	T-value (P-value)	***iaaM-ACC deaminase*** [y]	Relative height (%)	T-value (P-value)
1189.9	**B2**	584.2	0.675 (0.548)ns	**C1**	1093.6	-0.038 (0.971)ns
	B6	1443.6	-1.014 (0.357)ns	**C4**	1107.5	-0.418 (0.694)ns
	B8	941.9	-0.105 (0.923)ns	**C5**	1199.1	0.070 (0.947)ns
	B11	938.4	-0.099 (0.928)ns	**C7**	966.7	0.318 (0.764)ns
	B14	697.8	0.418 (0.704)ns	**C13**	1357.1	-0.967 (0.388)ns
	B17	739.5	0.301 (0.779)ns	**C14**	1112.3	0.108 (0.918)ns
	B22	1353.9	-0.835 (0.442)ns	**C15**	963.8	-0.144 (0.893)ns
	B23	1517.6	-1.368 (0.265)ns	**C16**	1618.0	-0.526 (0.618)ns
	B24	1329.2	-0.923 (0.424)ns	**C17**	913.6	-0.040 (0.969)ns
	B25	859.1	0.072 (0.947)ns	**C18**	1131.5	-0.0236 (0.982)ns
	B26	1227.6	-0.583 (0.581)ns	**C19**	1164.7	-0.155 (0.885)ns
				C20	832.5	0.131 (0.904)ns

[z]*iaaM*: GRP-*iaaM* transgenic plants.
[y]*iaaM-ACC deaminase*: GRP-*iaaM*-35S-*ACC deaminase* transgenic plants.
***: significant at 0.1% (P < 0.001); **: significant at 1% (0.001<P< 0.01); *: significant at 5% (0.01<P<0.05); ns: not significant.

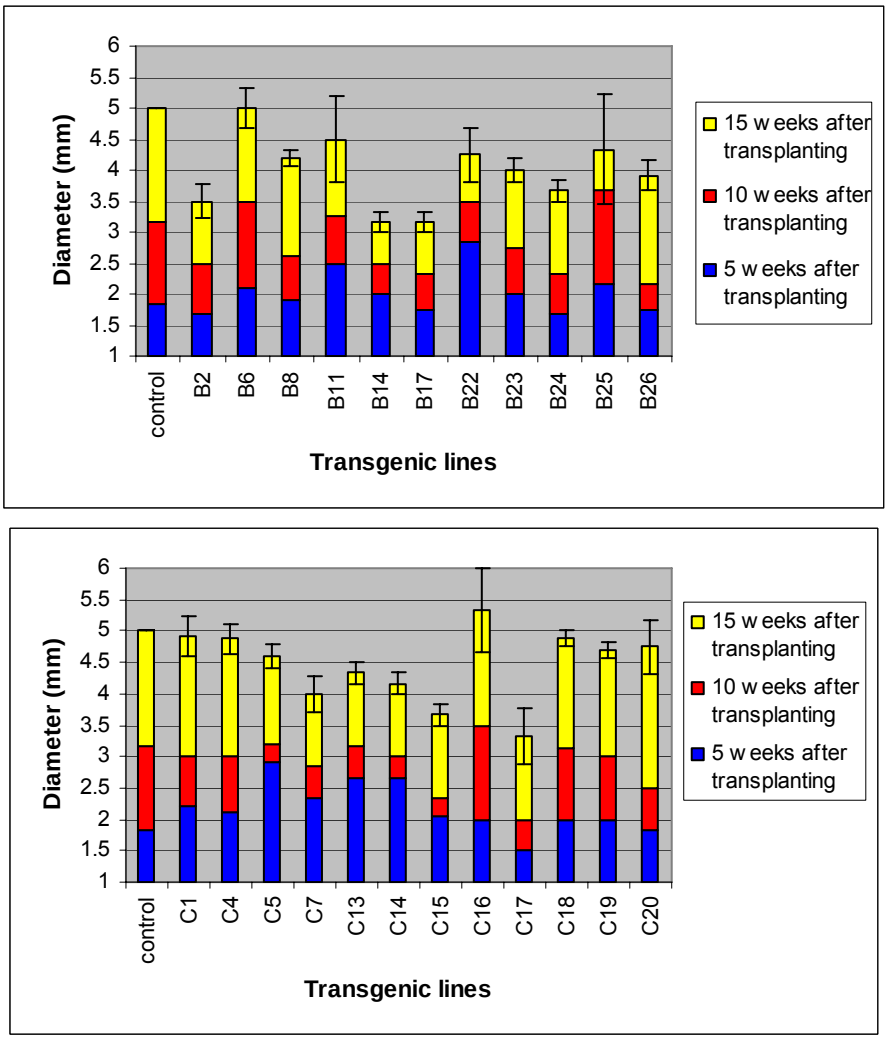


Figure 8. The means of diameters of transgenic and non-transgenic plants at 5, 10, and 15 weeks after transplanting in 2003. Control: non-transgenic plant; B2, B6, B8, B11, B14, B17, B22, B23, B24, B25, B26: transgenic plants expressing the GRP-*iaaM* gene; C1, C4, C5, C7, C13, C14, C15, C16, C17, C18, C19, C20: transgenic plants expressing the GRP-*iaaM*-35S-*ACC deaminase* genes. The bar means standard error.

Table 4. Results of T-test of diameters between transgenic and non-transgenic plants 15 weeks after transplanting in 2003.

Diameter of control (mm)	***iaaM***[z]	Diameter (mm)	T-value (P-value)	***iaaM-ACC deaminase***[z]	Diameter (mm)	T-value (P-value)
5.0	**B2**	3.5	0.195 (0.858)ns	**C1**	5.1	-0.327 (0.755)ns
	B6	5.0	-0.970 (0.404)ns	**C4**	4.9	-0.884 (0.442)ns
	B8	4.2	-0.360 (0.744)ns	**C5**	4.6	0.306 (0.770)ns
	B11	4.0	-0.190 (0.862)ns	**C7**	4.0	0.465 (0.658)ns
	B14	3.2	0/463 (0.675)ns	**C13**	4.3	-0.463 (0.675)ns
	B17	3.2	0.463 (0.675)ns	**C14**	4.3	0.302 (0.773)ns
	B22	4.3	-0.380 (0.725)ns	**C15**	3.7	0.661 (0.951)ns
	B23	4.0	-0.200 (0.856)ns	**C16**	5.3	-0.133 (0.899)ns
	B24	3.7	-0.410 (0.698)ns	**C17**	3.3	0.314 (0.769)ns
	B25	4.3	-0.380 (0.719)ns	**C18**	4.9	-0.944 (0.928)ns
	B26	3.9	-0,130 (0.905)ns	**C19**	4.7	-0.113 (0.915)
				C20	4.8	0.107 (0.918)ns

[z]*iaaM*: GRP-*iaaM* transgenic plants.
[y]*iaaM-ACC deaminase*: GRP-*iaaM*-35S-*ACC deaminase* transgenic plants.
***: significant at 0.1% (P < 0.001); **: significant at 1% (0.001<P< 0.01); *: significant at 5% (0.01<P<0.05); ns: not significant.

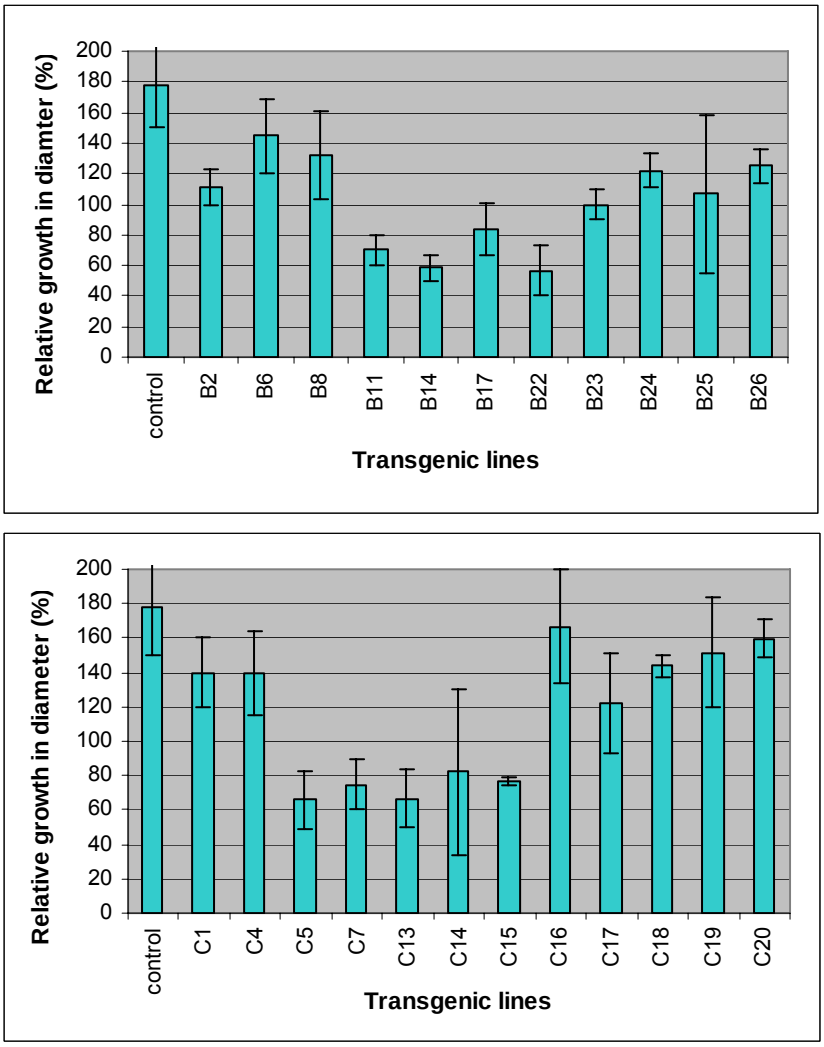


Figure 9. The means of relative growth in diameter of transgenic and non-transgenic plants in 2003. Control: non-transgenic plant; B2, B6, B8, B11, B14, B17, B22, B23, B24, B25, B26: transgenic plants expressing the GRP-*iaaM* gene; C1, C4, C5, C7, C13, C14, C15, C16, C17, C18, C19, C20: transgenic plants expressing the GRP-*iaaM*-35S-*ACC deaminase* genes. The bar means standard error.

Table 5. Results of T-test of relative growth in diameter between transgenic and non-transgenic plants in 2003.

Relative diameter of control (%)	***iaaM***[z]	Relative diameter (%)	T-value (P-value)	***iaaM-ACC deaminase***[y]	Relative diameter (%)	T-value (P-value)
177.8	**B2**	111.1	0.446 (0.686)ns	**C1**	140.0	0.296 (0.779)ns
	B6	144.7	-0.208 (0.845)ns	**C4**	139.6	-0.110 (0.914)ns
	B8	132.0	0.024 (0.982)ns	**C5**	65.83	1.680 (0.084)ns
	B11	70.0	1,277 (0.292)ns	**C7**	75.0	1.452 (0.220)ns
	B14	58.3	1.521 (0.226)ns	**C13**	66.67	1.298 (0.264)ns
	B17	83.3	0.973 (0.386)ns	**C14**	91.75	1.061 (0.333)ns
	B22	56.7	1.500 (0.208)ns	**C15**	77.27	1.152 (0.333)ns
	B23	100.0	0.671 (0.550)ns	**C16**	166.7	0.122 (0.907)ns
	B24	122.2	0.223 (0.838)ns	**C17**	122.2	0.196 (0.853)ns
	B25	106.7	0.374 (0.723)ns	**C18**	143.8	0.324 (0.759)ns
	B26	125.0	0.167 (0.878)ns	**C19**	151.7	0.115 (0.912)ns
				C20	159.7	-0.530 (0.634)ns

[z]*iaaM*: GRP-*iaaM* transgenic plants.
[y]*iaaM-ACC deaminase*: GRP-*iaaM*-35S-*ACC deaminase* transgenic plants.
***: significant at 0.1% (P < 0.001); **: significant at 1% (0.001<P< 0.01); *: significant at 5% (0.01<P<0.05); ns: not significant.

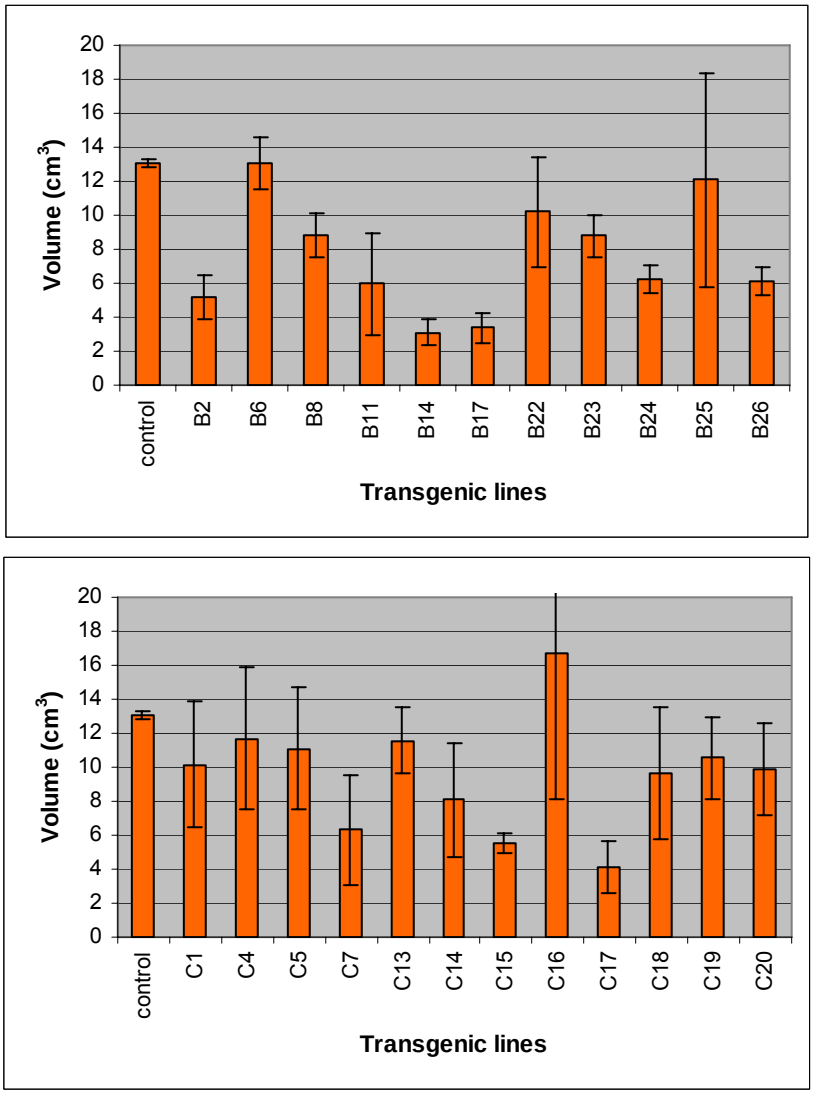


Figure 10. The means of volumes of transgenic and non-transgenic plants 15 weeks after transplanting in 2003. Control: non-transgenic plant; B2, B6, B8, B11, B14, B17, B22, B23, B24, B25, B26: transgenic plants expressing the GRP-*iaaM* gene; C1, C4, C5, C7, C13, C14, C15, C16, C17, C18, C19, C20: transgenic plants expressing the GRP-*iaaM*-35S-*ACC deaminase* genes. The bar means standard error.

Table 6. Results of T-test of volumes between transgenic and non-transgenic plants 15 weeks after transplanting in 2003.

Volume of control (cm^3)	***iaaM*** [z]	Volume (cm^3)	T-value (P-value)	***iaaM-ACC deaminase*** [y]	Volume (cm^3)	T-value (P-value)
13.08	**B2**	5.20	6.009 (0.009)**	**C1**	14.23	-0.454 (0.674)ns
	B6	13.06	0.324 (0.759)ns	**C4**	14.57	-0.493 (0.656)ns
	B8	8.86	3.408 (0.019)*	**C5**	15.51	-1.706 (0.163)ns
	B11	7.80	0.451 (0.671)ns	**C7**	8.90	0.815 (0.446)ns
	B14	3.09	11.111 (<0.001)***	**C13**	11.56	0.773 (0.521)ns
	B17	3.40	9.987 (0.002)**	**C14**	10.75	1.415 (0.293)ns
	B22	10.20	1.040 (0.408)ns	**C15**	5.52	10.316 (<0.001)***
	B23	8.79	3.506 (0.025)*	**C16**	22.24	-0.843 (0.447)ns
	B24	6.22	7.790 (0.001)**	**C17**	4.12	5.860 (0.028)*
	B25	12.07	0.239 (0.834)ns	**C18**	14.45	-0.989 (0.396)ns
	B26	6.14	7.018 (<0.001)***	**C19**	12.66	0.464 (0.662)ns
				C20	9.87	1.324 (0.316)ns

[z]*iaaM*: GRP-*iaaM* transgenic plants.
[y]*iaaM-ACC deaminase*: GRP-*iaaM*-35S-*ACC deaminase* transgenic plants.
***: significant at 0.1% (P < 0.001); **: significant at 1% (0.001<P< 0.01); *: significant at 5% (0.01<P<0.05); ns: not significant.

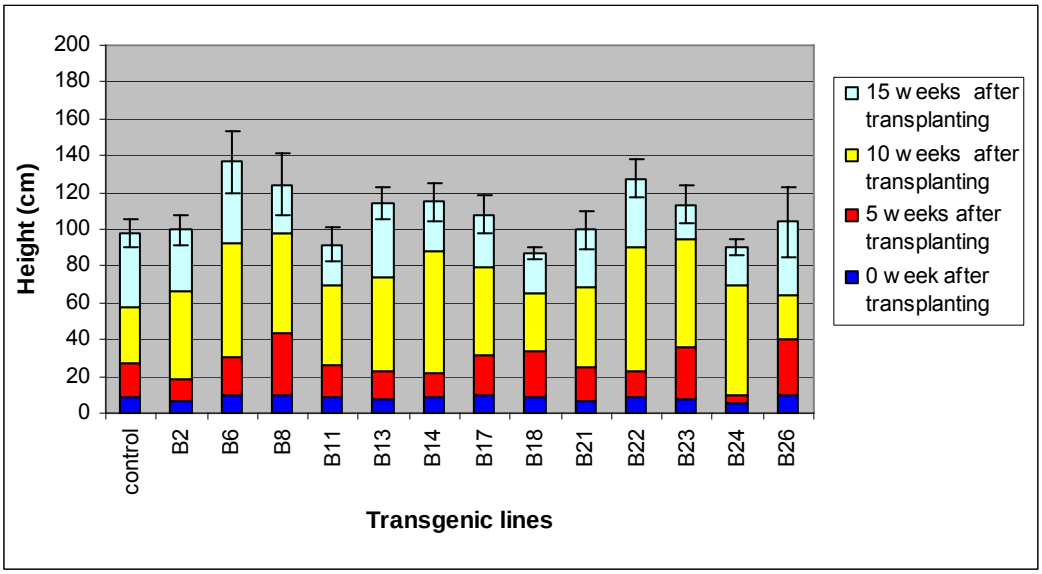
10 and Table 6). The differences could be shown between each group by a method of linear contrast, and Table 7 shows that the GRP-*iaaM*-35S-*ACC deaminase* transgenic groups had significantly more growth in height, diameter, and volume than the GRP-*iaaM* transgenic plants. The GRP-*iaaM* transgenic plants had significantly less growth in diameter and relative growth in diameters than non-transgenic control, which resulted in less growth in volume than non-transgenic plants in the linear contrast (Table 7).

In 2004, the GRP-*iaaM* transgenic plants were not significantly different in height from the non-transgenic control, but the GRP-*iaaM*-35S-*ACC deaminase* transgenic lines C7, C11, C13, and C18 were significantly taller than the control (Fig. 11 and Table 8). The GRP-*iaaM* transgenic line B22 and the GRP-*iaaM*-35S-*ACC deaminase* transgenic lines C7, C13,and C18 had more relative growth in height than non-transgenic control (Fig. 12 and Table 9). The diameters of both transgenic plants were not significantly different from non-transgenic control except the GRP-*iaaM*-35S-*ACC deaminase* transgenic line C18 which was significantly thicker than the control (Fig. 13 and Table 10), but the relative growth in diameters were not significantly different from the control (Fig. 14 and Table 11). The transgenic lines B22, C13, and C18 had larger volumes than the non-transgenic control (Fig 15 and Table 12). Linear contrast showed the GRP-*iaaM*-35S-*ACC deaminase* transgenic plants had better growth in height, relative height, and volume than the GRP-*iaaM* transgenic plants and the control (Table 13).

Table 7. Linear contrasts in heights, diameters, and volumes of transgenic and non-transgenic plants in 2003.

Treatments	H15	RH	D15	RD	Vol
Control	66.7	1189.9	5.0	177.8	13.08
iaaM[y]	57.7	1094.4	4.04	106.3	7.95
iaaM-ACC deaminase[x]	71.4	1125.8	4.54	112.1	12.40
Control vs *iaaM*	(0.192)ns	(0.997)ns	(0.004)**	(0.006)**	(0.049)*
Control vs *iaaM-ACC deaminase*	(0.503)ns	(0.716)ns	(0.183)ns	(0.025)*	(0.798)ns
iaaM vs *iaaM-ACC deamnise*	(<0.001)***	(0.326)ns	(<0.001)***	(0.150)ns	(<0.001)***

[z] H0, H5, H10, and H15: means of heights at 0, 5, 10, and 15 weeks after transplanting; RH: mean of relative heights; D5, D10, and D15: means of diameters at 5, 10, and 15 weeks after transplanting; RD: mean of relative diameters; Vol: mean of volumes.
[y] *iaaM*: GRP-*iaaM* transgenic plants.
[x] *iaaM-ACC deaminase*: GRP-*iaaM*-35S-*ACC deaminase* transgenic plants.
***: significant at 0.1% (P < 0.001); **: significant at 1% (0.001<P< 0.01); *: significant at 5% (0.01<P<0.05); ns: not significant.



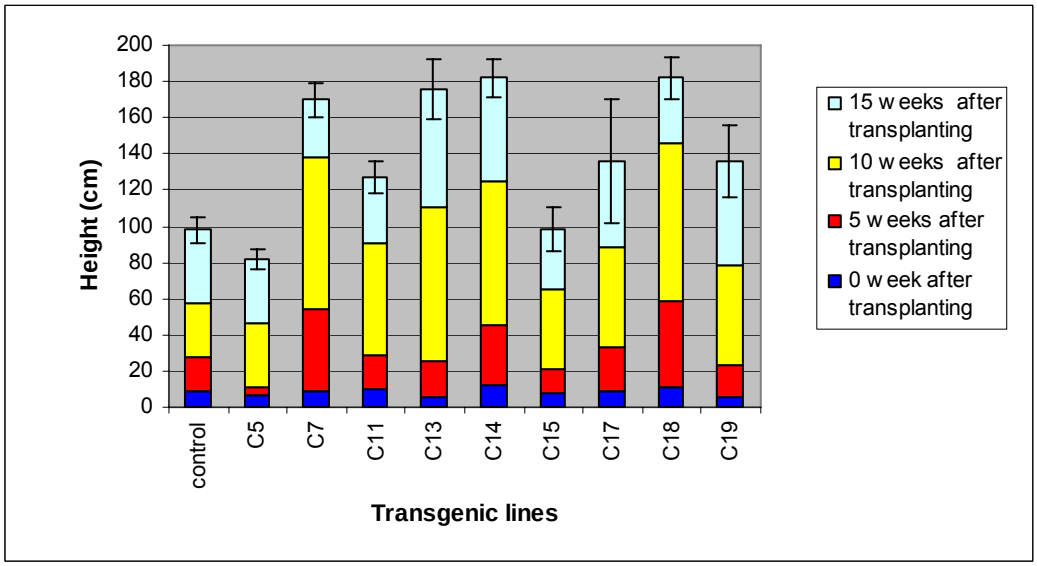


Figure 11. The means of heights of transgenic and non-transgenic plants at 0, 5, 10, and 15 weeks after transplanting in 2004. Control: non-transgenic plant; B2, B6, B8, B11, B13, B14, B17, B18, B21, B22, B23, B24, B26: transgenic plants expressing the GRP-*iaaM* gene; C5, C7, C11, C13, C14, C15, C17, C18, C19: transgenic plants expressing the GRP-*iaaM*-35S-*ACC deaminase* genes. The bar means standard error.

Table 8. Results of T-test of heights between transgenic and non-transgenic plants 15 weeks after transplanting in 2004.

Height of control (cm)	***iaaM*** [z]	Height (cm)	T-value (P-value)	***iaaM-ACC deaminase***[y]	Height (cm)	T-value (P-value)
99.2	**B2**	99.6	-1.217 (0.245)ns	**C5**	81.3	-0.158 (0.877)ns
	B6	136.5	0.343 (0.742)ns	**C7**	169.7	-5.148 (<0.001)***
	B8	124.4	-2.042 (0.075)ns	**C11**	127.5	-2.800 (0.019)*
	B11	91.8	-0.258 (0.7999)ns	**C13**	175.5	-4.347 (0.002)*
	B13	114.1	-2.047 (0.059)ns	**C14**	181.9	-1.354 (0.213)ns
	B14	115.0	0.694 (0.510)ns	**C15**	98.2	-0.998 (0.348)ns
	B17	107.8	-0.865 (0.398)ns	**C17**	135.8	-1.505 (0.229)ns
	B18	86.9	-0.505 (0.625)ns	**C18**	181.9	-5.475 (<0.001)***
	B21	99.6	-1.121 (0.281)ns	**C19**	136.2	-2.300 (0.083)ns
	B22	127.7	-2.712 (0.022)ns			
	B23	113.3	-1.888 (0.088)ns			
	B24	90.5	-1.168 (0.268)ns			
	B26	103.9	-1.011 (0.345)ns			

[z]*iaaM*: GRP-*iaaM* transgenic plants.
[y]*iaaM-ACC deaminase*: GRP-*iaaM*-35S-*ACC deaminase* transgenic plants.
***: significant at 0.1% (P < 0.001); **: significant at 1% (0.001<P< 0.01); *: significant at 5% (0.01<P<0.05); ns: not significant.

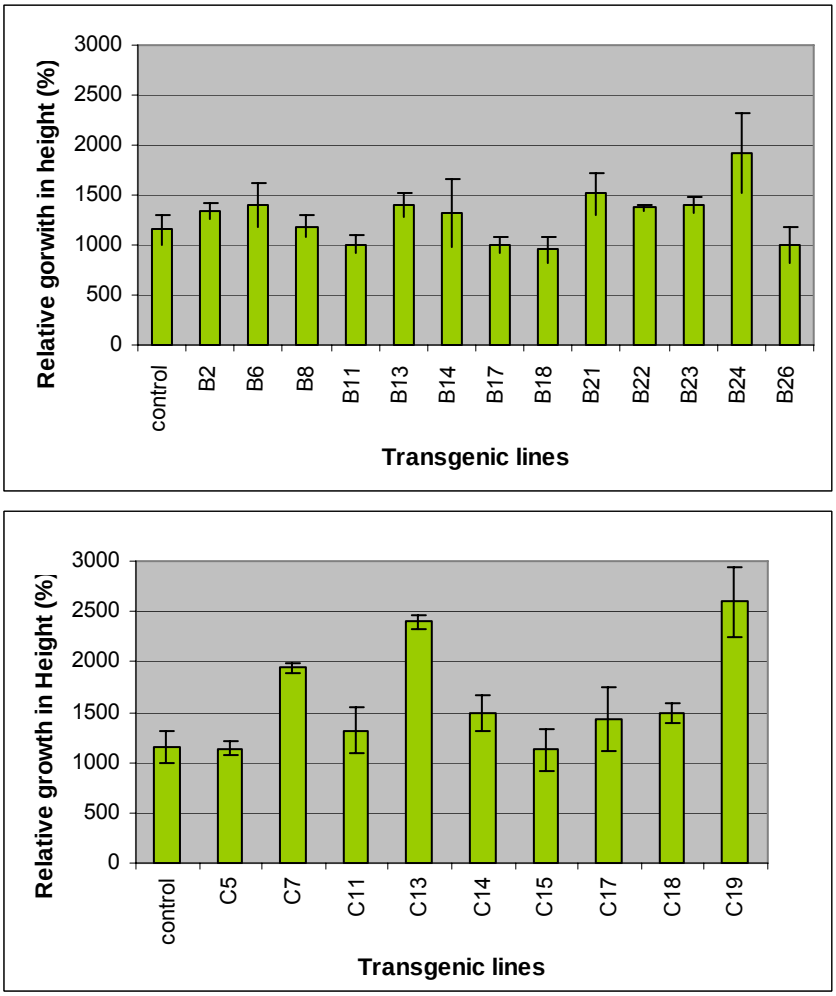


Figure 12. The means of relative growth in height of transgenic and non-transgenic plants in 2004. Control: non-transgenic plant; B2, B6, B8, B11, B13, B14, B17, B18, B21, B22, B23, B24, B26: transgenic plants expressing the GRP-*iaaM* gene; C5, C7, C11, C13, C14, C15, C17, C18, C19: transgenic plants expressing the GRP-*iaaM*-35S-*ACC deaminase* genes. The bar means standard error.

Table 9. Results of T-test of relative growth in height between transgenic and non-transgenic plants in 2004.

Relative height of control (%)	***iaaM*** [z]	Relative height (%)	T-value (P-value)	***iaaM-ACC deaminase*** [y]	Relative height (%)	T-value (P-value)
1151.94	**B2**	1341.4	-1.954 (0.074)ns	**C5**	1136.8	-1.037 (0.322)ns
	B6	1393.9	-0.020 (0.984)ns	**C7**	1940.4	-5.047 (0.001)**
	B8	1189.5	-1.167 (0.266)ns	**C11**	1320.5	-1.300 (0.250)ns
	B11	1003.0	0.009 (0.993)ns	**C13**	2762.2	-8.783 (<0.001)***
	B13	1396.4	-2.058 (0.057)ns	**C14**	1482.8	-0.383 (0.709)ns
	B14	1317.1	-1.021 (0.354)ns	**C15**	1123.1	-0.720 (0.499)ns
	B17	992.9	0.120 (0.906)ns	**C17**	1435.2	-1.363 (0.2445)ns
	B18	952.3	-0.132 (0.897)ns	**C18**	1485.9	-2.575 (0.024)*
	B21	1512.1	-2.058 (0.062)ns	**C19**	3193.3	-2.665 (0.117)ns
	B22	1371.4	-2.255 (0.048)*			
	B23	1404.9	-2.275 (0.054)ns			
	B24	1924.0	-2.254 (0.110)ns			
	B26	1006.9	-0.318 (0.757)ns			

[z]*iaaM*: GRP-*iaaM* transgenic plants.
[y]*iaaM-ACC deaminase*: GRP-*iaaM*-35S-*ACC deaminase* transgenic plants.
***: significant at 0.1% (P < 0.001); **: significant at 1% (0.001<P< 0.01); *: significant at 5% (0.01<P<0.05); ns: not significant.

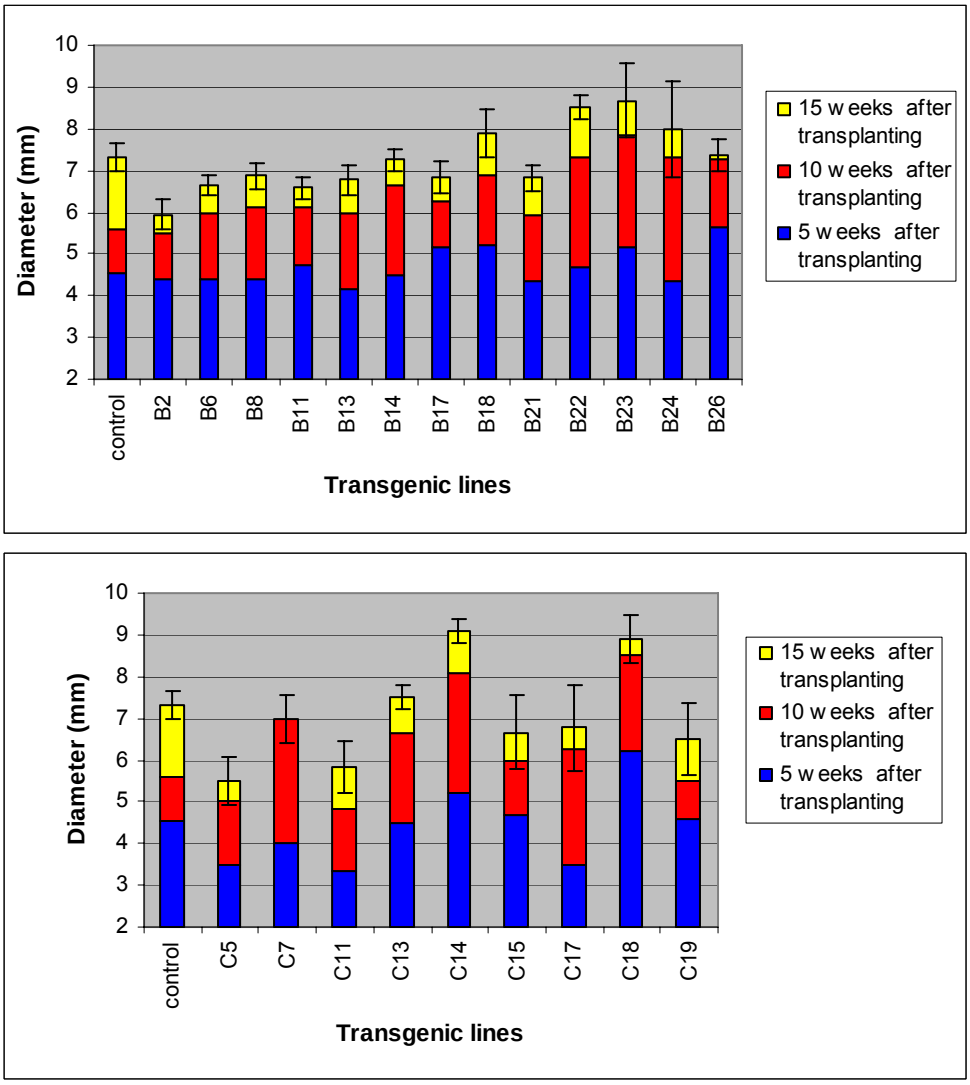


Figure 13. The means of diameters of transgenic and non-transgenic plants at 5, 10, and 15 weeks after transplanting in 2004. Control: non-transgenic plant; B2, B6, B8, B11, B14, B17, B18, B21, B22, B23, B24, B26: transgenic plants expressing the GRP-*iaaM* gene; C5, C7, C11, C13, C14, C15, C17, C18, C19: transgenic plants expressing the GRP-*iaaM*-35S-*ACC deaminase* genes. The bar means standard error.

Table 10. Results of T-test of diameters between transgenic and non-transgenic plants 15 weeks after transplanting in 2004.

Diameter of control (mm)	***iaaM***[z]	Diameter (mm)	T-value (P-value)	***iaaM-ACC deaminase***[y]	Diameter (mm)	T-value (P-value)
7.3	**B2**	5.9	-0.081 (0.937)ns	**C5**	5.5	0.300 (0.770)ns
	B6	6.6	0.827 (0.427)ns	**C7**	7.0	-0986 (0.346)ns
	B8	6.9	-0.966 (0.355)ns	**C11**	5.8	0.014 (0.989)ns
	B11	6.6	-0.163 (0.863)ns	**C13**	7.5	-1.565 (0.149)ns
	B13	6.8	-0.859 (0.409)ns	**C14**	9.1	-0.330 (0.748)ns
	B14	7.3	-1.341 (0.210)ns	**C15**	6.7	-0.601 (0.560)ns
	B17	6.8	-0.236 (0.816)ns	**C17**	6.8	-0.640 (0.542)ns
	B18	7.9	-1.756 (0.103)ns	**C18**	8.9	-2.965 (0.010)*
	B21	6.8	-0.928 (0.373)ns	**C19**	6.5	-0.488 (0.639)ns
	B22	8.5	-2.514 (0.031)ns			
	B23	8.7	-2.096 (0.069)ns			
	B24	8.0	-1.399 (0.211)ns			
	B26	7.4	-1.412 (0.186)ns			

[z]*iaaM*: GRP-*iaaM* transgenic plants.
[y]*iaaM-ACC deaminase*: GRP-*iaaM*-35S-*ACC deaminase* transgenic plants.
***: significant at 0.1% (P < 0.001); **: significant at 1% (0.001<P< 0.01); *: significant at 5% (0.01<P<0.05); ns: not significant.

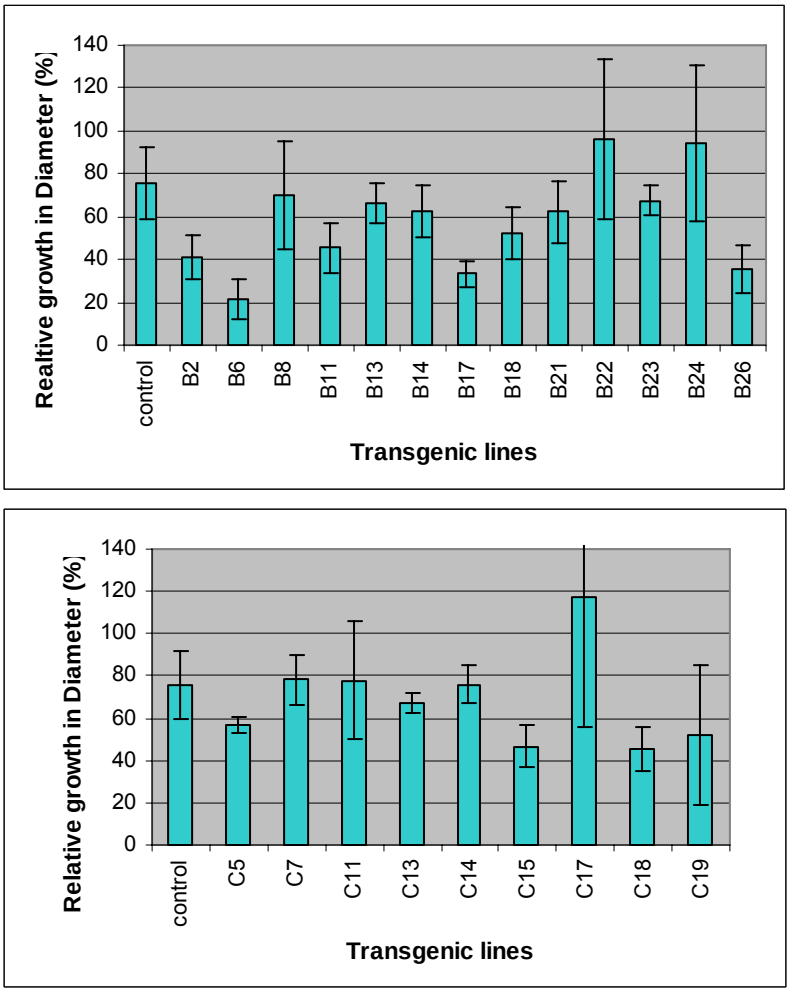


Figure 14. The means of relative growth in diameter of transgenic and non-transgenic plants in 2004. Control: non-transgenic plant; B2, B6, B8, B11, B14, B17, B18, B21, B22, B23, B24, B26: transgenic plants expressing the GRP-*iaaM* gene; C5, C7, C11, C13, C14, C15, C17, C18, C19: transgenic plants expressing the GRP-*iaaM*-35S-*ACC deaminase* genes. The bar means standard error.

Table 11. Results of T-test of relative growth in diameter between transgenic and non-transgenic plants in 2004.

Relative diameter of control (%)	***iaaM*[z]**	Relative diameter (%)	T-value (P-value)	***iaaM-ACC deaminase*[y]**	Relative diameter (%)	T-value (P-value)
75.7	**B2**	41.0	1.005 (0.331)ns	**C5**	56.5	0.238 (0.816)ns
	B6	52.6	1.231 (0.239)ns	**C7**	78.3	-0.8799 (0.400)ns
	B8	70.0	-0.310 (0.767)ns	**C11**	77.8	0.0691 (0.948)ns
	B11	46.4	0.1888 (0.378)ns	**C13**	67.2	-0.391 (0.704)ns
	B13	66.4	-0.309 (0.762)ns	**C14**	76.0	0.280 (0.783)ns
	B14	67.7	-0.105 (0.918)ns	**C15**	46.7	0.718 (0.489)ns
	B17	33.2	1.729 (0.110)ns	**C17**	117.6	-0.894 (0.466)ns
	B18	52.4	0.403 (0.694)ns	**C18**	45.1	0.797 (0.430)ns
	B21	62.2	-0.074 (0.942)ns	**C19**	52.1	0.228 (0.835)ns
	B22	96.1	-0.878 (0.445)ns			
	B23	67.4	-0.710 (0.491)ns			
	B24	94.4	-0.847 (0.459)ns			
	B26	35.6	1.242 (0.238)ns			

[z]*iaaM*: GRP-*iaaM* transgenic plants.
[y]*iaaM-ACC deaminase*: GRP-*iaaM*-35S-*ACC deaminase* transgenic plants.
***: significant at 0.1% (P < 0.001); **: significant at 1% (0.001<P< 0.01); *: significant at 5% (0.01<P<0.05); ns: not significant.

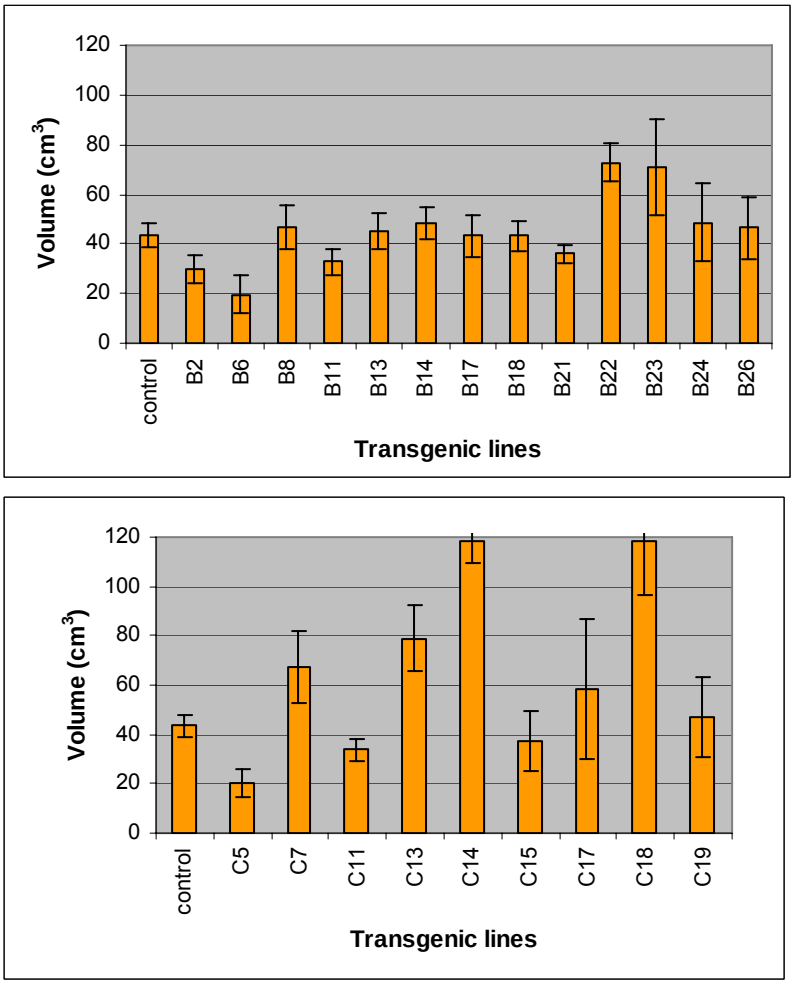


Figure 15. The means of volumes of transgenic and non-transgenic plants 15 weeks after transplanting in 2004. Control: non-transgenic plant; B2, B6, B8, B11, B14, B17, B18, B21, B22, B23, B24, B26: transgenic plants expressing the GRP-*iaaM* gene; C5, C7, C11, C13, C14, C15, C17, C18, C19: transgenic plants expressing the GRP-*iaaM*-35S-*ACC deaminase* genes. The bar means standard error.

Table 12. Results of T-test of volumes between transgenic and non-transgenic plants 15 weeks after transplanting in 2004.

Volume of control (cm^3)	***iaaM*** [z]	Volume (cm^3)	T-value (P-value)	***iaaM-ACC deaminase*** [y]	Volume (cm^3)	T-value (P-value)
43.5	**B2**	29.7	-0.383 (0.707)ns	**C5**	20.4	0.607 (0.559)ns
	B6	48.0	-0.435 (0.674)ns	**C7**	67.4	-2.531 (0.086)ns
	B8	47.0	-1.780 (0.113)ns	**C11**	33.8	-0.867 (0.406)ns
	B11	32.8	-0.425 (0.676)ns	**C13**	78.8	-3.423 (0.042)*
	B13	45.2	-1.859 (0.078)ns	**C14**	118.1	-2.440 (0.045)ns
	B14	48.2	-0.939 (0.375)ns	**C15**	37.4	-0.798 (0.470)ns
	B17	43.2	-1.091 (0.290)ns	**C17**	58.4	-1.110 (0.383)ns
	B18	43.1	-1.765 (0.101)ns	**C18**	118.2	-3.979 (0.011)**
	B21	35.9	-1.169 (0.263)ns	**C19**	47.2	-1.187 (0.321)ns
	B22	72.6	-4.306 (0.005)*			
	B23	70.6	-1.247 (0.280)ns			
	B24	48.6	-0.575 (0.596)ns			
	B26	46.3	-0.719 (0.495)ns			

[z]*iaaM*: GRP-*iaaM* transgenic plants.
[y]*iaaM-ACC deaminase*: GRP-*iaaM*-35S-*ACC deaminase* transgenic plants.
***: significant at 0.1% ($P < 0.001$); **: significant at 1% ($0.001<P< 0.01$); *: significant at 5% ($0.01<P<0.05$); ns: not significant.

Table 13. Linear contrasts in heights, diameters, and volumes of transgenic and non-transgenic plants in 2004.

Treatments	H15	RH	D15	RD	Vol
Control	99.2	1152.9	7.3	75.7	4348.4
iaaM[y]	106.6	1252.2	6.9	55.6	4335.2
iaaM-ACC deaminase[x]	148.2	1728.3	7.3	67.6	7134.5
Control vs *iaaM*	(0.417)ns	(0.455)ns	(0.782)ns	(0.435)ns	(0.547)ns
Control vs *iaaM-ACC deaminase*	(<0.001)***	(<0.001)***	(0.939)ns	(0.950)ns	(0.009)**
iaaM vs *iaaM-ACC deamnise*	(<0.001)***	(<0.001)***	(0.742)ns	(0.231)ns	(0.001)***

[z] H0, H5, H10, and H15: means of heights at 0, 5, 10, and 15 weeks after transplanting; RH: mean of relative heights; D5, D10, and D15: means of diameters at 5, 10, and 15 weeks after transplanting; RD: mean of relative diameters; Vol: mean of volumes.
[y] *iaaM*: GRP-*iaaM* transgenic plants.
[x] *iaaM-ACC deaminase*: GRP-*iaaM*-35S-*ACC deaminase* transgenic plants.
***: significant at 0.1% (P < 0.001); **: significant at 1% (0.001<P< 0.01); *: significant at 5% (0.01<P<0.05); ns: not significant.

4.3. Analyses of the auxin and ethylene levels of the transgenic plants

The amount of IAA was measured from young leaves of ten GRP-*iaaM* transgenic lines, thirteen GRP-*iaaM*-35S-*ACC deaminase* transgenic lines, and non-transgenic plants to determine the expression of the *iaaM* gene. The average IAA content in the control plants was 1.36 ng/g and the IAA levels in transgenic plants ranged from 0.98 ng/g to 1.56 ng/g (Tables 14 and 15).

The amount of ethylene was also measured from mature leaves of five GRP-*iaaM* transgenic lines, six GRP-*iaaM*-35S-*ACC deaminase* transgenic lines, and non-transgenic plants to compare the effect of the inserted *ACC deaminase* gene on the ethylene production of the transgenic hybrid aspens. The average ethylene content in control plants was 0.154 ng/g and the ethylene levels in transgenic plants ranged from 0.102 ng/g to 0.211 ng/g (Tables 14, 15, and Fig. 16).

In order to figure out a relation between height, relative height, diameter, relative diameter, or volume and auxin or ethylene content, linear correlation was performed. Figure 16 shows a negative correlation between ethylene content and heights of the GRP-*iaaM*-35S-*ACC deaminase* transgenic lines in 2003 and 2004. The heights of GRP-*iaaM*-35S-*ACC deaminase* transgenic lines in 2003 and 2004 showed a significant negative correlation (R^2=0.5054 and R^2=0.4754) with ethylene content (Fig.17).

Table 14 . The IAA and ethylene content in transgenic and non-transgenic plants having the GRP-*iaaM* gene[z].

Inserted gene	Line	2003			2004			IAA/ FW (ng/g)	Ethylene/ FW (ng/ g)
		n	Height (cm)	Diameter (mm)	n	Height (cm)	Diameter (mm)		
Non-transgenic		3	66.7 ± 1.3	5.0 ± 0.1	8	97.8 ± 7.2	7.2 ± 0.4	1.36 ±0.05	0.154 ± 0.04
GRP-*iaaM*	B2	3	51.8 ± 4.8	3.5 ± 0.3	8	99.6 ± 7.9	5.9 ± 0.4	1.56[y]	-
	B6	5	66.0 ± 4.2	5.0 ± 0.3	4	136.5 ± 16.8	7.7 ± 0.7	1.47	-
	B8	5	62.6 ± 5.8	4.2 ± 0.1	4	124.4 ± 16.5	6.9 ± 0.4	-[x]	-
	B11	3	59.0 ± 6.3	4.0 ± 0.5	11	91.8 ± 9.0	6.8 ± 0.3	1.37	-
	B13	-	-	-	13	114.1 ± 8.5	6.8 ± 0.3	-	-
	B14	3	38.0 ± 5.8	3.2 ± 0.2	4	115.0 ± 10.2	7.3 ± 0.3	-	-
	B17	3	41.8 ± 7.2	3.2 ± 0.2	9	107.8 ± 10.3	6.8 ± 0.4	-	-
	B18	-	-	-	5	86.9 ± 2.7	7.9 ± 0.6	1.16 ± 0.04	-
	B21	-	-	-	6	99.6 ± 10.8	6.8 ± 0.3	1.32 ± 0.16	-
	B22	3	67.0 ± 8.7	4.3 ± 0.4	3	127.7± 10.9	8.5 ± 0.3	1.37 ± 0.12	0.165 ± 0.02
	B23	4	68.6 ± 2.8	4.0 ± 0.2	3	113.3 ± 10.4	8.7 ± 0.9		0.180 ± 0.04
	B24	3	58.3 ± 2.0	3.7 ± 0.2	3	90.5 ± 4.3	8.0 ± 1.2	1.27 ± 0.15	0.197 ± 0.01
	B25	3	67.8 ± 9.9	4.3 ± 0.9	-	-	-	1.31	0.164 ± 0.01
	B26	6	49.4 ± 4.3	3.9 ± 0.3	4	103.9 ±19.3	7.9 ± 0.9	1.52 ± 0.04	0.190 ± 0.03

[z]: The heights and diameters in transgenic plants expressing the GRP-*iaaM* gene were measured 15 weeks after transplanting from cuttings. IAA was measured from young fresh leaves and ethylene was measured from mature leaves.

[y]: Had no replications, thus the standard errors are not included.

[x]: Indicates the data not collected.

Table 15. The IAA and ethylene content transgenic and non-transgenic plants having the GRP-*iaaM*-35S-*ACC deaminase* genes[z].

Inserted gene	Line	2003			2004			IAA/ FW (ng/g)	Ethylene/ FW (ng/ g)
		n	Height (cm)	Diameter (mm)	n	Height (cm)	Diameter (mm)		
Non-transgenic		3	66.7 ± 1.3	5.0 ± 0.1	10	103.0 ± 10.2	7.2 ± 0.4	1.36 ± 0.05	0.154 ± 0.04
GRP-*iaaM*-35S-*ACC deaminase*	C1	5	67.2 ± 4.0	4.9 ± 0.3	-	-[x]	-	1.15[y]	-
	C4	4	75.3 ± 7.8	4.9 ± 0.2	-	-	-	1.24	-
	C5	5	92.7 ± 2.8	4.6 ± 0.2	3	81.8 ± 5.6	5.5 ± 0.6	1.39 ± 0.01	-
	C7	3	63.3 ± 10.9	4.0 ± 0.3	3	169.7 ± 9.6	7.7 ± 1.2	1.27	-
	C11	-	-	-	3	127.5 ± 9.0	5.8 ± 0.6	1.18	-
	C13	3	77.0 ± 8.0	4.3 ± 0.2	3	175.5 ± 16.5	7.5 ± 0.3	1.01	-
	C14	3	78.3 ± 9.4	4.2 ± 0.2	5	181.9 ± 10.7	9.1 ± 0.3	1.31	0.102 ± 0.04
	C15	3	52.1 ± 2.6	3.7 ± 0.2	3	98.2 ± 11.8	6.7 ± 0.9	1.17	-
	C16	3	90.8 ± 12.8	5.3 ± 0.7	-	-	-	1.29	0.136 ± 0.02
	C17	3	41.7 ± 9.1	3.3 ± 0.4	3	135.5 ± 34.5	6.8 ± 1.0	0.98	0.211 ± 0.03
	C18	4	76.8 ± 3.9	4.9 ± 0.1	5	181.9 ± 11.6	8.9± 0.6	1.44 ± 0.15	0.155 ± 0.02
	C19	5	72.7 ± 2.5	4.7 ± 0.1	3	136.2 ± 19.9	6.5 ± 0.9	1.56 ± 0.08	0.177 ± 0.03
	C20	3	52.8 ± 5.6	4.8 ± 0.4	-	-	-	1.21 ± 0.17	0.178 ± 0.05

[z]: The heights and diameters in transgenic plants expressing the GRP-*iaaM*-CaMV35S-*ACC deaminase* genes were measured 15 weeks after transplanting from cuttings. IAA was measured from young fresh leaves and ethylene was measured from mature leaves.

[y]: Had no replications, thus the standard errors are not included.

[x]: Indicates the data not collected.

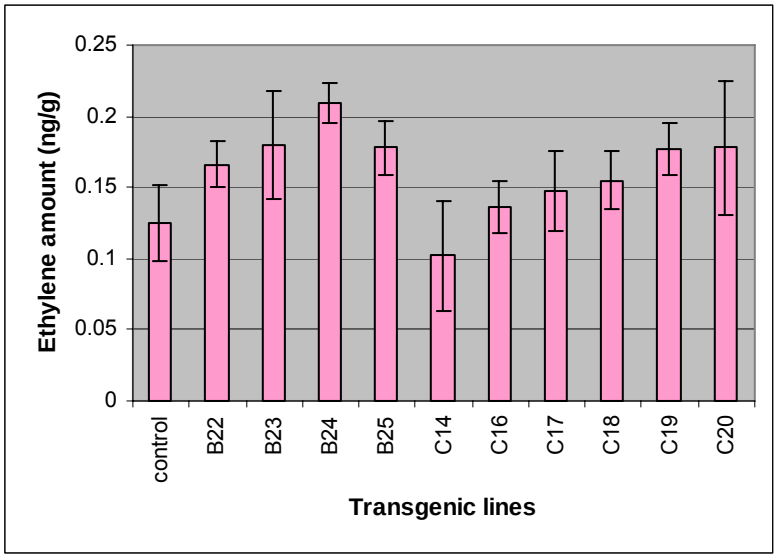


Figure 16. The ethylene content transgenic and non-transgenic plants. Control: non-transgenic control; B22, B23, B24, B25: transgenic plants expressing the GRP-*iaaM* gene; C14, C16, C17, C18, C19, C20: transgenic plants expressing the GRP-*iaaM*-35S-*ACC deaminase* genes. The bar means standard error.

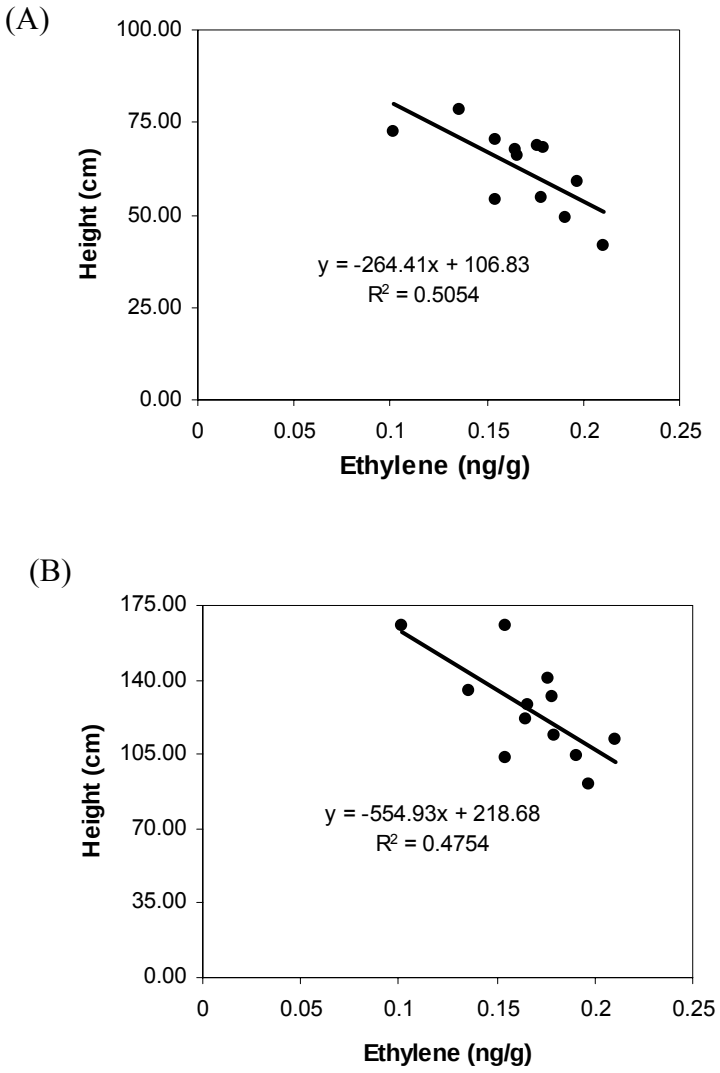


Figure 17. Linear correlation between the heights at 15 weeks after transplanting and ethylene content of transgenic plants and non-transgenic plants. The negative correlation between the heights and ethylene content was shown in 2003 (A) and 2004 (B).

CHAPTER V

DISCUSSION

5.1. Transformation and clonal plant propagation

Fourteen transgenic lines expressing the GRP-*iaaM* gene and thirteen transgenic lines expressing the GRP-*iaaM*-35S-*ACC deaminase* gene were produced by the transformation of a hybrid aspen with pGI or pGIA through two experiments with the transformation efficiency of 10.83 -15.0%. This was significantly lower (about 50%) than the efficiency reported by Dai et al. (2003). This lower frequency was primarily because shoots were harvested only in four weeks after shoot initiation. In Dai's procedures, shoots were regenerated from four to ten weeks.

Of all transformed plants, eight of fourteen GRP-*iaaM* transgenic lines (57.14%) and four of thirteen GRP-*iaaM*-35S-*ACC deaminase* transgenic lines (30.76%) showed a single copy of the inserted gene (Table 1). Most transgenic plants did not exhibit the significant differences in height and diameter growth from non-transgenic control *in vitro* (Fig 2). The low expression of the transgene might be due to multiple copies of the inserted gene. Multiple copies of the transgene and DNA methylation are often involved in the transgene inactivation. The insertion of multiple copies of the transformed gene has shown lower expression (Hobbs et al., 1990; Linn et al., 1990; Flavell, 1994; Araki et al., 2000).

After completing the experiments in 2003, stems of the transgenic plants were cut and rooted in 2004. All rooted successfully and grew faster than the cuttings of 2003. The mean height of the tallest transgenic line in 2003 was 81.9 cm, but plants in 2004 grew up to 181.9 cm (Figs. 6 and 11, Tables 14 and 15). These results may be caused from that the cuttings in 2003 were from the apical shoots but the cuttings in 2004 were from stems of plants. In addition, the fact that the stem cuttings in 2004 were processed one month earlier than those of 2003 might make them grow more vigorously in a more favorable environment. The height of plants 0 weeks after transplanting in 2003 were 4.17 ~ 7.6 cm, and the height of plants in 2004 were 4.9 ~11.6 cm (data not shown). The plants from shoot tip cuttings in 2003 were weak and took longer time to root. This condition appeared to predispose these plants to attack by fungal pathogens, which was successfully treated with fungicide but may have also led to reduced growth in the 2003 experiment.

5.2. The effect of the *iaaM* gene on the growth of the transgenic aspen

Accelerated tree growth in a short rotation farming system is required to compensate for a reduction in land area available for wood production and to satisfy a growing demand for wood (Tzfira et al., 1999). Since auxin plays a vital role in plant growth and development, the *iaaM* gene has potential to promote tree growth (Davies, 1987). Transgenic herbaceous plants expressing the *iaaM* gene have shown elongated stem in petunia (Klee et al., 1987) and tobacco (Kares et al., 1992) and elongated hypocotyl in

Arabidopsis (Romano et al., 1993; van der Graaff et al., 2003). The alteration of growth and development was mainly associated with elevated an auxin level in the transgenic plants. The correlation between plant height and IAA content was also shown in the report of Alekseeva et al. (2004). The height of transgenic tobacco plants expressing the *iaaM* gene was positively correlated with auxin levels. While herbaceous plants have been shown the positive effect of the *iaaM* gene on height, the transgenic aspens showing elevated level of IAA amount showed decreased height growth (Tuominen et al., 1995). Tuominen et al. (2000) also reported that transgenic aspens having the *iaaM* gene were shorter or similar in height to wild type plants, even though the transgenic plants contained higher amounts of IAA than the wild type. The results indicate that the elevated auxin by genetic modification might not accelerate the growth of tree height. This study also showed that the transgenic plants had similar or less growth in height than the non-transgenic plants. Five transgenic lines having the *iaaM* gene were shorter than non-transgenic control in 2003 and were not significantly different from non-transgenic plants in 2004 (Figs. 6 and 11, Tables 2 and 8).

In this research, the IAA levels in the transgenic plants were not much different from those of the non-transgenic control, but three lines of the GRP-*iaaM* transgenic plants and two lines of the GRP-*iaaM*-35S-*ACC deaminase* transgenic plants contained slightly higher amounts of IAA than the non-transgenic control (Tables 14 and 15). It is contrary with previous research which showed distinctly higher amounts of auxin in the *iaaM*-transgenic aspens than the wild type plants (Tuominen et al., 1995, 2000). The lack of difference of IAA levels between transgenic and non-transgenic plants in this study might

be due to the limited measurement performed in only young leaves. If the *iaaM* gene was expressed specifically in vascular tissues, IAA needs to be measured in other tissues such as vascular tissues and cambium tissues. In fact, transgenic aspen expressing the *iaaM* gene driven by a vascular tissue specific promoter (Tuominen et al., 1995) showed different IAA levels in mature leaves, mature stem, and roots, and Tuominen et al. (2000) also extracted IAA from the cambium region. Therefore, the characterization of IAA concentration in various tissues in the current study may elucidate the change of IAA content of transgenic aspen expressing the *iaaM* gene. The lack of difference in the IAA level may be also explained by incomplete transcription or translation. Even though the *iaaM* gene was inserted into the aspen chromosome successfully, it is possible that the gene is not to be transcribed into RNA or translated into proper proteins. Additional northern blot or RT-PCR could also elucidate whether the inserted genes are expressing in the transgenic plants.

5.3. The effect of the *ACC deaminase* gene on the growth of the transgenic aspen

Plant growth and development is known to be altered by auxin and ethylene. Furthermore, overproduction of auxin stimulates the production of ethylene (McKeon and Yang, 1987), but it is often unclear if the morphological or anatomical effects of auxin are due to auxin alone, ethylene alone, or interaction of both (Romano et al., 1993). To uncouple these effects, Romano et al. (1993) hybridized transgenic plants producing elevated level of IAA by the *iaaM* gene with plants producing reduced level of ethylene

by the *ACC deaminase* gene. They found that most of the effects were primarily controlled by auxin rather than ethylene, but ethylene is partially responsible for the inhibition of stem elongation. In addition, it has been reported that the application of ethylene inhibited shoot growth (Davies, 1987) and that the distinct effect of ethylene is reduction in stem elongation (Taiz and Zeiger, 1998). Transgenic tobacco showing the highest ethylene production displayed significant dwarf morphology, resembling the phenotype of a wild-type plant exposed to excess ethylene (Araki et al., 2000). Hansen and Grossmann (2000) also reported that auxin-induced ethylene inhibited tomato height growth.

To effectively uncouple these effects in trees, the transgenic aspens expressing the *ACC deaminase* gene in conjugation with the *iaaM* gene were produced in this research. Interestingly, all four GRP-*iaaM* transgenic plants had higher ethylene content than non-transgenic control and all GRP-*iaaM*-35S-*ACC deaminase* transgenic plants showed decreased or similar ethylene amounts with the non-transgenic control (Tables 14, 15, and Fig. 16). That the transgenic plants did not have distinctly higher ethylene levels than non-transgenic control is not surprising because the GRP-*iaaM* transgenic plants did not exhibit much difference in auxin concentration from the non-transgenic plants. It appears that since distinct over-production of auxin amount was not observed, ethylene overproduction was not triggered by auxin in this experiment. If the GRP-*iaaM* gene was expressed at low levels due to use of the vascular specific promoter, the expression of the GRP-*iaaM*-35S-*ACC deaminase* gene might show characteristics of the plants transformed with the CaMV35S-*ACC deaminase* gene alone because the cauliflower

mosaic virus 35S promoter is known to drive strong and constitutive expression (Klee et al., 1987; Benfey and Chua, 1990; Romano et al., 1993). In this study, several GRP-*iaaM* transgenic lines showed slightly higher levels of ethylene than non-transgenic control, which might be due to the effect of elevated IAA levels by low expression of the *iaaM* gene. In contrast, most GRP-*iaaM*-35S-*ACC deaminase* transgenic lines showed lower or similar levels of ethylene than non-transgenic control (Tables 14, 15, and Fig. 16). These transgenic lines can be explained in that the slightly elevated auxin by the *iaaM* gene triggered the production of ethylene, but the *ACC deaminase* reduced the slightly elevated ethylene levels. However, more measurements of ethylene content might reveal the expression of the *ACC deaminase* gene because there was much variation of ethylene content in each transgenic and non-transgenic line.

The modified auxin and ethylene levels could affect the growth of the transgenic plants having the GRP-*iaaM*-35S-*ACC deaminase* gene. Two lines in 2003 and four lines in 2004 of the GRP-*iaaM*-35S-*ACC deaminase* transgenic plants were significantly higher than the non-transgenic plants while the GRP-*iaaM* transgenic plants were shorter or similar in height to non-transgenic plants (Figs. 6 and 11, Tables 2 and 6). The GRP-*iaaM*-35S-*ACC deaminase* transgenic plants having modified IAA and ethylene levels had more growth in height and volume than the GRP-*iaaM* transgenic plants in both 2003 and 2004 linear contrasts (Tables 7 and 13). A negative correlation between ethylene amounts and heights of the GRP-*iaaM*-35S-*ACC deaminase* transgenic plants in 2003 and 2004 was shown (Fig. 17). The larger heights and volume in the GRP-*iaaM*-35S-*ACC deaminase* transgenic plants may be due to reduced ethylene by the *ACC deaminase*

gene, and the growth of GRP-*iaaM* transgenic plants may be disturbed by elevated ethylene triggered by elevated auxin even though measurement did not show the significant differences of IAA content. This result indicates that a decreased ethylene level in the transgenic plants could affect the growth of plants. Hansen and Grossmann (2000) proposed that auxin-induced ethylene triggers abscisic acid (ABA) biosynthesis and ABA results in the inhibition of plant growth.

Interestingly, the GRP-*iaaM*-35S-*ACC deaminase* transgenic plants did not exhibit the differences in diameter even though they had more growth in height in 2004. This may be due to the reduced ethylene level, because ethylene is important in the growth of stem and wood formation (Eklund, 1991; Eklund and Little, 1994).

CHAPTER VI

CONCLUSION AND FUTURE PERSPECTIVES

The modified auxin and ethylene level could affect the growth of the transgenic plants having GRP-*iaaM*-35S-*ACC deaminase*. Several lines of the GRP-*iaaM*-35S-*ACC deaminase* transgenic plants were significantly taller than the non-transgenic plants, while the GRP-*iaaM* transgenic plants were shorter or similar height compared to the non-transgenic plants. The GRP-*iaaM*-35S-*ACC deaminase* transgenic plants showed more growth in height, and volume than the GRP-*iaaM* transgenic plants in the linear contrast, and a negative correlation between ethylene amounts and heights of the GRP-*iaaM*-35S-*ACC deaminase* transgenic plants was shown. The larger heights and volume of the GRP-*iaaM*-35S-*ACC deaminase* transgenic plants may be due to reduced ethylene by the *ACC deaminase* gene, and the growth of GRP-*iaaM* transgenic plants may be disturbed by elevated ethylene triggered by elevated auxin.

This result indicates that an increased auxin level might have a negative effect on the growth of trees resulting from elevated ethylene levels triggered by elevated auxin, but a decreased ethylene level in addition to an increased auxin level might have a positive effect on the growth of the trees. In this study, the auxin level is only slightly elevated in the transgenic plants, but if the promoter is substituted for a strong promoter in the GRP-*iaaM*-35S-*ACC deaminase* transgenic plants, the transgenic plants have potential for breeding woody plants which are taller and have a larger volume, which is beneficial to the wood industry.

Although the results in this research did not show a clear positive effect of the GRP-*iaaM* and the GRP-*iaaM*-35S-*ACC deaminase* genes within the time period of evaluation, the long-term effect remains to be seen, especially for those plants exhibiting reduced ethylene. These plants should be planted in the field and be evaluated for the long term effect of the genes. Emphasis should be placed on the secondary growth and possible wood property alterations, as some of the chemical components have been shown to vary (Labbé et al., 2005).

LIST OF REFERENCES

LIST OF REFERENCES

Alekseeva, V.V., Rukavtsova, E.B., Bobreshova, M.E., Lozhnikova, V.N., and Bur'yanov, Ya. I. 2004. Production and analysis of tobacco transgenic plants expressing the *Agrobacterium* gene for tryptophan monooxygenase. Russian J. Pant Physiol. 51: 541-546.

Aloni, R. 1987. Differentiation of vascular tissues. Annu. Rev. Plant Physiol. 38:179-204.

Altman, A. 2003. From plant tissue culture to biotechnology: scientific revolutions, abiotic stress tolerance, and forestry. In vitro Cell Dev. Biol. Plant 39:75-84.

Andersson-Gunnerås, S., Hellgren, J.M., Björklund, S., Regan, S., Moritz, T, and Sundberg, B. 2003. Asymmetric expression of a poplar ACC oxidase controls ethylene production during gravitational induction of tension wood. The Plant J. 34:339-349.

Araki, S., Matsuoka, M., Tanaka, M., and Ogawa, T. 2000. Ethylene formation and phenotype analysis of transgenic tobacco plants expressing a bacterial ethylene-forming enzyme. Plant Cell Physiol. 41:327-334.

Avsian-Kretchmer, O., Cheng, J.C., Chen, L., Moctezuma, E., and Sung, Z.R. 2002. Indole-acetic-acid distribution coincides with vascular differentiation pattern during Arabidopsis leaf ontogeny. Plant Physiol. 130:199-209.

Bandurski, R.S., Cohen, J.D., Slovin, J.P., and Reinecke, D.M. 1995. Auxin biosynthesis and metabolism. In: Plant hormones and their role in plant growth and development, Ed., P.J. Davies. Kluwer Academic Publishers, pp.1-12.

Bartel, B. 1997. Auxin Biosynthesis. Annu. Rev. Plant Physiol. Plant Mol. Bio. 48:51-66.

Benfey, P.N. and Chua, N.H. 1990. The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. Science 250: 959-966.

Bennett, M.D. and Leitch, I.J. 1997. Nuclear DNA amounts in angiosperms- 583 new estimates. Annals of Botany 80:169-196.

Brunner, A.M., Mohamed, R., Melian, R., Sheppard, L.A., Rottman, W.H., and Strauss, S.H. 1998. Genetic engineering of sexual sterility in shade trees. J. Arboriculture 24:263-272.

Buchanan, B.B., Gruissem, W., and Jones, R.L. 2000. Biochemistry and molecular biology of plant. American Soc. Plant Physiol., pp.312-454.

Chaffey, N. 1999. Cambium: old challenges-new opportunities. Trees 13:138-151.

Cheliak, W.M. and Rogers, D.L. 1990. Integrating biotechnology into tree improvement programs. Can. J. For. Res. 20:452-463.

Collett, C.E., Harberd, N.P., and Leyser, O. 2000. Hormonal interaction in the control of *Arabidopsis* hypocotyl elongation. Plant Physiol. 124:553-561.

Confalonieri, M., Beleghi, B., Balestrazzi, A., Negri, S., Facciotto, G., Schenone, G., and Delledonne, M. 2000. Transformation of elite white poplar (*Populus alba* L.) cv. 'Villafranca' and evaluation of herbicide resistance. Plant Cell Rep. 19:978-982.

Dai, W., Cheng, Z.M., and Sargent, W. A. 2003. Plant regeneration and *Agrobacterium*-mediated transformation of two elite aspen hybrid clones form in vitro leaf tissues. In Vitro Cell. Dev. Bio. Plant 39:6-11.

Davies, P.J. 1987. The plant hormones: Their nature, occurrence, and functions. In: Plant hormones and their role in plant growth and development, Ed., P.J. Davies. Kluwer Academic Publishers, pp.1-12.

Delledonne, M., Allegro, G., Belenghi, B., Balestrazzi, A., Picco, F., Levine, A., Zelasco, S., Calligari, P., and Confalonieri, M. 2001. Transformation of white poplar (*Populus alba* L.) with a novel *Arabidopsis thaliana* cysteine preteinase inhibitor and analysis of insect pest resistance. Molecular Breeding 7:35-42.

Dickmann, D.I. and Stuart, K.W. 1983. The Culture of Poplars. Michigan State university. pp.1-22.

Dinus, R.J., Payne, P., Sewell, M.M., Chiang, V.L., and Tuskan, G. A. 2001. Genetic modification of short rotation poplar wood: properties for ethanol fuel and fiber. Critical Rev. Plant Sci. 20:51-69.

Donahue, R.A., Davis, T.D., Micher, C.H., Rieneschneider, D.E., Carter, D.R., Marquardt, P.E., Sankhla, N., Sankhla, D., Haissig, B.E., and Isebrands, J.G. 1994. Growth, photosynthesis, and herbicide tolerance of genetically modified hybrid poplar. Can. J. For. Res. 24:2377-2383.

Doyle, J.J and Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissues. Pytochem. Bull. 19:11-15.

Dumas, B., Van Doorsselaere, J., Gielen, J., Legrand, M., Fritig, B., Van Montagu, M., and Inze, D. 1992. Nucleotide sequence of a complementary DNA encoding O-methyltransferase from poplar. Plant Physiol. 98:796-797.

Eklund, L. 1991. Hormone levels in the cambial region of *Picea abies* during the onset of cambial activity. Physiol. Plant 82:385-388.

Eklund, L. and Little, C.H.A. 1994. Interaction between indole-3-acetic acid and ethylene in the control of tracheid production in detached shoots of *Abies balsamea.* Tree Physiol. 15:27-34.

Evans, M.L. 1985. The action of auxin on plant cell elongation. Crit. Rev. Plant Sci. 2:317-365.

Fenning, T.M. and Gershenzon, J. 2002. Where will the wood come from? Plantation forests and the role of biotechnology. Trends in Biotech. 20:291-296.

Ficcadenti, N., Sestili, S., Pandolfini, T., Cirillo, C., Lepnardo, R., and Spena, A. 1999. Genetic engineering of parthenocarpic fruit development in tomato. Molecular Breeding 5:463-370.

Fillatti, J.J., Sellmer, J., McCown, B., Haissig, B., and Cmai, L. 1987. *Agrobacterium*-mediated transformation and regeneration of *Populus*. Mol. Gen. Genet. 206:192-199.

Fladung, M., Grossmann, K., and Ahuja, M. R. 1997. Alterations in hormonal and developmental characteristics in transgenic *Populus* conditioned by the *rolC* gene from *Agrobacterium rhizogenes*. J. Plant Physiol. 150:420-427.

Flavell, R. B. 1994. Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc. Natl. Acad. Sci. USA 91:3490-3496.

Follin, A., Inze, D., Budar, F., Genetello, C., van Montagu, M., and Schell, J. 1985. Genetic evidence that the tryptophan 2-mono-oxygenase gene of *Pseudomonas savastanoi* is functionally equivalent to one of the T-DNA genes involved in plant tumor formation by *Agrobacterium tumefaciens*. Mol. Gen. Genet. 201:178-185.

Giri, C.C., Shyamkumar, B., and Anjaneyulu, C. 2004. Progress in tissue culture, genetic transformation and applications of biotechnology to trees: an overview. Trees 18:115-135.

Grünward, G., Deutsch, F., and Eckstein, D. 2000. Wood formation in *rolC* transgenic aspen trees. Trees 14:297-304.

Grünward, C., Ruel, K., Joseleau, J.P., and Fladung, M. 2001. Morphology, wood structure and cell wall composition of *rolC* transgenic and non-transformed aspen trees. Trees 15:503-517.

Hamilton, A.J., Lycett, G.W., and Grierson, D. 1990. Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants. Nature 346:284-287.

Han, K.H., Gordon, M.P., and Strauss, S.H. 1997. High frequency transformation of cottonwoods (genus *Populus*) by *Agrobacterium rhizogenes*. Can. J. For. Res. 27:464-470.

Han, K. H., Melian, R., Ma, C., and Strauss, S.H. 2000. An *Agrobacterium* transformation protocol effective on a variety of cottonwood hybrids (genus *Populus*). Plant Cell Rep. 19:315-320.

Han, K.H. 2001. Molecular biology of secondary growth. J. Plant Biotech. 32:45-57.

Hansen, H. and Grossmann, K. 2000. Auxin-induced ethylene triggers abscisic acid biosynthesis and growth inhibition. Plant Physiol. 124:1437-1448.

Hellgren, J.M., Olofsson, K., and Sundberg, B. 2004. Patterns of auxin distribution during gravitational induction of reaction wood in poplar and pine. Plant Physiol. 135:212-220.

Herschbach, C. and Kopriva, A. 2000. Transgenic trees as tools in tree and plant physiology. Trees 16:250-261.

Hood, E. E., Gelvin, S. H., Melchers, L. S., and Hoekma, A. 1993. A new *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res. 2:208-218.

Honma, M. and Shimomura, T. 1978. Metabolism of 1-aminocyclopropane-1-carboxylic acid. Agric. Biol. Chem. 42:1825-1831.

Hobbs, S.L., Kpodar, P., and DeLong, C.M. 1990. The effect of T-DNA copy number, position and methylation on reporter gene expression in tobacco transformants. Plant Mol. Biol. 15:851-864.

Hu, W.J., Harding, S.A., Lang, J., Popko, J.L., Ralph, J., Stokke, D.D., Tsal, C.J., and Chiang, V.L. 1999. Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees. Nat. Biotech. 17: 808-812.

Ingemarsson, B.S.M., Lundqvist, E., and Eliasson, L. 1991. Seasonal variation in ethylene concentration in the wood of *Pinus sylvestris* L. Tree Physiol. 8: 273-279.

Kares, C., Depicker, A., Debreyne, P., Crouzet, P., and Otten, L. 1992. Transgenic tobacco plants with heat-inducible IAA synthesis genes. Progress in Plant Growth Regulation, pp. 713-723.

Keller, B., Schmid, J., and Lamb, C.J. 1989. Vascular expression of a bean cell wall glycine-rich protein-β-glucuronidase gene fusion in transgenic tobacco. EMBO J. 8:1309-1314.

Keller, B. and Baumgartner, C. 1991. Vascular-specific expression of the bean GRP 1.8 gene is negatively regulated. Plant Cell 3:1051-1061.

Kende, H. and Zeevaart, J.A.D. 1997. The five 'classical' plant hormones. Plant Cell 9: 1197-1210.

Kieber, J.J. 1997. The ethylene response pathway in *Arabidopsis*. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 277-296.

Klee, H.J., Horsch, R.B., Hinchee, M.A., Hein, M.B., and Hoffman, L. 1987. The effects of overproduction of two *Agrobacterium tumefaciens* T-DNA auxin biosynthetic gene products in transgenic petunia plants. Genes Dev. 1:86-96.

Klee, H.J., Hayford, M.B., Kretzmer, K.A., Barry, G.F., and Kishore, G.M. 1991. Control of ethylene synthesis by expression of a bacterial enzyme in transgenic tomato plants. Plant Cell 3:1187-1193.

Klee, H.J. and Lanahan, M.B. 1995. Transgenic plants in hormone biology. In: Plant hormones physiology, biochemistry, and molecular biology. Ed., P.J. Davies, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp.340-353.

Kleiner, K.W., Ellis, D.D., McCown, B.H., and Raffa, K.F. 1995. Field evaluation of transgenic poplar expressing a *Bacillus thuringiensis cryl A(α) d-endotoxin* gene against forest tent caterpillar (Lepidoptera:Lasiocampidae) and gypsy moth (Lepodoptera:Lymantriidae) following winter dormancy. Environ. Entomol. 24:1358-1364.

Labbé, N., Rials, T.G., Kelley, S.S., Cheng, Z.M., Kim, J.Y., and Li,Y. 2005. FT-IR imaging and py-MBMS: new tools to investigate wood tissues. Wood Science and Technol. 39:61-76.

Lapierre, C., Pollet, B., Petit-Conil, M., Toval, G., Romero, J., Pilate, G., Laple, J.C., Boerjan, W., Ferret, V., Nadai, V.D., and Jouanin, L. 1999. Structural alterations of lignins in transgenic poplars with depressed cinnarnyl alcohol dehydrogenase or caffeic acid O-methyltrasnferase activity have an opposite impact on the efficiency of industrial kraft pulping. Plant Physiol. 119:153-163.

Li, B.L., Wycloff, G.W., and Einspahr, D.W. 1993. Hybrid aspen performance and genetic gains. North. J. Appl. For. 10:117-122.

Linn, F., Heidmann, I., Saedler, H., and Meyer, P. 1990. Epigenetic changes in the expression of the maize A1 gene in *Petunia hybrida*: role of numbers of integrated gene copies and state of methylation. Mol. Gen. Genet. 222:329-336.

Little, C.H.A. and Pharis, R.P. 1995. Hormonal control of radcal and longitudinal growth in the tree stem. In: Plant stems: physiology and functional morphology. Ed., B.L. Gartner, Academic Press, San Diego, pp.281-319.

Little, C.H.A. and Savidge, R.A. 1987. The role of plant growth regulators in forest tree cambial growth. Plant Growth Regul. 6:137-169.

Lloyd, G and McCown, B. 1980. Commercially-feasible micopropagation of mountain laurelo, *Kalmia latifolia*, by use of shoot-tip culture. Proc. Int. Plant Prop. Soc. 30:421-427.

McKeon, T. and Yang, S.F. 1987. Biosynthesis and metabolism of ethylene. In: Plant hormones and their role in plant growth and development, Ed., P.J. Davies Boston, Martinus Nijhoff, pp.94-112.

Miller, A.R., Pengelly, W.L, and Roberts, L.W. 1984. Induction of xylem differentiation in *Lactuca* by ethylene. Plant Physiol. 75:1165-1166.

Nilsson, O., Moritz, T., Sundberg, B., Sandberg, G., and Olsson, O. 1996. Expression of the *Agrobacterium rhizogenes rolC* gene in a deciduous forest tree alters growth and development and leads to stem fascination. Plant Physiol. 112:493-502.

Normanly, J., Slovin, J.P., and Cohen, J.D. 1995. Rethinking auxin biosynthesis and metabolism. Plant Physiol. 107:323-329.

O'Neill, S.D. 1997. Pollination regulation of flower development. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48:547-574.

Oeller, P.W., Li, M.W., Taylor, L.P., Pike, D.A., and Theologis, A. 1991. Reversible inhibition of tomato fruit senescence by antisense RNA. Science 254:437-439.

Olson, D.C., White, J.A., Edelman, L., Harkins, R.N., and Kende, H. 1991. Differential expression of two genes for 1-aminocyclopropane-1-carboxylate synthase in tomato fruit. Proc. Natl. Acad. USA 88:5340-5344.

Ringli, C., Keller, B., and Ryser, U. 2001a. Glycine-rich proteins as structural components of plant cell walls. Cell. Mol. Life Sci. 58:1430-1441.

Ringli, C., Hauf, G., and Keller, B. 2001b. Hydrophobic interaction of the structural protein GRP 1.8 in the cell wall of protoxylem elements. Plant Physiol.125:673-682.

Rishi, A. S., Nelson, N.D., and Goyal, A. 2001. Genetic modification for improvement of *Populus*. Physiol. Mol. Biol. Plants 7:7-21.

Romano, C.P., Hein, M.B., and Klee, H.J. 1991. Inactivation of auxin in tobacco transformed with the indoleacetic acid lysine synthetase gene of *Pseudomonas-savastanio*. Genes Dev. 5:438-446.

Romano, C.P., Cooper, M.L., and Klee, H.J. 1993. Uncoupling auxin and ethylene effects in transgenic tobacco and *Arabidopsis* plants. Plant Cell 5:181-189.

Rotino, G.L., Perri, E., Zottini, M., Sommer, H., and Spena, A. 1987. Genetic engineering of parthenocarpic plants. Nat. Biotech. 15:1398-1401.

Savidge, R.A. 1988. Auxin and ethylene regulation of diameter growth in trees. Tree Physiol. 4:401-414.

Sheehy, R., Honma, M., Yamada, M., Sasaki, T., Martineau, B., and Hiatt, W.R. 1991. Isolation, sequence and expression in *Escherichia coli* of *Pseudomonas* sp. Strain ACP gene encoding 1-aminocyclopropane-1-carboxylate deaminase. J. Bacteriol. 173: 5260-5265.

Shinshi, H., Usami, S., and Ohme-Takagi, M. 1995. Identification of an ethylene-responsive region in the promoter of a tobacco class I chitinase gene. Plant Mol. Biol. 27:923-932.

Sitbon, F., Hennion, S., Sundberg, B., Little, C.H.A., Olsson, O., and Sandberg, G. 1992. Transgenic tobacco plants coexpressing the *Agrobacterium tumefaciens iaaM* and *iaaH* genes display altered growth and indoleacetic acid metabolism. Plant Physiol. 99:1062-1069.

Stockhaus, J., Schlue, U., Koczor, M., Chitty, J.A., Taylor, W.C., and Westhoff, P. 1997. The promoter of the gene encoding the C_4 form of phosphoenolpyruvate carboxylase directs mesophyll-specific expression in transgenic C_4 *Flaveria* spp. Plant Cell 9:479-489.

Sundberg, B. 1990. Influence of extraction solvent (buffer, methanol, acetone) and time of the quantification of indole-3-actic acid in plants. Physiol. Plant 78:293-297.

Sundberg, B., Tuominen, H., and Little, C.H.A. 1994. Effects of the indole-3-acetic acid (IAA) transport inhibitors N-1-naphthylphthalamic acid and morphactin on endogenous IAA dynamics in relation to compression wood formation in 1-year-old *Pinus sylvestris* (L.) shoots. Plant Physiol. 106:469-476.

Tsai, C.J., Popko, J.L., Mielker, M.R., Hu, W.J., amd Podila, G.K. 1998. Suppression of O-methyltrasferase gene by homologous sense transgene in quaking aspen causes red-brown wood phenotypes. Plant Physiol. 117:101-112.

Taiz, L. and Zeiger, E. 1991. Plant physiology. The Benjamin/Cummings Publishing Company, Inc.

Taiz, L. and Zeiger, E. 1998. Plant physiology. Sinauer Associates Publishers. Sunderland, MA.

Trebitsh, T., Staub, J.E., and O'Neill, S.D. 1997. Identification of a 1-aminocyclopropane-1-carboxylic acid synthase gene linked to the female (F) locus that enhances female sex expression in cucumber. Plant Physiol. 113:9870-995.

Tuominen, H., Ostin, A., and Sandberg, B. 1994. A novel metabolic pathway for indole-3-acetic acid in apical shoots of *Populus tremula* (L.) x *Populus tremuloides* (Michx.). Plant Physiol. 106:1511-1520.

Tuominen, H., Sitbon, F., Jasobsson, G., Sandberg, G., Olsson, O., and Sundberg, B. 1995. Altered growth and wood characteristics in transgenic hybrid aspen expressing *Agrobacterium tumefaciens* T-DNA indolacetic acid-biosynthetic genes. Plant Physiol. 109:1179-1189.

Tuominen, H., Puech, L., Fink, S., and Sundberg, B. 1997. A radial concentration gradient of indole-3-acetic acid is related to secondary xylem development in *Populus*. Plant Physiol. 115:577-585.

Tuominen, H., Puech, L., Regan, S., Fink, S., Olsson, O., and Sundberg, B. 2000. Cambial-region-specific expression of the *Agrobacterium iaa* genes in transgenic aspen visualized by a linked *uidA* reporter gene. Plant Physiol. 123:531-542.

Tuskan, G.A. 1998. Short-rotation forestry: What we know and what we need to know. Biomass Bioenergy 14:307-315.

Tuskan, G.A., Difazio, S.P., Teichmann, T. 2004. Poplar genomics is popular: The impact of the poplar genome project on the research. Plant Biology 6:2-4.

Tzfira, T., Yarnitzky, O., Vainstein, A., and Altman, A. 1996. Highly efficient transformation and regeneration of transgenic aspen plants through shoot-bud formation in root culture, and transformation of *Pinus halepensis*. Somatic Cell Gen. Mol. Gen. of Trees, pp. 125-130.

Tzfira, T., Jensen, C.S., Wang, A., Zuker, A., Vinocur, B., Altman, A., and Vainstein, A. 1997a. Transgenic *Populus tremula*: a step-by-step protocol for its *Agrobacterium*-mediated transformation. Plant Mol. Biol. Rep.15:219-235.

Tzfira, T., Jensen, C.S., Vainstein, A., and Altman, A. 1997b. Improved rooting ability and root-system performance in transgenic aspen plants. Biology of Root Formation, Plenum Press, New York, pp. 181-186.

Tzfira, T., Vinocur, B., Altman, A., and Vainstein, A. 1998a. Rol-Transgenic *Populus tremula*: root development, root-borne bud regeneration and *in vitro* propagation efficiency. Trees 12:464-471.

Tzfira, T., Zuker, A., and Altman, A. 1998b. Forest-tree biotechnology: Genetic transformation and its application to future forests. Trends in Biotech. 16:439-446.

Tzfira, T., Vainstein, A., and Altman, A. 1999. Rol-gene expression in transgenic aspen (*Populus tremula*) plants results in accelerated growth and improved stem production index. Trees 14:49-54.

Uggla, C., Moritz, T., Sandberg, G., and Sundberg, B. 1996. Auxin as a positional signal in pattern formation on plants. Proc. Natl. Acad. Sci. 93:9282-9286.

Uggla, C., Mellerowiez, E.J., and Sundberg, B. 1998. Indole-3-acetic acid controls cambial growth in scots pine by positional signaling. Plant Physiol. 117: 113-121.

van der Graff, E., Boot, K., Granbom, R., Sandberg, G., and Hooykaas, P.J.J. 2003. Increased endogenous auxin production in *Arabidopsis thaliana* caused both earlier described and novel auxin-related phenotypes. J. Plant Growth Regul. 22:240-252.

Weigle, D. and Nilsson, O. 1995. A developmental switch sufficient for flower initiation in diverse plants. Nature 337:495-500.

Yamamoto, F. and Kozlowski, T.T. 1987. Effects of ethrel on growth stem anatomy and ethylene production of *Pinus densiflora* seedlings. J. Exp. Bot. 38:293-310.

Yang, S.F. and Hoffman, N.E. 1984. Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. 35:155-189.

Ye, Z.H. 2002. Vascular tissue differentiation and pattern formation in plants. Annu. Rev Plant Biol. 53:183-202.

VITA

Joo Young Kim was born in Seoul, South Korea on September 20, 1972. She graduated from Seomoon Women's High School in spring 1988, and achieved a Bachelor of Agriculture degree in spring 1996 and a Master of Agriculture degree in spring 1998 in the department of Horticultural Science, College of Natural Resources at Korea University (Seoul, Korea).

After coming to Fargo, North Dakota, United States of America, she entered the Graduate School in the department of Plant Sciences of North Dakota State University (Fargo, ND) in fall 2000. She transferred to the University of Tennessee (Knoxville, TN) in fall 2002 and had studied in the department Plant Sciences by fall 2004. Before graduation, She moved to Florida and have worked in the department of Environmental Horticulture since March 2005. She acquired Master of Sciences in the department of Plant Sciences at University of Tennessee (Knoxville, TN) in spring 2007.